
Appropriately Timing Seed Germination-New Players and Upstream Controllers

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under the supervision of Professor Josette Masle

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Declaration

This thesis does not contain any material previously submitted for the award of any other degree or diploma at any university. It includes original research conducted by me, with some collaborative contributions as appropriately acknowledged. Specifically, in Chapter 2, the work on *ERf*-mediated ABA-GA signalling and *GL1* identification was carried out in collaboration with Dr. Amrit K. Nanda and Dr. Abdeljalil El Habti. In Chapter 4, the construction of the CRISPR/Cas9 vector and the characterization of primary transformants were performed in collaboration with Dr. Rachel Ikawa and Dr. Deyun Qiu. To the best of my knowledge, the content is original and has not been previously published or written by others, except where proper citations are provided.

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Abstract

Environmental change and abiotic stresses are challenging limiting factors of food production and sustainability. Seed germination is a most critical developmental phase change, particularly under abiotic stress conditions such as drought or salinity, to which it is especially vulnerable, with severe consequences on productivity. The timing of germination plays a major role in setting the timeline of the plant developmental cycle, and in conditioning seedling survival and adaption to unfavourable environments until completion of the plant developmental cycle and successful reproduction. Germination is a point of no return for the emerging seedling, with the need to fast become autotrophic once directly exposed to the external environment. Over recent decades, great progress has been made in understanding seed dormancy, its establishment during maturation on the mother plant, and the factors controlling its release after shedding. In contrast, little is known about the genetic determinants of germination in non-dormant seeds, especially under abiotic stresses. How seeds perceive changes in their environment, and how those signals are transmitted to their inner living tissues to trigger and pace germination is ill-understood.

The Arabidopsis *ERECTA* family *AtERf* comprises three leucine-rich repeat receptor-like kinases (LRR-RLKs): *ERECTA* (*ER*) and its two paralogous, *ERECTA-LIKE 1* (*ERL1*) and *ERECTA-LIKE 2* (*ERL2*). It is a demonstrated master regulator of various aspects of aerial plant growth and development, including organ shape, size or stomatal and vascular patterning, and is also involved in responses to environmental stresses, and some immune responses. A recent study (Nanda et al., 2019) uncovered another, unsuspected function of the Arabidopsis *ERECTA* family in the seed, especially in the sensitivity of seed germination to salinity and osmotic stress. The germination of seeds with reduced *ERECTA* family signalling was found to be normal under optimal conditions but slowed down, delayed or even halted by salinity and osmotic stress, through largely reversible mechanisms, so that when conditions became more favourable again, germination generally resumed, without significant loss of seed viability. That adaptive function was shown to be primarily under maternal control, and to involve the three family members, with specificity, and a dominant role of the *ER* gene.

The present study followed from that work, with a two-fold aim: a) Better understand the physiological and molecular bases of the maternal inheritance of the *ERf*-mediated germination phenotype; b) Test conservation across species of the newly discovered *ERf* regulatory function in Arabidopsis seed germination, with a focus on barley (*Hordeum vulgare*, *Hv*), a major staple food and pillar of the brewing industry; and fundamentally, also a representative of grain cereals, which produce caryopses as reproduction units, i.e. fruits containing a single seed enclosed within the pericarp, and specialised highly resistant outer envelopes.

The first Chapter of this thesis describes the discovery of a novel molecular interactor of the Arabidopsis *AtERf* - the MYB family transcription factor *AtGL1*- previously unknown to be acting in seeds, nor to interact with the extensively documented *ERf* signalling pathways in aerial organs. *GL1* is here shown to synergistically interact with the *ERf* members, in a gene combination-dependent manner, to regulate the initiation, progression, and timing of germination completion under saline conditions. Its function is primarily maternally controlled, via the seed coat, through properties determined during seed embryogenesis and also the subsequent differentiation of the seed coat during seed maturation, and the formation of its mucilage.

A second Chapter reports on a genome-wide analysis of *ERf*- and *GL1*-mediated changes in transcriptional networks during embryogenesis and the full expansion of the seed cavity, as well as the early differentiation of the seed specialised mucilage excreting epidermal cells, through RNAseq analysis of developing siliques sequentially over the first 6 days of seed development. The results indicate that wide-spread *ERf* and *GL1* separately act and also interact in the transcriptional control of hormone signalling, transcription factors, cell wall biogenesis, the formation of cuticular and wax compounds, stress response pathways. They also converge towards the conclusion of a) synergistic and also in part specific, complementary functions of the *AtERf* and *GL1* at the transcriptional level; and b) that *GL1* is not integral to the *ERf* pathway but acts through an intersecting pathway.

The last part of the thesis presents the analysis of a range of barley mutant lines obtained through CRISPR-Cas9 editing of the putative barley *HvERf* and *HvGL1* orthologues. They show that these genes, separately and together, exert a major regulatory role in vegetative development, fertility and the anatomical and structural characteristics of the maternal seed enclosing tissues, and in the regulation of seed germination under salinity but also constitutively.

In conclusion, the findings reported in this thesis overall highlight the potential of the barley *ERECTA* genes and putative orthologue to *AtGL1* for genetic improvement, especially with respect of more uniform germination within seed cohorts, and regulation of its timing to mitigate risks of germination failure or seedling death, thus enhancing yield. Furthermore, manipulating *ERf/GL1* expression and/or functions offer novel tools for potentially mitigation risks during of premature germination on the mother plant, that makes seeds improper to brewing

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Chapter 1

General Introduction

Environmental changes and stresses are the most limiting factors in crop production and improvement ([Lobell et al., 2011](#)). Seed germination is a critical phase in the plant life cycle, particularly under abiotic stress conditions such as salinity, which can severely affect crop yield and productivity. Germination starts soon after a non-dormant seed starts absorbing water from its surrounding environment and metabolism gets reactivated. The timing of germination plays a major role in setting the timeline of the plant developmental cycle, and conditioning seedling survival and adaption to unfavourable environments until completion of its life cycle with successful reproduction. Germination is a point of no return for the emerging embryo, with the need to become autotrophic quickly once exposed to the external environment.

Yield loss due to germination failure or early seedling death is a critical issue in crop species around the world, especially in areas prone to drought or salinity stresses. Seed fitness is also a major component of biodiversity in natural communities, that helps to overcome abiotic and biotic stress during the germination process ([Thompson et al., 2002](#); [Larios et al., 2014](#); [DeMalach et al., 2019](#); [E. Ling, 2019](#); [Mao et al., 2019](#); [Huang et al., 2023](#)). Understanding the genetic regulation of salt tolerance during seed germination can contribute to the development of crop varieties with enhanced stress tolerance, an urgent necessity in the face of global climate change. Over recent decades, great progress has been made in understanding seed dormancy, its establishment during maturation on the mother plant, and the factors controlling its release after shedding. In contrast, little is known about the genetic control of germination in non-dormant seeds under different abiotic stresses, including salinity and drought-induced osmotic stress. A complex network of endogenous controllers (stored mRNAs and proteins, Reactive Oxygen Species, plant growth regulators, especially abscisic acid (ABA) and gibberellins (GA), transcription factors.) are involved, as well as local conditions in the environment surrounding the seed. However, how seeds perceive changes in their environment and how those signals are transmitted to their inner living tissues to trigger germination is ill-understood.

After completion of embryogenesis and embryo morphogenesis, seeds undergo a complex process of maturation and desiccation as well as dormancy acquisition on the mother plant and then use different dispersal mechanisms to find their way home to establish as a seedling and later a fully developed plant. After it finds its way, the dry seed begins to take up water and initiates germination and activates its cellular machinery ([Crowe, 1992](#)); reviewed in ([Bentsink & Koornneef, 2008](#)). Through the evolutionary process, all angiosperms have evolved mechanisms to produce covered seeds, where the

inner embryo is surrounded by a resistant, structurally complex dead envelope of maternal origin (reviewed in ([Linkies et al., 2010](#))). The development, structure, biochemical attributes and physiology of the embryo surrounding tissues remain species-specific ([Weitbrecht et al., 2011a](#)) and are little understood. In this study, we used *Arabidopsis thaliana*, a well-known member of the Brassicaceae family, as the main model plant ([Meinke et al., 1998](#)). The *Arabidopsis thaliana* model system provides invaluable insights into the genetic and molecular mechanisms underlying plant development and stress responses ([Meinke et al., 1998](#); [Koornneef & Meinke, 2010](#)); (reviewed in ([Ferjani et al., 2023](#))). In *Arabidopsis*, the mature seed coat (Testa) is only separated from the embryo by a live single-cell thick endosperm layer ([Iwanowska et al., 1994](#); [Debeaujon & Koornneef, 2000](#)) (Figure 1-1 B). In many other species, among which cereals, the embryo is also in close contact with a residual nourishing endosperm of dead cells, containing accumulated reserves which fuel embryo growth upon seed imbibition ([Olsen, 2004](#)). In *Arabidopsis*, the developing embryo absorbs the whole endosperm during morphogenesis on the mother plant, apart from the residual aleurone endosperm layer. Most of the reserves – mostly lipids and proteins- are accumulated in the cotyledons of the mature embryo. The endosperm arises from the fertilisation of the ovule central cell and is, therefore, a triploid tissue (2 copies of the maternal genome, one copy of the paternal genome), while the diploid embryo arises from fertilisation of the egg cell. There are several phases of endosperm development starting with nuclearisation, followed by cellularisation, differentiation, maturation and cell death ([Mansfield, 1990](#); [Berger, 1999](#); [Olsen, 2004](#)). These processes begin in the micropylar region and later spread to the chalazal region ([Muller et al., 2006](#); [D. Yan et al., 2014](#)) which is the region of attachment of the fertilised ovule and young derived seed to the maternal silique walls, via the funiculus. Seed development on the mother plant plays a vital role in seed vigour and viability. The process of seed development, encompassing embryogenesis and maturation, significantly impacts the quality of the seeds sown, especially under abiotic stress conditions (reviewed in ([Escudero et al., 2024](#))).

1.1 The effects of salinity stress on seed germination

1.1.1 General overview of *Arabidopsis* seed germination

Germination in *Arabidopsis* is a two-step process strictly defined as the time when the embryo first breaks through its covering tissues as they weaken through the action of cell wall degrading enzymes regulated by inter-signalling between the seed's three compartments and of the increasing force exerted by the expanding turgid embryo ([Bewley et al., 1994](#)). Germination follows a triphasic water uptake dynamic, consisting of a rapid uptake-phase I, a plateau-phase II, and then a third phase III of

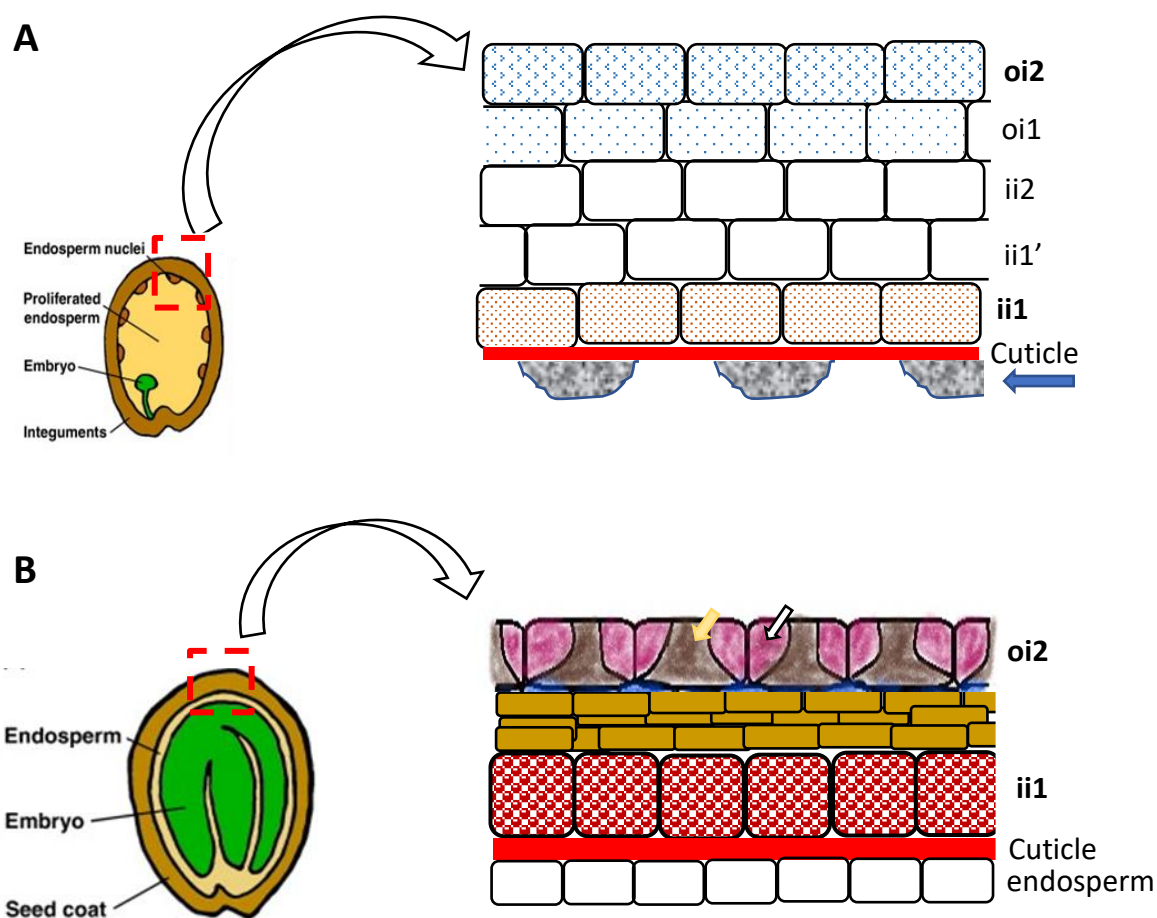


Figure 1-1. A&B. Arabidopsis developing (A) and mature seed (B) and maternally derived embryo covering layers. **A.** Developing Arabidopsis seed ('1-2 days after flower opening) and its developing seed coat. Two outer integument (oi2 and oi2) and three inner integument layers (ii2, ii1', and ii1), endosperm associated cuticle and developing free nuclear endosperm (blue arrow) of Arabidopsis developing seed coat were shown in the illustration. **B.** Arabidopsis seed covering layers at maturity. The outer epidermal cells of the Arabidopsis seed coat bearing the columella (yellow arrow), made from secondary cell wall materials, and mucilage pockets (white arrow with black margins). All the other three integument layers in between the outer epidermis (oi2) and pigment layer (ii1) were crushed during the seed coat differentiation. ii1 originated cuticle was transferred to the endosperm at the later stage of seed development.

important uptake of water to fulfil the requirement of cotyledon and radicle expansion preceding endosperm rupture and exposure of the embryo to the external environment ([Bewley et al., 1994](#)). Arabidopsis seed germination begins with the rapid uptake of water from its surroundings and the quasi-immediate secretion of the highly hydrophilic pectin-rich mucilage from the seed coat oi2 epidermal cells by rupture of their outer tangential primary cell walls. This mucilage encapsulates the seed and facilitates rapid water uptake and transfer to the inner living tissue, especially when there is limited water availability in the surrounding environment, necessary for reactivation of metabolism

and embryonic cell proliferation during the following phase (II) when seed water content ceases to increase and terminates with the rupture of seed coat or testa (Testa Rupture-TR) by the radicle. The last phase of rapid water uptake sustains embryo expansion until the growing radicle breaks through the endosperm (Endoderm Rupture -ER), marking the completion of germination ([Karssen, 1976](#); [Leubner-Metzger, 1995](#); [Bewley, 1997](#); [Bentsink & Koornneef, 2008](#)). In a strict sense, this is determined by the balance of the growth potential of the embryo and the mechanical resistance of the embryo-covering tissues, endosperm and testa. Seeds start absorbing the water mainly through the micropyle, rapidly swell, changing in both size and shape ([Bewley, 1997](#)); (reviewed in ([Masubelele et al., 2005](#); [Weitbrecht et al., 2011b](#))).

Mutants affected in cell proliferation show delayed radicle emergence but were not shown to be affected in the final germination rate ([Barrôco et al., 2005](#)). Absciscic acid (ABA) and Gibberellic Acid (GA) phytohormones play a crucial role in controlling the arabidopsis seed two-step germination. ABA maintains dormancy and delays endosperm weakening, and counteracts the germination promoting role of GA, thus delaying/inhibiting the germination ([Garciaarrubio et al., 1997](#)); reviewed in ([Kermode, 2005](#)); ([Muller et al., 2006](#)). In addition to its roles in seed dormancy and germination, ABA is also involved in adaptation to abiotic stresses, such as drought, cold and salt ([Xiong & Zhu, 2002](#)); (Reviewed in ([X. Zhang et al., 2022](#)));([Yadav et al., 2023](#)). As ABA accumulates in response to abiotic stress, it is often referred to as a plant stress hormone. The decreased sensitivity of ABA-deficient and insensitive mutants to salt and osmotic stress during germination has demonstrated that ABA inhibits germination in response to abiotic stress ([Xiong et al., 2001](#); [Xiong & Zhu, 2002](#)).

1.1.2 The effects of salinity stress on seed germination; a focus on crops

Salinity stress is one of the most severe challenges to achieving optimal crop yields globally. Increasing soil salinity is attributed to factors such as elevated temperatures, increased evapotranspiration, and reduced precipitation (reviewed in ([Chele et al., 2021](#))). Poor irrigation practices, inadequate drainage, and decreased rainfall further contribute to this issue, particularly in arid and semi-arid regions ([Lodeyro & Carrillo, 2015](#)); reviewed in ([van Zelm et al., 2020](#)). Crops experience salinity stress at every stage of their life cycle, including seed germination. Due to the vast structural and biochemical differences between seeds of various crop species, there are notable interspecies variations in responses to salinity stress during germination and early seedling growth. Nevertheless, all studied species have shown sensitivity to salinity during germination to varying degrees across species and cultivars. Numerous studies have reported reduced germination percentages and slower germination speeds under salinity stress in a wide range of crops, including barley ([El Goumi et al., 2014](#); [Askari et](#)

al., 2016; Al-Tardeh et al., 2023), rice (Khan MSA, 1997; Ologundudu, 2014), wheat (Datta, 2009; Hmissi et al., 2023), pepper (Yildirim, 2006), soybean (Hosseini et al., 2002; Canavar, 2023), and lentils (Foti et al., 2019), or Arabidopsis (W. Yuan et al., 2016). This illustrates the global challenge salinity stress poses to crop germination and yield, despite the inherent differences among species.

Salinity stress can negatively affect seed germination either through ionic stress due to the accumulation of sodium (Na^+) and chloride ions (Cl^-), causing an imbalance in nutrient uptake and toxicity, or through osmotic stress, due to reduced water uptake during imbibition (Zhu, 2001; Munns, 2002; Murillo-Amadot et al., 2002; Khajeh-Hosseini et al., 2003; Lowry et al., 2009; Isayenkov & Maathuis, 2019). Low water potential and abundance of Na^+ and Cl^- ions alter metabolism or inhibit certain steps in metabolic sequences of germination (Younis et al., 1993). Decreased water potential enhances oxidative stress by inducing the production of reactive oxygen species (ROS), and intracellular damage (Ibrahim, 2016). Salt stress also disturbs the seed ABA- GA hormonal balance during germination and delays germination due to reduced GA and elevated ABA (Lopez-Molina et al., 2001; Kim & Park, 2008; Yuan et al., 2011). Besides ABA and GA, other plant hormones play a major role in salinity signalling during seed germination, such as ethylene signalling, which has been shown to promote seed germination under salt stress in Arabidopsis (Reviewed in (Riyazuddin et al., 2020)). Through natural selection, plants developed various adaptations, including developmental and hormonal variation, to protect themselves from salinity stress (Flowers TJ, 1977; Greenway & Munns, 1980; Ehret et al., 1990; Zhu, 2003). Few genes such as ion antiporters NHX1, SALT OVERLY SENSITIVE (SOS) (Liu et al., 2000; Zhang & Blumwald, 2001; Qiu et al., 2002). ABA biosynthesis genes ABI3, ABA3 in upper ground parts (Giraudat et al., 1992; Xiong & Zhu, 2002; Ren et al., 2010) and naturally occurring quantitative trait Loci's (QTL's) during germination and early seedling growth (Galpaz & Reymond, 2010; DeRose-Wilson & Gaut, 2011; Mwando et al., 2020) have been identified in previous studies of salt tolerance. However, salinity sensing during seed germination is still poorly understood, and the signalling pathways have yet to be discovered. A recent study by Nanda et al. (2019) has established that the Arabidopsis *ERECTA* gene family is involved in sensing salinity during germination and has a germination-promoting role under salt and osmotic stress.

1.2 The *ERECTA* gene family (*ERf*) and its functional importance on seed germination under salt and osmotic stress

1.2.1 The Arabidopsis *ERECTA* gene family (*ERf*)

Arabidopsis Receptor-Like Kinases (RLKs) is a large group of more than 600 members, structurally characterised by the presence of an extracellular interacting domain, a transmembrane domain and an intracellular kinase domain ([Genome, 2000](#); [Shiu & Bleecker, 2001](#)). The leucine-rich repeat-receptor-like kinases (LRR-RLKs) represent the largest subgroup of RLKs with approximately 235 members and contain well-conserved leucine rich motif tandem repeats ([Shiu & Bleecker, 2001](#); [Hosseini et al., 2020](#)). The plant RLKs are involved in sensing the environment mostly through small ligands that bind to the extracellular interacting domain; those ligands are mostly small secreted peptides, often 10-20 amino acids in length. These peptides can derive from various sources, including plant hormones and pathogen-associated molecular patterns (PAMs) ([Becraft, 2002](#); [Diévert & Clark, 2004](#); [Vert et al., 2005](#); [Matsubayashi & Sakagami, 2006](#); [De Smet et al., 2009](#); [Uchida et al., 2012](#); [Hosseini et al., 2020](#)). Despite the large number of LRR-RLKs in the Arabidopsis genome, only a handful of them have been assigned a function.

ERECTA (*ER*) is a well well-known LRR-RLK and has two close homologues in Arabidopsis, named *ER-LIKE* genes (*ERL1* and *ERL2*) ([Shpak et al., 2004](#)). Their extracellular domain contains leucine-rich motifs, and in Arabidopsis, *ER* and *ERL2* have 20 LRRs, and *ERL1* has 21 LRRs ([Torii et al., 1996](#); [Kosentka et al., 2017](#)). Phylogenetic analyses of the *ER* gene family (*ERf*) revealed the presence of *ERf* homologues in every species investigated, including bryophytes, suggesting that the *ERf* appeared early during plant land colonisation. The *ERf* is then thought to have split in two, *ER* and *ER-LIKE*, prior to the appearance of monocots and dicots ([Masle et al., 2005](#); [Villagarcia et al., 2012a](#)). In Arabidopsis, *ERL1* and *ERL2* are immediate paralogues of *ER*, presenting 72% sequence similarity with *ER* and 83% sequence similarity with each other ([Shpak et al., 2004](#)). The *ERECTA* family (*ERf*) sequence contains a unique structure of 26 introns, some of which play a key role in precise gene expression and regulation ([Karve et al., 2011](#)) and mRNA stability ([Wong & Schmitz, 2022](#)).

Mitogen-activated protein kinases (MAPK) signalling cascades are commonly, but not exclusively, triggered by LRKs, capable of perceiving external signals at the cell surface and transducing them inside the cell. MAPKs are serine/threonine-specific (ser/thr) kinases and are involved in the phosphorylation of a wide range of substrates. Typically, MAPK cascade signalling is initiated by the activation of a MAPKK kinase (MAPKKK/MKKK) which, in turn, phosphorylates downstream MAPK kinase kinases (MAPKK/MKK), which then activate one or several downstream MAPKs, leading to the phosphorylation

of a range of downstream targets, including transcription factors that alter gene expression, and other proteins of various kinds, and ultimately regulating multiple downstream cellular responses. Several MAPKs can be involved in each tier of the cascade, with partially overlapping or redundant roles, and different spatial and temporal patterns of activation so as to diversify signalling and bring specificity ([Jonak et al., 2002](#); [Pedley & Martin, 2005](#); [Colcombet & Hirt, 2008](#); [Pitzschke et al., 2009](#); [Sanchez-Rodriguez et al., 2009](#); [Tena et al., 2011](#); [Xu & Zhang, 2015](#); [Thulasi Devendrakumar et al., 2018](#); [M. Zhang et al., 2018](#); [Wang & Gou, 2020](#)). The high number of Arabidopsis MAPKs suggests specialisation ([Ichimura et al., 2002](#); [Jonak et al., 2002](#)). However, functional studies of several MAPKs over the last decade revealed that a given MAPK can regulate diverse physiological processes, and how specificity is achieved is still not well known. For example, MPK3 and MPK6 have been identified as important players in plant responses to various stress stimuli, such as oxidative stress, low temperature, dehydration, touch, wounding, hyper-osmotic stress and pathogen recognition ([Kovtun et al., 2000](#); [Nuhse et al., 2000](#); [Ichimura et al., 2002](#)) (reviewed in ([Sun & Zhang, 2022](#))); ([Xu & Zhang, 2015](#); [M. Zhang et al., 2018](#)) and are also involved in plant development, reproduction, stomatal patterning and hormone signalling ([Bush & Krysan, 2007](#); [Wang et al., 2007](#); [Hord et al., 2008](#); [Yoo et al., 2008](#)) (reviewed in ([Zheng et al., 2018](#); [Sun & Zhang, 2022](#))); ([Siodmak et al., 2023](#)). Exactly how these kinases are capable of transducing specific signals in response to different endogenous or exogenous stimuli is still unknown. Several plant MAPKs such as MPK11, MPK5, MPK12, MPK13, and MPK4 have also been implicated in responses to environmental stimuli of biotic or abiotic origin, or the perception of endogenous growth and development stimuli (reviewed in ([De Zélicourt et al., 2016](#))); ([Thulasi Devendrakumar et al., 2018](#); [Siodmak et al., 2023](#)).

The *Erf* signalling is activated by ligand binding to the *Erf* extracellular leucine-rich-repeat receptor, and this triggers MAPK phosphorylation cascades that regulate multiple cellular functions involved in plant development and morphogenesis, tissue patterning, depending on ([Meng et al., 2012](#)). Most of the *Erf* ligands identified so far belong to the *EPIDERMAL PATTERNING FACTOR* (*EPF*) and *EPIDERMAL PATTERNING FACTOR-LIKE* (*EPFL*) families ([Hara et al., 2007](#); [Hara et al., 2009](#); [Uchida et al., 2012](#); [Sopena-Torres et al., 2018](#)). A recent study shows that plant U-box ubiquitin E3 ligases PUB30 and PUB 31 are necessary as the integral component of *Erf* signalling, thereby optimising the *Erf* signalling in developmental processes ([Chen et al., 2023](#)).

1.2.2 The *ERf* is a master regulator of plant vegetative and reproductive development, and germination under salinity and osmotic stress

The *ERECTA* family has been extensively studied and is known for its functions in numerous aspects of plant development, especially stomatal development, vascular patterning, reproductive development, cellular proliferation and plant morphogenesis, and also its role in the regulation of water use efficiency, abiotic stress responses and immune responses to some pathogens ([Torii et al., 1996](#); [Berger & Altmann, 2000](#); [Masle et al., 2005](#); [Shpak et al., 2005](#); [Bemis et al., 2013](#); [Shen et al., 2015](#); [Jorda et al., 2016](#); [Cai et al., 2017](#); [Sopena-Torres et al., 2018](#); [H. Y. Cai, M. N. Chai, et al., 2021](#); [H. Y. Cai, Y. M. Huang, et al., 2021](#); [Cai et al., 2023](#)). Knockout *er* mutants develop rounder leaves with reduced petioles and most strikingly, present a reduced, erect stature, a compact inflorescence with a clustering of flowers supported by short pedicels, and that, upon fertilisation, develop into short, blunt siliques, increased stomatal density ([Rédei, 1992](#); [Torii et al., 1996](#); [Douglas et al., 2002](#); [Shpak et al., 2004](#); [Douglas & Riggs, 2005](#); [Masle et al., 2005](#); [Bemis, 2013](#)). In contrast, *erl1* and *erl2* knockout mutations of either of the two *ER* paralogues, *ERL1* and *ERL2*, have alone no effect on vegetative or reproductive development, but in combination with *er*, uniquely enhance growth and patterning defects, through major roles in cell proliferation and the determination cell fate, especially in the epidermis and the stomatal lineage ([Shpak et al., 2004](#); [Shpak et al., 2005](#); [Pillitteri, 2007](#)).

Moreover, loss of function of all three genes in the *er erl1 erl2* mutant induces even more severe and extensive phenotypes, including extreme dwarfism, reduced lateral organ size, stomatal clustering, and totally abnormal flower development resulting in sterility. This large body of data overall gives ample evidence of partial redundancy but also functional specificity within the family, and synergistic roles of its three members tailored to the regulation of a range of targets involved in diverse physiological functions ([Tisne et al., 2011](#); [Wu et al., 2022](#)). The study of *Arabidopsis* ecotypes, as well as *of er* mutants, including Landsberg *er* (*Ler*), has shown the importance of *ER* in biological processes relating to plant adaptation and micro-environmental canalization of development ([Hall et al., 2007](#)). *ER* has been found to be a QTL hotspot for multiple traits, including, but not limited to, cell expansion and division ([Tisné et al., 2008](#)), transpiration efficiency ([Masle et al., 2005](#)), seed mineral concentrations ([Waters & Grusak, 2008](#)), as well as resistance to pathogens ([Godiard et al., 2003](#); [Llorente et al., 2005](#); [Jorda et al., 2016](#); [Takahashi et al., 2016](#)). Overlaps between the *ERf* pathway and several hormonal signalling pathways have been reported (most prominently auxin, but also gibberellin, cytokinin and Brassinosteroids signalling (BR)) ([Woodward et al., 2005](#); [Hu et al., 2011](#); [Ragni et al., 2011](#); [Chen et al., 2013](#); [Uchida & Tasaka, 2013](#); [X. Qu et al., 2017](#); [H. Cai et al., 2021](#); [Sarnowska et al., 2023](#)). *ER* has also been shown to positively regulate plant transpiration efficiency in

Arabidopsis through both restricting leaf stomatal conductance via lower stomatal density, and improving leaf photosynthetic capacity ([Masle et al., 2005](#)). The *ERf* role in the control of water loss by transpiration has been demonstrated in a range of species (e.g. tomato & rice ([Shen et al., 2015](#)), poplar ([H. Li et al., 2021](#)), etc). Overall, the large body of studies on the *ERECTA* family has identified it as a master regulator of plant development and defense in aerial organs.

A recent study by ([Nanda et al., 2019](#)) established a novel role for the Arabidopsis *ERECTA* gene family, in the seed, and its sensitivity to salinity and osmotic stress. The germination of seeds with reduced *ERECTA* family signalling was found to be normal under optimal conditions, but slowed down, delayed, or even halted by salinity and osmotic stress, through largely reversible mechanisms and when conditions became more favourable again, germination resumed, without significant loss of seed viability. This was the first time that the *ERECTA* receptor kinases were implicated in seed germination and the direct signalling of abiotic stress during that crucial developmental phase change. Except for the *er105 erl1* double mutant, loss of function of *ER*/*and* *ERL* showed no effect on testa rupture or endosperm rupture under the optimal conditions (0mM NaCl). The loss-of-function *er105*, *er105 erl1*, *er105 erl2*, *er105 erl1/seg erl2* are hypersensitive to salinity, in increasing degree, and to osmotic stress, whereas *erl1*, *erl2* and *erl1 erl2* show WT germination phenotypes ([Nanda et al., 2019](#)) Salinity appears to be signalled through its ionic effects, and also through osmotic effects at very high salinity levels ([Nanda et al., 2019](#)). Furthermore, the *ERf* function in regulating sensitivity to salinity during germination in seeds was shown to be primarily maternally controlled by the embryo surrounding tissues, seed coat and endosperm, from germination assays of F1 seeds derived from reciprocal crosses of *er erl1*. and WT flowers. These experiments also revealed a function of the *ERf* on seed size and shape, also maternally controlled, and provided evidence of some partial overlap with the *ERf* function on germination. The salt-hypersensitive *er erl1*, *er erl2* and *er erl1/seg erl2* seeds were also characterised by smaller size and showed shape differences, but the *er* seeds were wild type like, and *erl1 erl2* seeds were significantly larger ([Nanda et al., 2019](#)).

The underlying mechanisms of these *ERf*-mediated seed phenotypes remain mostly unknown, apart from unambiguous evidence that they largely take place during the development of the seed on the mother plant, primarily of its maternal tissues, and affect germination through a control on the properties of the maternal tissues, the seed coat especially, and the ABA-GA signalling loop during germination, involving all three *ERf* family members in a synergistic, non-redundant manner ([Nanda et al., 2019](#)). Otherwise, a recent study reported that the *ER* regulates the cell proliferation in the outer seed coat integument of the seed and that this caused a reduction of seed size ([Wu et al., 2022](#)), via a cascade involving MAPK-DA1 and UBIQUITIN SPECIFIC PROTEASE 15 (UBP15) ([Nanda et al., 2019](#); [Wu et al., 2022](#)).

1.2.3 Maternal regulation of *ERf*-mediated salt hypersensitive germination phenotype.

The Arabidopsis seed maternal tissues

The *Arabidopsis* seed coat originates from the two ovular integuments ([Leon-Kloosterziel et al., 1994](#)), the outer integument has two layers named, from outside to inside, outer (oi2) and inner (oi1). The inner integument consists of two layers at the chalazal and micropylar end -ii2 and ii1-, and at the opposite distal curving zone presents a third layer, ii1' ([Beeckman et al., 2000](#); [Haughn & Chaudhury, 2005](#); [Loubery et al., 2018](#)) (Figure 1-1). Upon fertilisation, seed coat integument layers proceed through active cell proliferation and expansion up to the late heart stage of the embryo about 4-DAFO (Days After Flower Opening), by then are clearly defined and distinguishable. Each of the 5 layers then undergoes extensive differentiation and specific structural and biochemical modifications during the seed maturation and desiccation phases, in parallel to embryo morphogenesis and expansion and endosperm development. From 2 DAFO, the innermost (ii1) seed coat layer produces and accumulates proanthocyanidin (PAs) in the vacuoles ([Beeckman et al., 2000](#); [Debeaujon & Koornneef, 2000](#); [Haughn & Chaudhury, 2005](#); [Loubery et al., 2018](#)), a type of oligomeric flavonoid, which gets oxidised during seed desiccation, giving the mature seed coat its brown colour ([Haughn & Chaudhury, 2005](#)) (Figure 1-1 B). At about 4 DAFO, the outer integument layer 2 (oi 2) starts to synthesise mucilage polysaccharides that are secreted into the apoplast. Through that process, the cytoplasm is progressively displaced, and finally disappears. A volcano-shaped structure, the columella, builds up in the centre of the cells, made up of secondary cell wall materials, and is flanked by mucilage pockets (Figure 1-1 B). In the mature seed, the pectinous-rich mucilage forms a thin lining on the epidermal cell inner walls ([Western et al., 2000](#); [Haughn & Chaudhury, 2005](#); [Haughn & Western, 2012](#); [North et al., 2014](#)).

Recent studies have established the presence in the *Arabidopsis* seed of an endosperm-associated cuticle ([De Giorgi et al., 2015](#); [Ingram & Nawrath, 2017](#); [Loubery et al., 2018](#)) and an embryo-associated cuticle ([Molina et al., 2008](#)). There are also indications of another external cuticle covering the seed coat, together with waxes ([Ingram & Nawrath, 2017](#)). Notably, the endosperm-associated cuticle is of maternal origin, produced from the ii1 layer ([Loubery et al., 2018](#)), and appears to be later transferred into the space between the seed coat and the endosperm. Its exact role is yet to be established, but obviously, it is likely very relevant to the permeability of the endosperm to solutes from the environment.

The testa and mucilage of the Arabidopsis seed are primary regulators of the ERF-mediated salt-hypersensitive germination phenotype.

The pectin-rich mucilage swells and is secreted to the outer environment as soon as the seed touches the water, and the mucilage capsule surrounding the seed facilitates the water uptake during the imbibition and keeps the seed hydrated throughout germination (reviewed in ([Arsovski, 2010](#)) ([Western, 2012](#)). That mucilage appeared to not affect the percentage germination or the germination rate of WT Arabidopsis seeds or seeds of mutant seeds defective in mucilage synthesis when there was ample water available in the seed surroundings during imbibition ([Penfield et al., 2001](#); [Yang et al., 2010](#)). However, the percentage of germination in the mucilage-defective mutants was reduced under conditions of low water potential ([Penfield et al., 2001](#)).

The Arabidopsis mucilage consists of two “domains”. One, commonly referred to as the inner adherent mucilage, that is tightly bound to the columella of each epidermal cell through cellulose fibrils (“rays”) ([Vaughan & WHITEHOUSE, 1971](#); [Willats et al., 2001](#); [Harpaz-Saad et al., 2011](#); [Mendu et al., 2011](#); [Sullivan et al., 2011](#)) and an outer non-adherent (loosely arranged) and highly hydrophilic layer ([Western et al., 2000](#); [Arsovski et al., 2009](#)). Biochemical analysis of the Arabidopsis mucilage showed that the outer mucilage largely consists of unbranched rhamnogalacturonan I (RG I) backbone and more than 80-90% sugars made from galacturonic acid (GalA) and rhamnose (Rha) ([A. Macquet et al., 2007](#); [Naran et al., 2008](#); [Arsovski et al., 2009](#); [Voiniciuc, Schmidt, et al., 2015](#); [Dean et al., 2017](#)). Similar to the outer mucilage, the inner mucilage predominantly contains unbranched RG I, with however the presence of branched RG I, and sugars such as arabinans, galactans, arabinoxylans, and arabinogalactans showed increased quantity in outer mucilage compared to inner mucilage ([A. Macquet et al., 2007](#); [Huang et al., 2011](#); [Walker et al., 2011](#); [Dean et al., 2017](#)).

Nanda et al. (2019) study reported that the removal of the non-adherent mucilage in *erf* mutant seeds partially alleviates much of their hypersensitivity to salt during germination, indicating that the maternal *oi2* epidermis of the seed coat plays an important, but not exclusive, role in the *ERF*-mediated salt sensitive germination phenotype. There appeared to be no genetic difference in the amount of mucilage excreted under salinity, with a similar increase compared to seeds imbibed in non-saline media. However, biochemical analysis of the composition of the non-adherent mucilage revealed compositional changes between the *erf* salt-hypersensitive mutants and WT, notably elevated uronic acids concentrations and decreased xylose/rhamnose, suggesting altered pectin branching and increased potential sites for ion (Ca^{+2} , Na^{+}) binding. It was proposed that the *ERECTA* family plays a role in the synthesis, deposition and architecture of the in particular through affecting pectin cross-linking, and thereby affecting the biophysical properties of the mucilage in an environment ionic content-dependent manner. However, many questions remain: How do the *ERF* influence the maternal

tissues and their salinity-dependent roles during germination? What is the *ERf* molecular pathway involved? And is this novel Arabidopsis *ERECTA* family function in seed germination conserved across species, especially among crops?

1.3 Functional conservation of *ERECTA* gene family across crop species; a focus on cereals

Extensive studies of the Arabidopsis *ERECTA* family have been done over the past decades, but although *ERECTA* orthologues are found across the plant kingdom the functional characterisation of *ERECTA* family genes in species than Arabidopsis is very limited, thus restricting their potential use for improving desirable agronomical traits. Phylogenetic studies show that *ERECTA* family genes are highly conserved across agronomically important crops, including cereals such as rice, wheat and barley in terms of copy number and gene structure ([Huang, 2013](#); [Shen et al., 2015](#); [Zakhrabekova et al., 2015](#); [Y. Zhang et al., 2018](#)). Rice *ERECTA* genes have similar spatial expression patterns as Arabidopsis ([Y. C. Zhang et al., 2018](#)) and knocking-out (KO) of *OsER* restricted stem elongation and the size of the inflorescence ([Y. C. Zhang et al., 2018](#)). Consistent with similar observations in barley *ER* KO (*ert-m*) ([Zakhrabekova et al., 2015](#)), and tomato ([Zhao et al., 2023](#)), overexpression of *ER* in rice and tomato was shown to increase heat tolerance ([Qi et al., 2004](#); [Shen et al., 2015](#)), and knock-out of a putative wheat *ER* orthologue indicated a role of that gene in promoting the tolerance of abiotic stresses such as drought, salinity and temperature ([Huang, 2013](#)). Consistently, over-expression of a putative wheat *ER* ligand of the EPF family was reported to have a positive effect on water use efficiency via a reduced number of stomata ([Dunn et al., 2019](#)). Overexpression of maize *ER* orthologue increased drought tolerance, growth rate and organ size ([Guo M, 2011](#)) ([Zakhrabekova et al., 2015](#); [Zhao et al., 2023](#)). Truncation of tomato *ERECTA* protein induced increased stomatal density, reduced leaf surface area, reduced number of leaves, and shift in flowering time, and increased tolerance to water stress ([Villagarcia et al., 2012a](#)). Moreover, a recent study in tomatoes reported a constitutive role of *SIER* in promoting seed germination, thus extending our earlier discovery of Arabidopsis *ERECTA* family function in the regulation of germination ([Nanda et al., 2019](#)) to another species, and suggested that the action of *SIER* was mediated via auxin signalling in interaction with ABA ([Chen et al., 2024](#)). Altogether, these studies suggest some conservation of *ERECTA* functions across diverse crop species, and of potentially great interest for breeding programs targeting important agronomical traits such as yield, flowering time, germination, and tolerance of environmental stresses such as water and temperature stress. However, the above studies all focused on the *ERECTA* member, none on the family as a whole. And our recent discovery of the Arabidopsis *ERf* function in the regulation of seed

germination under salinity and osmotic stress ([Nanda et al., 2019](#)) is yet to be investigated in other species with different seed types, especially crop species.

1.4 Thesis aims

My thesis follows from earlier work in the Masle lab that established that the *ERECTA* gene family was active in Arabidopsis seeds, and acted to control the timing and progression of germination in an environment-conditional manner ([Nanda et al., 2019](#)). Reduction of *ERf* signalling delayed germination or even halted it under salinity or drought-induced osmotic stress and kept the seed viable until favourable environmental conditions returned, then allowing resumption of the germination process. That function was shown to be primarily maternally controlled, through unidentified properties of the maternal tissues, primarily the seed coat ([Nanda et al., 2019](#)). I have followed two main research directions in this thesis:

1.4.1 AIM 1- Investigate the maternal inheritance of the *ERf*-mediated salt-hypersensitive germination phenotype

Aim 1. a. *Probe the role of a putative novel transcription factor in the *ERf*-mediated control of seed germination*

In the course of earlier experiments in the Masle lab, it was noticed that seeds of the glabrous *er 105 gl1* mutant tended to germinate somewhat more slowly than its *er105 GL1 counterparts*, while no such trend was apparent for single mutant *gl1* seeds compared to WT. *GLABRA 1 (GL1)* is an R2R3 MYB transcription factor required for the initiation of trichomes in interaction with bHLH (basic Helix-Loop-Helix) *GL3* or *ENHANCER OF GLABRA3 (EGL3)*, and the WD40-repeat protein *TRANSPARENT TESTA GLABRA 1 (TTG1)*, forming a regulatory complex that activates the transcription of target genes essential for trichome development. Studies have shown that mutations in *GL1* result in a significant reduction or complete absence of trichomes ([Herman & Marks, 1989](#); [Oppenheimer et al., 1991](#); [Larkin et al., 1994b](#)). The role of MYB transcription factors in trichome development is conserved across various plant species, highlighting the evolutionary significance of *GL1* ([Arteaga et al., 2021](#)); (reviewed in ([Han et al., 2022](#))); ([Kabir et al., 2024](#)). *GL1* has also been implicated in the regulation of plant senescence ([Eckstein et al., 2019](#)), and together with *GL1*, *GL3* and *TTG1*, in leaf cuticle development and systemic acquired resistance (SAR) in Arabidopsis ([Xia et al., 2010](#)). Gibberellic acid (GA) and cytokinin upregulate *GL1* expression and trichome formation in Arabidopsis ([Perazza et al., 1998](#); [Gan](#)

[et al., 2006](#); [Gan et al., 2007](#); [Maes et al., 2008](#)); (reviewed in ([J. X. Li et al., 2021](#))). Although extensive research has been done on *TTG1* on its role in seed coat development and mucilage synthesis ([Koornneef, 1981](#); [Koornneef, 1982](#); [Western et al., 2001](#)), to date, *GL1* is only known to act in aerial organs and primarily in the specification of trichomes. The above serendipitous observations led us to speculate that *GL1* might be involved in the regulation of seed germination, possibly in interaction with the *ERECTA* family. In the first chapter of this thesis, I tested that hypothesis.

Aim 1. b. Probe the respective contributions of the seed coat and the endosperm in the ERF-GL1 maternal control of germination

As described previously, the Arabidopsis seed coat is a completely maternal diploid tissue, and the endosperm is mostly of maternal origin also, with two copies of the maternal genome against only one copy of the paternal genome. The endosperm is a living tissue in direct contact with the seed coat -its only separation of the environment and intense communication between endosperm, seed coat and embryo, takes place to orchestrate germination. In this thesis, I used a genetic approach to test the contribution of the endosperm to the *ERf* function in seed germination, taking advantage of the epidermal-specific *ATML1* promoter to restore the expression of *AtER* in *erf* mutant backgrounds, only in cells with epidermal identity ([P. Lu et al., 1996](#); [Sessions et al., 1999](#)). *ATML1*, an HD-ZIP class IV transcription factor in Arabidopsis, plays a crucial role in epidermal cell identity and development (Reviewed in ([Verma et al., 2022](#))). I obtained results that unambiguously establish that *ER* expression in the seed epidermis is sufficient to recapitulate WT *maximum* germination percentage under salt stress, and to also fully recapitulate WT germination *kinetic*, *apart in* or nearly so,

Aim 1. c. Probe the implication of the ERF and GL1 in seed coat development and differentiation during seed embryogenesis on the mother plant

Nanda et al. (2019) suggested a model in which the *ERf* proteins influence the swelling, architecture, and permeability properties of the seed coat mucilage and cell walls, and their biophysical modifications during imbibition and germination in a saline environment. This is expected to, in turn, modify the biochemical and mechanical stimuli perceived by the underlying endosperm and embryo ([Nanda et al., 2019](#)). Seed coat properties of mature seeds, such as cell number, cell wall thickness and cell dimensions, are determined during seed development on the mother plant. I used a range of microscopy techniques to investigate changes in the anatomy, morphology and surface characteristics, of the seed coat, associated with loss of *ERf* or/and *GL1* functions, focusing on both the mature seed

coat and the developing seed coat, during the initial morphogenetic phase of seed development when seed size, and seed coat patterning are determined, and the differentiation of the outer epidermal layer and its specialised mucilage secreting cells is initiated. Using a range of microscopic techniques, I compared across a developmental series, *erf* and WT seeds in a *GL1* or *gl1* background, as well as *erf* seeds expressing the *ERECTA* gene under the control of the epidermal-specific ATML1 promoter. I identify functions of the *ERf* on cell proliferation, cell expansion and growth anisotropy, secondary cell wall thickening, columella morphology, and surface characteristics, which involve the three *ERf* members, in a non-redundant manner and some interactions with *GL1*. Remarkably, complementation with *ERg* only, and only in the epidermis abolished most of the *erf/gl1* induced developmental effects. In addition, I uncovered a novel role of the *ERf* and *GL1* in regulating the endosperm-associated cuticle deposition.

1.4.2 AIM 2- Investigate the *ERf*- and *GL1*-mediated changes in transcriptional networks during early seed development

The *ERECTA* LRR-RK are among the best characterised plant LRR-RKs among the more than 200 present in Arabidopsis, which constitutes the largest subfamily of membrane-bound cell-surface receptors with an intra-cellular kinase domain (RLKs) that plant use to perceive and transduce external cues to tune cellular responses to constantly changing conditions ([Torii et al., 1996](#); [Lease et al., 2001](#); [Shpak et al., 2004](#); [Kosentka et al., 2017](#)). As master regulators of multiple aspects of plant development, as diverse as plant architecture, floral development, stomatal patterning or vascular differentiation, the *ERECTA* kinases are capable of perceiving multiple ligands, some shared, some unique ([Hara et al., 2009](#); [Kondo et al., 2010](#); [Lee et al., 2012](#)); reviewed in ([Torii, 2012](#); [Shpak, 2013](#)). All ligands identified to date are small peptides of the EPIDERMAL PATTERNING FACTOR (EPFs), or EPF-Like family (EPFLs). Ligand binding triggers downstream signalling, involving co-receptors such as TMM for the regulation of stomatal patterning, and SOMATIC EMBRYOGENESIS KINASES (SERKs), among which SRK4 or SERK3/BAK1 (Brassinosteroid Associated Kinase). Seemingly shared immediate downstream components across *ERECTA* signalling pathways in several aspects of plant development, and even immune responses include a cascade of mitogen-activated protein kinases, MEKKK YODA-MKK4/MLL5-MPK3/MPK6- as well as the BSK1/2 BR-Signalling Kinases ([Bergmann et al., 2004](#); [Wang et al., 2007](#); [Meng et al., 2012](#); [Meng et al., 2015](#)).

However, all these studies relate to aerial organs, during vegetative and floral development, none to a developmental phase change involving massive reprogramming such as seed germination, furthermore an adaptative largely maternally inherited function.

Next-generation and third-generation sequencing are emerging technologies that allow genome-wide investigations of transcriptional network dynamics in response to exogenous or endogenous cues. The transcriptome is a complete set of RNA molecules, including messenger RNA (mRNA), non-coding RNA, and other RNA species produced by the cell in an organism or a specific tissue. RNA Seq allows us to identify and quantify gene transcripts at a given developmental stage in a particular organ or tissue, under specific conditions. Given the total lack of information on the *ERECTA* family and *GL1*-mediated molecular pathway in seed germination, I used RNAseq as a first step, non-biased genome-wide approach to address that question. Based on the results presented in the previous Chapter (Chapter 2), I carried out an RNAseq analysis of developing seeds and enclosing siliques of wild type *Arabidopsis* and the *erf/gl1* mutants described in Chapter 2, across a time series covering the initial 6 days of seed development, when seed coat expansion and patterning are determined, and the epidermal cells start to differentiate into specialised mucilage secretory cells. My aim with a time series starting as soon as flower opening and pollination, and including 5 subsequent time points was to detect potential primary molecular effectors of the *ERf* and *GL1* controls on the distinctive constitutive properties of the seed coat and endosperm-associated cuticle, identified in Chapter 2.

1.4.3 AIM 3. From *Arabidopsis* to crops: Is the function of the *Arabidopsis ERECTA/ERECTA-like* and *GL1* genes in seed germination conserved in barley (*Hordeum vulgare*)?

Arabidopsis is a very powerful model system for laboratory experiments and represents only one of the numerous types of seeds produced by plants. My aim in this Chapter was to examine whether the critical functions of the *ERf* and *GL1* pathways in seed development and germination were conserved in other species.

Barley (*Hordeum vulgare* L.) belongs to the genus *Hordeum* and family Poaceae, known for its remarkable phenotypic and genetic diversity resulting from a long history of domestication (reviewed in ([Pourkheirandish & Komatsuda, 2007](#))). As the fourth-ranking cereal crop globally, barley is primarily utilised for animal feed and the brewing industry and as a staple crop mostly in Asian countries ([Sakellariou & Mylona, 2020](#)). Barley is known for its adaptability to various environmental conditions and is a rich source of starch, protein, and dietary fibres such as β -glucan ([Czuchajowska, 1998](#); [Izydorczyk, 2000](#)). The absence of maternally derived hull significantly increases the nutrient content in the seed ([Pejcz et al., 2017](#); [Sterna et al., 2017](#)). Barley, being a rich fibre source, particularly β -glucan, offers significant health benefits, including reduced risks of heart disease, diabetes, and hypertension ([Maheshwari et al., 2019](#)); reviewed in ([Tosh & Bordenave, 2020](#)).

Similar to other cereals, the barley grain is a caryopsis; at maturity, the seed coat (testa) and the pericarp are tightly fused. Unlike Arabidopsis, barley seeds have a large starchy endosperm that occupies two-thirds of the seed space, and the embryo consists of the embryo axis and the scutellum transfer tissue. The embryo axis consists of the shoot and root primordia, covered by coleoptile and coleorhiza, respectively. The aleurone layer, which is three-cell layers thick, covers the starchy endosperm, and thins to a single cell layer around the embryo, known as germ aleurone ([Bradbury, 1956](#); [Buttrose, 1963](#); [Sargent, 1980](#); [Gould FW, 1983](#); [Walbot, 1994](#)). In the barley caryopsis, the pericarp is tightly attached to the two-cell layer thick seed coat. At maturity, the lemma and palea (collectively known as the husk or hull) are tightly attached to the underlying pericarp by a cementing layer ([Harlan, 1920](#); [Gaines et al., 1985](#); [Evers & Millar, 2002](#); [Oikku et al., 2005](#); [Taketa et al., 2008](#)). Barley exhibits primary dormancy imposed by seed-covering tissues such as lemma, palea, pericarp, and testa, while secondary dormancy is induced by external environmental factors such as high moisture content ([Briggs, 1978](#)). Germination in barley begins with water uptake and is completed once the radicle protrudes from the covering tissues, through a two-step process ([Bewley, 1997](#)). The repair mechanisms for macromolecules commence shortly after water uptake ([Osborne et al., 1993](#)) involving the mobilisation of storage proteins and RNAs, reactivation and accumulation of growth regulators such as gibberellin, and initiation of protein biosynthesis ([Yamada, 1982](#); [Shewry et al., 1995](#); [Gallardo et al., 2001](#); [Vázquez-Ramos & de la Paz Sánchez, 2003](#)). This process is critical for the malting and brewing industry, where gibberellin transfer to the aleurone layer triggers enzyme production that breaks down starch into sugars. Thus, besides being crucial to biomass production and yield, timely germination is essential for maintaining brewing quality.

Barley is a high salinity tolerant plant due to successful breeding over time, although salinity tolerance varies among genotypes and growth stages ([Mano et al., 1996](#); [Mano & Takeda, 1997](#); [Munns, 2005](#); [Xue et al., 2009](#)). Barley hence serves as an excellent crop model to study germination and adaptative mechanisms to salinity in plants and grain cereals, the major sources of food worldwide ([Chen et al., 2007](#); [Wu et al., 2011](#); [Gorzolka et al., 2016](#)).

The barley *ERECTA* gene family has not been characterised. Phylogenetic studies indicate the presence of three *ER/ER*-like genes in the barley genome as in other cereals and many other species. A putative Arabidopsis *ER* orthologue, *HvER*, maps to chromosome 7 ([Zakhrabekova et al., 2015](#)). The morphology of an *HvER* mutant (*ert-m*) indicates a role in the regulation of plant stature and inflorescence rachis morphology ([Zakhrabekova et al., 2015](#)).

Arabidopsis *GL1* had no established orthologue in barley. Using sequence homology search, phylogenetic and synteny analysis, a putative unannotated candidate was chosen among the numerous incomplete sequences with the highest homology to *AtGL1*. I then used CRISPR/cas9 gene

editing to generate a range of mutant lines carrying knock-out *er/erl* and *gl1* mutations, separately and in various combinations. Successfully edited homozygous progeny were obtained, and physiologically characterised. The results demonstrate a crucial function of the *HvERf* family on seed germination, and seed development, but with also some specificity and also, excitingly, of the candidate putative *HvGL1* orthologue. They give readily available tools to breeders for barley genetic improvement and open novel, exciting avenues of research on the development and biology of the barley and cereal caryopses.

The next RESULTS section of this thesis is structured in three Chapters:

Chapter 2- Identification of the *GL1* Transcription Factor (TF) as an interacting partner of the *ERf* in the control of seed germination.

Chapter 3- The *ERf-GL1* regulatory molecular network during early seed development.

Chapter 4- *ERECTA* as a key target for the genetic improvement of BARLEY (*Hordeum vulgare*) seed germination under changing salinity.

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Chapter 2

Identification of the *GL1* Transcription Factor (TF) as an interacting partner of the *ERf* in the control of seed germination

2.1 Introduction

Germination begins with water uptake for seed imbibition and activation of embryonic cell expansion, and is completed with visible radicle protrusion through the seed coat and the endosperm ([Bewley et al., 1994](#); [Bewley, 1997](#)). The seed coat, or testa, is the direct interface between the environment and the live endosperm and embryo inner seed compartments. The Arabidopsis testa originates from the ovule two integuments, which upon fertilisation undergo active cell proliferation and expansion for 3-4 days, followed by complex differentiation in parallel to endosperm development and embryo expansion within ([Beeckman et al., 2000](#); [Western et al., 2000](#)). The outer integument is made of two layers, oi2 and oi1, for the most external one, and oi1 and the inner integument of also two layers at the seed chalazal and micropylar end, ii2 and ii1, but a third one at the seed distal end, ii1', formed from the periclinal division of ii1 post-fertilisation ([Beeckman et al., 2000](#); [Haughn & Chaudhury, 2005](#); [Loubery et al., 2018](#)). The micropyle is a micro-pore which accommodates the -passage of the pollen tube through the integuments of the mature ovule for pollen delivery to the germinal cells, while the chalazal cells are connective cells between the silique wall vasculature and the proximal end of the. At maturity, the seed coat integuments are highly differentiated layers, with specific properties ([Beeckman et al., 2000](#); [Western et al., 2000](#)) and roles during germination ([Debeaujon & Koornneef, 2000](#); [Finch-Savage & Leubner-Metzger, 2006](#); [Debeaujon et al., 2007](#)) (Figure 1-1, Chapter 1), as a result of structural and biochemical modifications during seed development and maturation.

Previous work of Masle uncovered that the *ERf* pathway, so far known for its role in multiple aspects of aerial plant development, regulates seed germination in an environment-conditional manner ([Nanda et al., 2019](#)). While being neutral under optimal conditions, reduced *ERf* signalling considerably delayed germination under salinity and hyperosmotic stress through, remarkably, a reversible process, moreover primarily under maternal control. The underlying mechanisms remained mostly unknown, except for a clear role of the gelatinous capsule of pectin-rich mucilage which, upon contact with the soil solution, immediately hydrates and expands, rupturing the enclosing cell outer primary anticlinal wall on each side of the columella, and diffusing around the seed, loosely connected to the its epidermis by an array of cellulose microfibrils. Analysis of the outer water soluble expelled from WT and *er erl1/seg erl2* triple mutant seeds indicated differences in xylose to rhamnose ratio, suggesting differences in pectin branching and cross-linking, and of the strength of mucilage attachment to the

seed coat ([Nanda et al., 2019](#)). In addition, the salt hypersensitive *erf* mutants, *er erf1*, *er erf2* and triple mutant were all characterised by smaller seeds, with thinner coats, indicating an overlap between the *ERf* functions in seed germination and determination of seed size, and showed modifications of seed shape. Altogether, these observations suggested an implication of the *ERf* in seed coat development and differentiation during seed development and maturation on the mother plant. The first aim of the experiments reported in this chapter was to probe this in more detail.

A second aim was to test the possibility that the maternal control of the *ERf* function in seed germination might also involve some direct role of the endosperm, a tissue carrying two copies of the maternal genome against only one copy of the paternal genome. Although reduced to only a one-cell thin layer, the residual endosperm layer constitutes the only live tissue of principally maternal origin in the *Arabidopsis* seed, and germination involves an intense inter-signalling between seed coat, endosperm and inner embryo. Hence, a second aim here, is to determine whether the primarily maternally controlled hypersensitivity of seed germination to salinity in mutants with reduced *ERf* signalling was affected by the seed coat alone or by both the seed coat and underlying endosperm. Using a genetic approach to separate between the two possibilities, I obtained results that unambiguously validate the first hypothesis: Complementation of salinity hypersensitive *erf* mutants by the native *ERg* under the control of the epidermal specific *ATML1* promoter recapitulated the germination phenotype of WT seeds.

Earlier observations in the Lab indicated a trend for seeds of the original glabrous *er105* knock-out mutant, *er105 gl1*, to germinate somewhat more slowly or more unevenly under water stress than seeds of the trichome bearing counterpart and extensively studied *er105 GL1*, while no such trend was apparent for single mutant *gl1* seeds. *gl1* is a null allele of *GLABRA1 (GL1)*, an R2R3-MYB transcription factor whose only documented function is in the induction of trichome development in aerial organs ([Herman & Marks, 1989](#); [Oppenheimer et al., 1991](#); [Larkin et al., 1994a](#)). However, our serendipitous observation with *er105 gl1* seeds led us to probe the possibility that *GL1* might play a role in seeds too, interact with the *ERf* in the regulation of seed germination. The third aim of the experiments reported in this Chapter was to directly investigate that hypothesis. The results reveal and unambiguously establish a role of *GL1* in both seed development and seed germination, in interaction with the *ERf*.

2.2 Material and Method

2.2.1 Plant materials

The *Arabidopsis* ecotype Col (Col-0, CS1093) was used as the wild-type (WT). The *ERECTA er105 gl1* loss-of-function mutant has been extensively used and described in the literature ([Torii et al., 1996](#); [Masle et al., 2005](#); [Bundy et al., 2012](#)). The *other single* mutant seeds used in this study were originally sourced from the SALK collection and were validated as previously described in Nanda et al. (2019). Two independent loss-of-function alleles were included, for each of *ER* (At2g26330), *ERL1* (At5g62230), *ERL2* (At5g07180) and *GL1* (At3g27920), namely: the *er105 gl1*, *erl1-2*, *erl2-1* alleles previously described (Torii et al., 1996; Shpak et al., 2004; Masle et al., 2005; Bundy et al., 2012; Bemis et al., 2013) and here coded *er gl1*, *erl1* and *erl2* for simplicity; and the *er2* (C3401) also previously described (Rédei, 1992; Lease et al., 2001; Masle et al., 2005; Hall et al., 2007), *erl1-5* (SALK_019567) and *erl2-2* (SALK_015275C) alleles; and *gl1-4* (SALK_039478C) alleles. The *er105* mutant (carrying *GL1*) and the *gl1* single mutant were obtained from the segregating progeny of the original *er105 gl1* mutant (Torii et al., 1996). The SALK lines were acquired from NASC (Alonso et al., 2003). The absence of residual target gene expression and the t-DNA insertion sites were verified before use (insertions located at 1516 bp from gene Start codon in *gl1-4*), Nanda et al. 2019. Double and triple *erf* mutants carrying *er105*, *erl1-2*, *erl2-1*; *erl2-2*, in both a *gl1* or *GL1* background were obtained through crosses. As the *er105 erl1-2 erl2-1* triple mutant is sterile, the segregating progeny of *er105 erl1-2/+ erl2-1* plants was used and is referred to in text and figures as *er erl1/seg erl2*. Seeds were propagated together at 21°C, 16 hours day length and a light intensity of 120-130 $\mu\text{mol m}^{-2}\text{s}^{-1}$ to obtain several fresh batches of seeds of the two sets of *erf GL1* and *erf gl1* mutants, from plants grown together, and then collected and stored together at 21°C room temperature. All seeds were harvested on the same day for all lines and then stored in a silica gel box at room temperature. The WT and *erf* mutant lines carrying the various combinations of *erf* mutations, *er105*, *erl1-2*, *erl2-1*, *er105 erl1-2*, *er105 erl2-1*, *erl1-2 erl2-1* and *er105 erl1-2/+ erl2-1*, *+/-GL1* were included in this study. Hereafter, they will be referred to as *er*, *erl1*, *erl2*, *er erl1*, *er erl2*, and *er erl1/seg erl2 +/-GL1* seeds for easy documentation.

2.2.2 Generation of transgenic expressing *AtML1 pro: ERg* in *erf* mutants carrying functional *GL1* or *gl1* null allele

Seeds expressing *AtML1: ERg: FLAG* in *er105 gl1* mutant background were obtained from the Uchida Lab, Tokyo, ([Uchida et al., 2012](#)) and propagated, with some flowers also being used as female parent for pollination with *ERf GL1*, *ERf gl1*, *erf GL1* and *erf gl1* pollen to generate progeny expressing the

transgene in either *erf GL1* or *erf gl1* backgrounds. F1 and F2 seeds collected from plants grown together, and stored together, were phenotypically characterised and screened by RT-PCR to verify the presence and effective expression of the transgene. Leaves from three-week-old seedlings were collected, snap-frozen in liquid nitrogen and ground using a Tissue Lyzer II (Quiagen). Total RNA was extracted using Qiagen mini-RNeasy kit according to the manufacturer's instructions along with DNase treatment to eliminate genomic DNA traces. Quantification of total RNA and total protein concentrations was performed with a Qubit4 fluorometer. 4µm of total RNA were used to obtain purified mRNA using TYGR Dynabeads^R oligo dT₂₅, and subsequently cDNA using 200u of M-MLV (Promega).

Primers:

ERg 6736f- CTTACGTGATGGGCACGATAGGT -forward,

FLAG rc- CTTGTCATCGTCATCCTTG TAGTC-reverse,

were used ([Uchida et al., 2012](#)) for the quantification of transgene expression. Transcript abundance was normalised using the geometric mean of transcript levels of four reference/calibrating genes (*APT1*, *At1g27450*; *PDF2*, *At1g13320*; *IP41*, *At4g34270*; and *AtbHLH* – *At4g38070*) and the Δ^{-Ct} calculation method ([Livak & Schmittgen, 2001](#)).

2.2.3 Germination assays

Mature seeds were collected and stored together for the same length of time after harvest, a minimum of one month, to release dormancy. Seeds were surface-sterilised using a 1:9 solution of 1.25% sodium hypochlorite:100% ethanol, rinsed twice with 100% ethanol and left to air dry before being plated on modified Hoagland's media (2 mM KNO₃, 5 mM Ca[NO₃]₂ .4H₂O, 2 mM MgSO₄ .7H₂O, 2 mM KH₂PO₄, 0.09 mM Fe-EDTA and micronutrients), at pH 5.8, with 0.7% Type M Agar (Sigma-Aldrich A4800) containing 0mM or 150mM NaCl. Seeds were stratified at 4°C, in the dark for two days, directly on plates, before being transferred to a growth chamber and exposed to continuous light of 100-115 µmol m⁻² s⁻¹ intensity and a constant temperature of 21°C. Some experiments included seeds on medium supplemented with 0µM, 1µM and 2µM ABA. The two germination steps, testa and endosperm rupture, were monitored for each individually, through daily observation under a binocular microscope, at 3h intervals until all the seeds completed germination on a control medium (0mM NaCl), and then 3 times to once daily for 150mM NaCl or ABA-treated plates, until no change in germination scores was observed. Data were plotted as percentages of seeds having testa or endosperm rupture as a function of incubation time post-stratification, or as T₅₀ values, corresponding

to the times (h post-stratification) when seeds reached 50% of testa or endosperm rupture ([Bewley & Black, 2013](#); [Nanda et al., 2019](#)).

2.2.4 Mature seed coat imaging

Desiccated mature dry seeds of *erf* mutants +/-*GL1* were used for this analysis. Seeds were maintained flat on a slide lined with translucent sticky tape, in a standardised position across all lines. The seeds were imaged at three dorsal locations, i.e., at the chalazal and micropyle ends, and mid-way between the two, using a Leica TCS-SP8 confocal microscope, with the following settings: gain `600, smart offset 0.1%, format 1024X1024 pixels, excitation at 405,458 and 514nm and emission acquisition window 593-681nm wave-lengths. Images were processed using the Fiji Image J software. Measurements were extracted from images captured from 6-8 seeds, and with independent seed batches. Optical Z-stacks through the seed coat (depth 28-30µm) were obtained using a Zeiss 780 confocal microscope at the Centre of Advanced Microscopy (CAM), ANU, with the same settings as above and autofluorescence spectra were extracted according to the Zeiss 780 demo manual.

2.2.5 Mucilage extraction and analysis

Mucilage extraction and analysis were carried out as described by Nanda et al.,2019. Mucilage extraction was performed on aliquots of 40 mg dry seeds. Each aliquot (n=4 per genotype per experiment) was suspended in 1ml Milli-Q water, followed by shaking at 500 rpm for 24 h at 4°C, vortex for 5 s, and centrifugation at 8000 g for 3 mins. 600 µl supernatant was recovered. Seeds were rinsed twice with 200 µl water, and 200 µl supernatant was recovered, each time after vigorous vortexing and centrifugation. The pooled supernatants (1ml total volume) was snap-frozen in liquid nitrogen and immediately lyophilised. The mucilage thus recovered was weighed on a 10⁻⁶ g precision balance. Given the observed genetic variation in seed size (see Text), sub-aliquots of a known number of seeds (at least 500) were weighed, imaged at high resolution and analysed for size with ImageJ before mucilage extraction, allowing for comparison of mucilage amounts per unit seed dry weight, and also average amount per individual seed in relation to seed size. The reduction of uronic acid methyl-esters and free uronic acids in the extracted mucilage were carried out following established protocols ([Kim & Carpita, 1992](#); [Pettolino et al., 2012](#)). The reduced polysaccharides were then hydrolysed, reduced, acetylated and subjected to GC/MS analysis as described ([Peng et al., 2000](#)).

2.2.6 Whole-mount imaging of Arabidopsis developing seeds using fluorescence microscopy

A fresh batch of plants was grown under the same conditions as above, and the flower pistils were marked daily using water-soluble ink, from flower opening (0 DAFO) to 20 days later (20 DAFO) i.e., the transition between the completion of seed morphogenesis and seed maturation phases. Ten developing siliques were collected daily over that 20-day period, slit open halfway and stored in 50% methanol, and 10% acetic acid overnight in the fridge and for a longer time (6-12 months) in this solution at 4°C, in the dark ([Truernit et al., 2008](#)). Once well cleared and ready to image, the siliques were carefully placed in a small petri plate and covered with a 1% SDS, 0.2 N NaOH solution overnight at room temperature, according to ([Hedhly et al., 2018](#)). They were then carefully and thoroughly rinsed in water and placed in a small new Petri plate with a 1:5 dilution of 12% commercial bleach, for 1-3 minutes depending on the developmental stage of the seed. The samples were then thoroughly washed to eliminate any trace of bleach and stained with 0.1% calcofluor white for 1h in the dark at 4°C, then rinsed for 5-10min, and stained with 0.001% Auramine O for 30min. The samples were then finally washed in 1M phosphate buffer for 1 min. Using a blunt-end needle, the siliques were carefully fully opened under a binocular microscope, and the seeds were placed on a slide under a coverslip with 1:1 glycerol: phosphate buffer. The seeds were imaged using a Leica DM5500B microscope., fitted with a Leica DFC310 FX camera. Images were processed using Image J (n=5 siliques, for each of the two biological replicates).

2.3 Results

2.3.1 Loss of function of *GL1* enhances the *ERf*-mediated seed germination sensitivity to salinity, in an *erf* mutation combination-dependent manner.

Although *GL1* has not previously been implicated in seeds nor reported to be expressed there ([Larkin et al., 1993](#)); <https://www.arabidopsis.org/servlets/TairObject?id=37734&type=locus>), qPCR analysis detected *GL1* transcripts in dry seeds, at very low abundance, and showed a strong induction of *GL1* expression during seed imbibition. *GL1* expression was also detected during the next two germination phases (Figure 2-1 A), but at much lower levels during the first phase leading to testa rupture, before a strong induction during the subsequent phase leading to endosperm rupture- the completion of germination. Remarkably, past imbibition, *GL1* expression was strongly downregulated in the presence of NaCl (Figure 2-1 A). These data indicate that *GL1* transcripts are present in the seed and that their abundance is regulated, somehow.

In order to assess their role in germination, *gl1*, *er erf1 gl1*, *er erf2 gl1* seeds and *er erf1/seg erf2 gl1* were compared to counterparts seeds with functional *GL1*, on 0mM or 150 mM NaCl. On 0 mM NaCl, *gl1* alone remained germination-neutral in WT, *erf1*, *erf2* and *erf1erf2* backgrounds, but caused small yet significant shifts in reaching the 50% and maximum % of endosperm and testa rupture in the *er erf1* and *er erf2* backgrounds, in opposite directions (advance and delay, respectively; (Figure 2-1 B&C). Although small in absolute value, these shifts were repeatedly observed, in several independent experiments such as shown (Figure 2-2 A&B. In stark contrast, in the presence of 150mM NaCl, *gl1* significantly enhanced the salt hypersensitivity of *er* seeds, and even more so that of *er erf1*, *er erf2* and *er erf1/seg erf2* seeds, to the point of precluding completion of germination in nearly all seeds of the latter two mutants (Figure 2-1 D&E; Figure 2-2 C-F). To unequivocally confirm that the differences in germination kinetics between *erf* mutants in WT or *gl1* background were effectively caused by the *gl1* mutation, *er erf1/seg erf2* was crossed with *gl1-4*, a T-DNA line containing an independent *GL1* knock-out insertion. Similar results were obtained for that independent *gl1-4* allele (Figure 2-3 A &B).

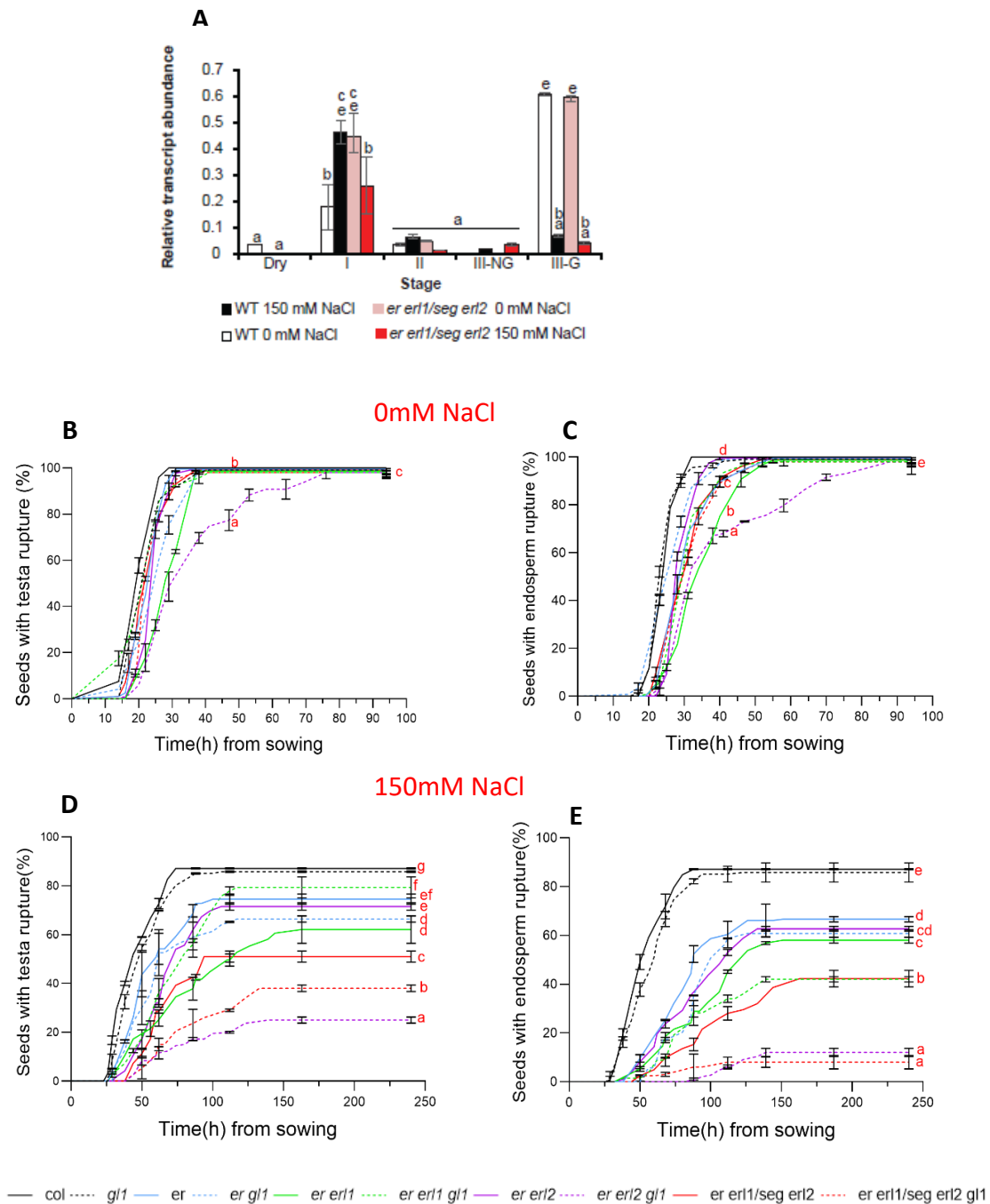


Figure 2-1: *GL1* interacts with the *ERf* in a combination dependent manner to reduce seed germination speed. **A.** *GL1* gene expression in WT dry seeds (“Dry”) and germinating seeds during the three germination phases. $n = 4$ independent seed pools per genotype and media, of 300 seeds each. Different letters indicate significant differences by two-way ANOVA and pair-wise test ($P < 0.001$). **B-E.** Percentage maximum testa and endosperm rupture of *ERf GL1* (Col), *ERf gl1* (*gl1*), *erf GL1* (*er*, *er erl1*, *er erl2*, *er erl1/seg erl2*) and *erf gl1* (*er gl1*, *er erl1 gl1*, *er erl2 gl1*, *er erl1/seg erl2 gl1*) seeds on NaCl-free media (**B and C**) and 150 mM NaCl media (**D and E**). Data are means ($\pm$ SE) $n = 3$ plates, 30 seeds per genotype in each plate; Experiment repeated 3 times. Statistically significant between germination percentage at a given time point between genotype are denoted by different letters by one-way ANOVA and Fisher’s LSD pairwise test ($P < 0.05$).

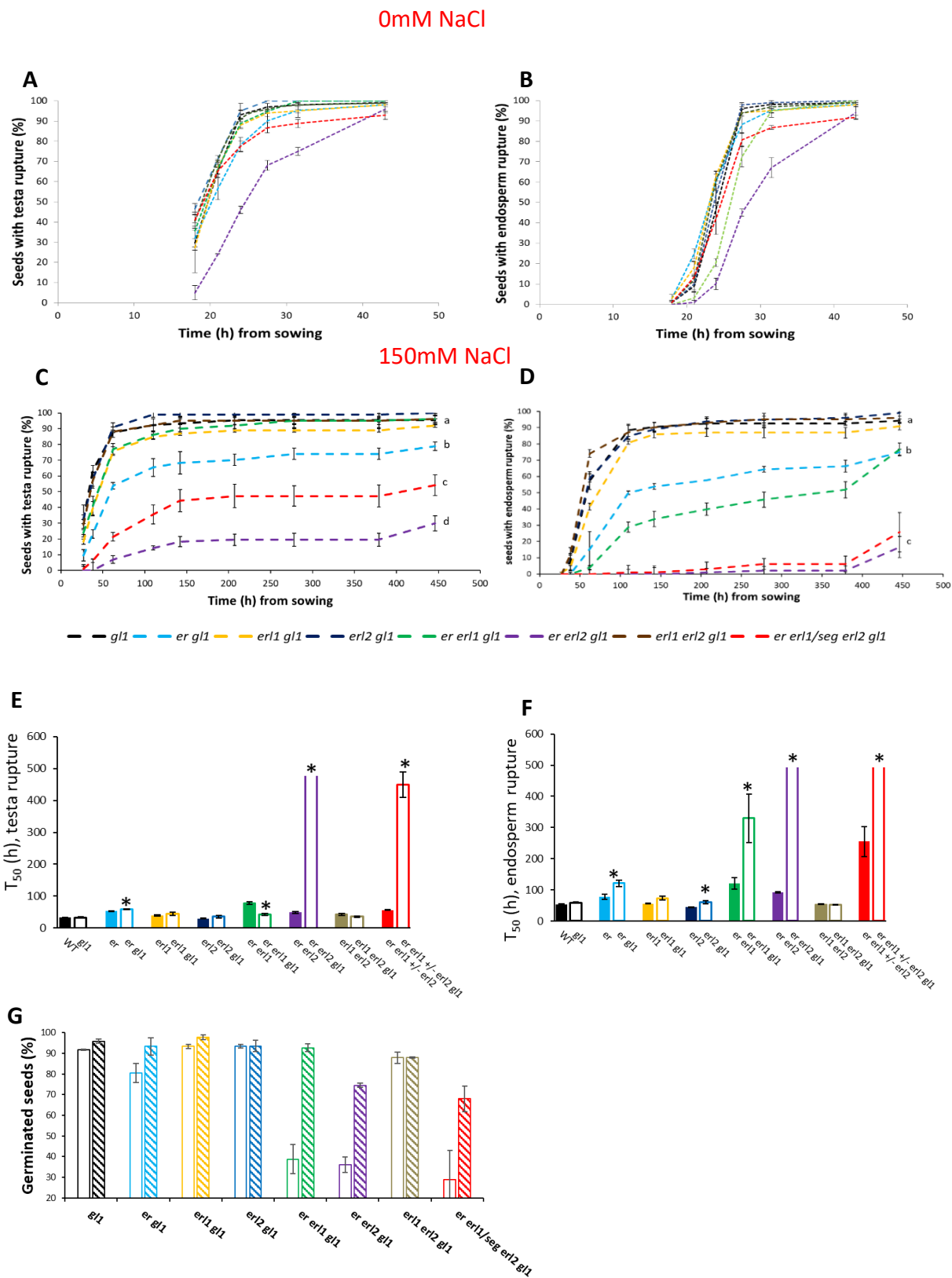


Figure 2-2: *GL1* interacts with the *ERf* in a combination-dependent manner to reduce seed germination speed- (second seed batch) A-D. Testa and endosperm rupture (%) of *gl1*, *er gl1*, *erl1 gl1*, *erl2 gl1*, *er erl1 gl1*, *er erl2 gl1*, *erl1 erl2 gl1*, *er erl1/seg erl2* seeds on NaCl-free media (A and B respectively) and 150 mM NaCl media (C and D respectively); Means and s.e.m. (n = 4 plates, 30 seeds per genotype in each plate; experiment repeated 3 times). Statistically significant between germination percentage at a given time point between genotype are denoted by different letters by one-way ANOVA and Fisher's LSD pairwise test (P < 0.05). **E&F.** T₅₀ (time taken to reach 50% testa and endosperm rupture) values (h post-stratification) on 150 mM NaCl media for testa rupture (E) and endosperm rupture (F), in WT and *erf* mutants carrying functional *GL1* (solid columns) or *gl1* (empty columns). Open bars with dashed contours denote undetermined T50 values, for mutants with still only 10-30% seeds with testa or endosperm rupture after 20 d incubation. Means and s.e.m. (n = 4 plates, 30 seeds per genotype per plate; experiment replicated 6 times). * Indicate significant differences by two-tailed paired t-tests for each genotype, comparing *gl1* vs *GL1* (P < 0.05). **G.** Proportions (%) of *gl1* and *erf gl1* seeds exhibiting endosperm rupture after 20 d incubation on 150 mM NaCl (empty bars) and 20 h later, once transferred to NaCl-free media (dashed bars).

In the *er erl1* background, *GL1* loss-of-function accelerated testa rupture but delayed the second germination step on saline medium (Figure 2-1 D&E and Figure 2-2 C-F). But in *er erl2 gl1* and *er erl1/seg erl2 gl1* seeds testa rupture was also dramatically delayed, or even prevented (Figure 2-1 D&E and Figure 2-2 C-F). This result highlights the specificity between *ERL1* and *ERL2* functional interactions with *GL1* in the control of seed germination sensitivity to salinity. Remarkably, however, the *erf gl1* seeds that failed to germinate even after an extended period of exposure to salinity still remained viable (Figure 2-2 G), as previously observed for *erf GL1* seeds ([Nanda et al., 2019](#)) and confirmed in the present experiments.

These data reveal a novel, unexpected function of the *GL1* transcription factor. They show that *GL1* is active in seeds, and synergistically interacts with the *ERf* to positively regulate germination under salinity stress. Furthermore, loss of *gl1* enhances the germination phenotypes induced by reduced *ERf* signalling, in an *erf* mutations-combination-dependent manner.

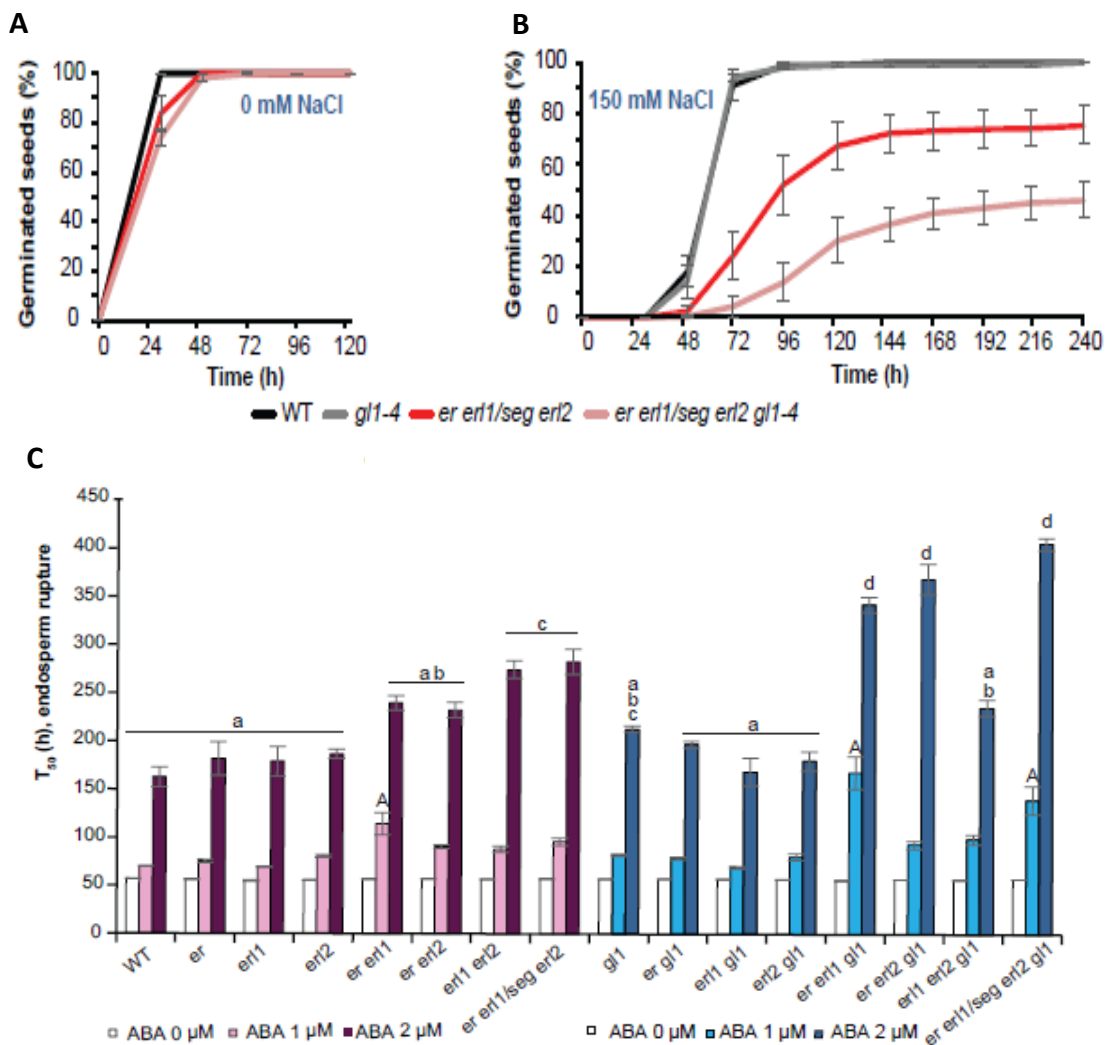


Figure 2-3: The *ERECTA* pathway intersects with a *GL1*-dependent pathway in regulating sensitivity to ABA during seed germination under salinity stress. A&B. Germination time-course of seeds carrying the *gl1-4* mutant allele in an *Erf* or triple mutant *er erl1/seg erl2* background under 0mM NaCl (A) and 150mM NaCl (B). C. Endosperm rupture T_{50} values for seeds carrying functional or non-functional *GL1* in 0mM NaCl media supplemented with 0, 1 or 2 μ M ABA. Means and s.e.m. ($n=4$ plates with 30 seeds per genotype per plate; experiment replicated 5 times). Different letters indicate significant differences between *GL1* and *gl1* seeds by two-way ANOVA and Tukey HSD pair-wise tests ($P < 0.001$), on media supplemented with 1 μ M ABA. Empty bars: 0 μ M ABA treatment; pale and deep purple bars: data obtained under 1 or 2 μ M ABA, respectively, for seeds carrying the native functional *GL1* allele; similarly, pale and deep blue bars represent data for the 1 and 2 μ M ABA treatments, respectively for *gl1* seeds.

2.3.2 Loss of *GL1* function enhances the sensitivity to ABA in salt-hypersensitive *ERf* mutants.

Seed germination is antagonistically regulated by the phytohormones GA and ABA ([Koornneef & Van der Veen, 1980](#); [Koornneef, 1982](#)). ABA is a potent seed germination inhibitor, well known to be upregulated by salinity and osmotic stress during germination ([Schopfer & Plachy, 1984](#); [Xu et al., 2011](#); [Yuan, 2011](#)). Earlier experiments ([Nanda et al., 2019](#)), showed that exogenous application of ABA caused a mild delay of testa rupture and a strong inhibition of endosperm rupture in the *er erl1*, *er erl2* double mutants and in the triple *er erl1/seg erl2* mutant, of different magnitude depending on the combination of *erf* mutations. In addition, the ABA-dependent *ABI3* and *ABI5* germination repressors ([Koornneef, 1982](#); [Giraudat et al., 1992](#); [Léon-Kloosterziel et al., 1996](#); [Finch-Savage & Leubner-Metzger, 2006](#); [Lefebvre et al., 2006](#); [Weitbrecht et al., 2011b](#)), and the interacting *RGL2* DELLA- protein-encoding gene were induced on saline media during imbibition and the first germination phase leading to testa rupture ([Nanda et al., 2019](#)). These differences in gene expression suggested that under salinity stress, loss of *ERf* function causes a shift in ABA-GA homeostasis. Together, these data led to the conclusion that the *ERf*-mediated regulation of germination involved interaction with the ABA- and GA-signalling pathways and homeostasis during germination.

I then next tested whether *GL1* might play a role too in that interaction. Germination assays of *er*, *erl1*, *erl2*, *er erl1*, *er erl2* and *er er1/seg erl2* carrying a functional *GL1* or null *gl1* allele were compared on 0mM NaCl media supplemented with various concentrations of ABA and germination was scored. On 0 μ M ABA, consistent with earlier experiments ([Nanda et al., 2019](#)), all *GL1* seeds from all genotypes had completed germination within 4 Days post-stratification (4 DPS). Exogenous application of ABA consistently inhibited germination, but to a different extent depending on genotype, as reported ([Nanda et al., 2019](#)). Loss of *GL1* function in the three most salinity-sensitive lines, *er erl1*, *er erl2* and *er erl1/seg erl2* enhanced the inhibitory function of ABA on seed germination compared to *erf GL1* counterparts, in an ABA dose-dependent manner (Figure 2-3 C). No such enhancing effect was observed for the *erl1 gl1*, *erl2 gl1*, *erl1 erl2 gl1*, or *er gl1*; all responded to salinity similarly to WT. Altogether, these data suggest that the *ERf-GL1* interaction in modulating seed germination sensitivity to salt involves ABA-dependent germination signalling pathways.

2.3.3 Parent of origin effect in the *ERf-GL1* interaction

Comparison of germination rates of F1 seeds derived from reciprocal crosses between WT and *erf* mutants indicated that the *ERf*-mediated control of seed germination sensitivity to salt was of maternal origin ([Nanda et al., 2019](#)). In a first step towards clarifying whether the enhanced hypersensitivity to salt-induced by *GL1* loss of function in *erf* background was of paternal and/or maternal origin, embryos were excised out of dry, mature, seeds, and cultured on NaCl-free or NaCl-supplemented media, and their relative growth rate was monitored over a 3-day period. Similar potential relative growth rates (0 mM NaCl) were observed irrespective of genotype at *ERf* loci, as well as similar growth inhibition on 150 mM NaCl media (Figure 2-4 A).

However, those results did not rule out the possibility that *in situ*, i.e., within intact seeds, *ERf*-dependent properties of the embryo *per se* might be involved., as germination is well-known to implicate a complex inter-signalling between seed compartments -testa-endosperm and embryo. To test that hypothesis, reciprocal crosses between *er erf2 gl1* and *gl1* were performed, and F1 seeds from ♀*gl1* x ♂*er erf2 gl1* or ♀*er erf2 gl1* x ♂*gl1* crosses were compared in germination assays. On both 0 and 150 mM NaCl media, F1 seeds from either cross germinated synchronously with the seeds of the maternal parent (Figure 2-4 B&C). These data indicate that the enhanced germination sensitivity to NaCl observed for *er erf2 gl1* compared to *er erf2* seeds is of maternal origin.

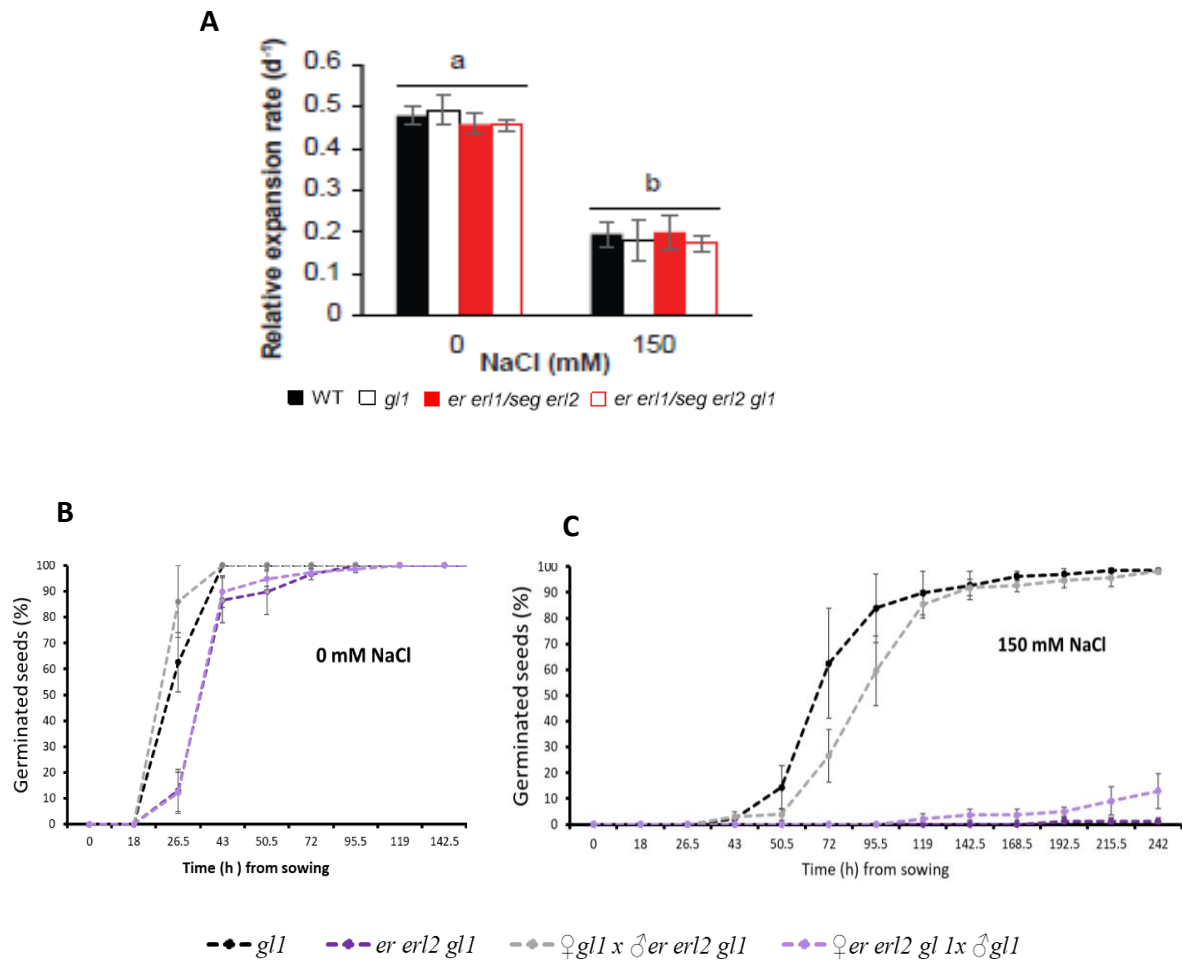


Figure 2-4: Maternal control of *ERF-GL1* interaction in salt hypersensitive germination. A. Relative expansion rates ($\text{mm}^2\ \text{mm}^{-2}\ \text{d}^{-1}$) of WT and *erl1/seg erl2* mature embryos carrying *GL1* or *gl1* on 0 or 150 mM NaCl, over a 72-h culture period following excision from dry seeds (means and s.e.m., $n=10$). Only the salinity effect was significant by two-way ANOVA. **B&C.** Germination time-course of *gl1* and *erl2 gl1* seeds, and F1 seeds derived from their reciprocal crosses under 0mM (**B**) and 150mM (**C**). Means and s.e.m. ($n=4$ plates with 30 seeds per genotype per plate; experiment replicated 5 times).

2.3.4 The *ERf-GL1* interaction in the control of seed germination is primarily controlled via the maternal seed coat

The next question was whether this maternal control of germination was solely exerted by the seed coat, or also involved some role of the predominantly maternal endosperm. The endosperm in the mature *Arabidopsis* seed is reduced to a 1 cell-thin layer, and is not amenable to *in situ* dissection. To separate its potential role from that of the seed coat, I used a genetic approach and created lines expressing the *ER* gene locus (*ERg*) i.e., the *ERf* member with a dominant role in the *ERf* function in seed germination, under the control of the well characterised *ARABIDOPSIS THALIANA MERISTEM L1 LAYER* (*AtML1*) epidermal-specific promoter ([P. Z. Lu et al., 1996](#); [Sessions et al., 1999](#); [Uchida et al., 2012](#)). Underlying my choice of an epidermal promoter was the well documented role of both the *ERf* and *GL1* as epidermal patterning genes in aerial organs - the former acting as master genes in the regulation of stomatal development, epidermal cell proliferation and expansion ([Shpak et al., 2004](#); [Shpak et al., 2005](#); [Pillitteri, 2007](#); [Tisne et al., 2011](#); [Meng et al., 2015](#); [X. Qu et al., 2017](#); [Wu et al., 2022](#)), the latter as a gene absolutely required for the induction of trichome initiation ([Herman & Marks, 1989](#); [Oppenheimer et al., 1991](#); [Larkin et al., 1994b](#)).

AtML1: ERg: FLAG er gl1 flowers expressing *ERg*; ([Uchida et al., 2012](#)) were used to pollinate *er erl1 gl1* and *er erl2 gl1* flowers (female recipients, ♀). The F1 progeny thus carried one segregating gene, *ERL1*, or *ERL2*, respectively. As a control, *Col(gl1)* (♀) was used as an additional maternal parent to generate lines expressing the same *AtML1: ERg: FLAG* transgene in epidermises lacking *GL1* function, in addition to the native *ERg*. F1 seeds were sown on soil and the F2 progeny was tested for the effective expression of the *AtML1: ERg: FLAG* transgene by qPCR analysis using previously validated primers ([Uchida et al., 2012](#)), using three weeks old leaves of selected F1 plants (F2 seeds) and derived F2 progeny (F3 seeds) (See Methods and Figure 2-5). As the originally received *AtML1: ERg: FLAG* seeds ([Uchida et al., 2012](#)) were in a *gl1* background I carried out similar crosses as above with *er erl1* and *er erl2*, carrying functional *GL1*. I visually selected trichome-bearing plants, with furthermore *ER* wild-type leaf morphology, and tested the effective expression of the *AtML1: ERg: FLAG* transgene by qPCR (Figure 2-5). Positive transgenic F1 plants in *er erl1(GL1)*, *er erl2(GL1)*, and *er (GL1)*, as well as control *Col (GL1)* backgrounds, were isolated, and the F2 progeny was collected for further analysis.

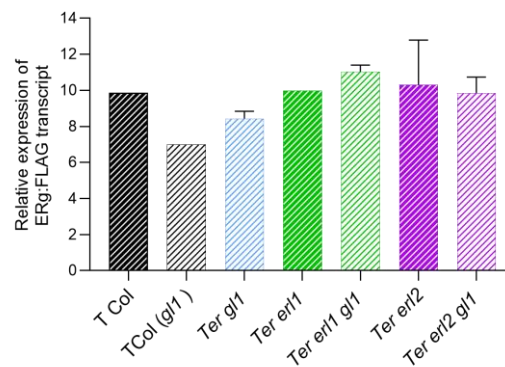
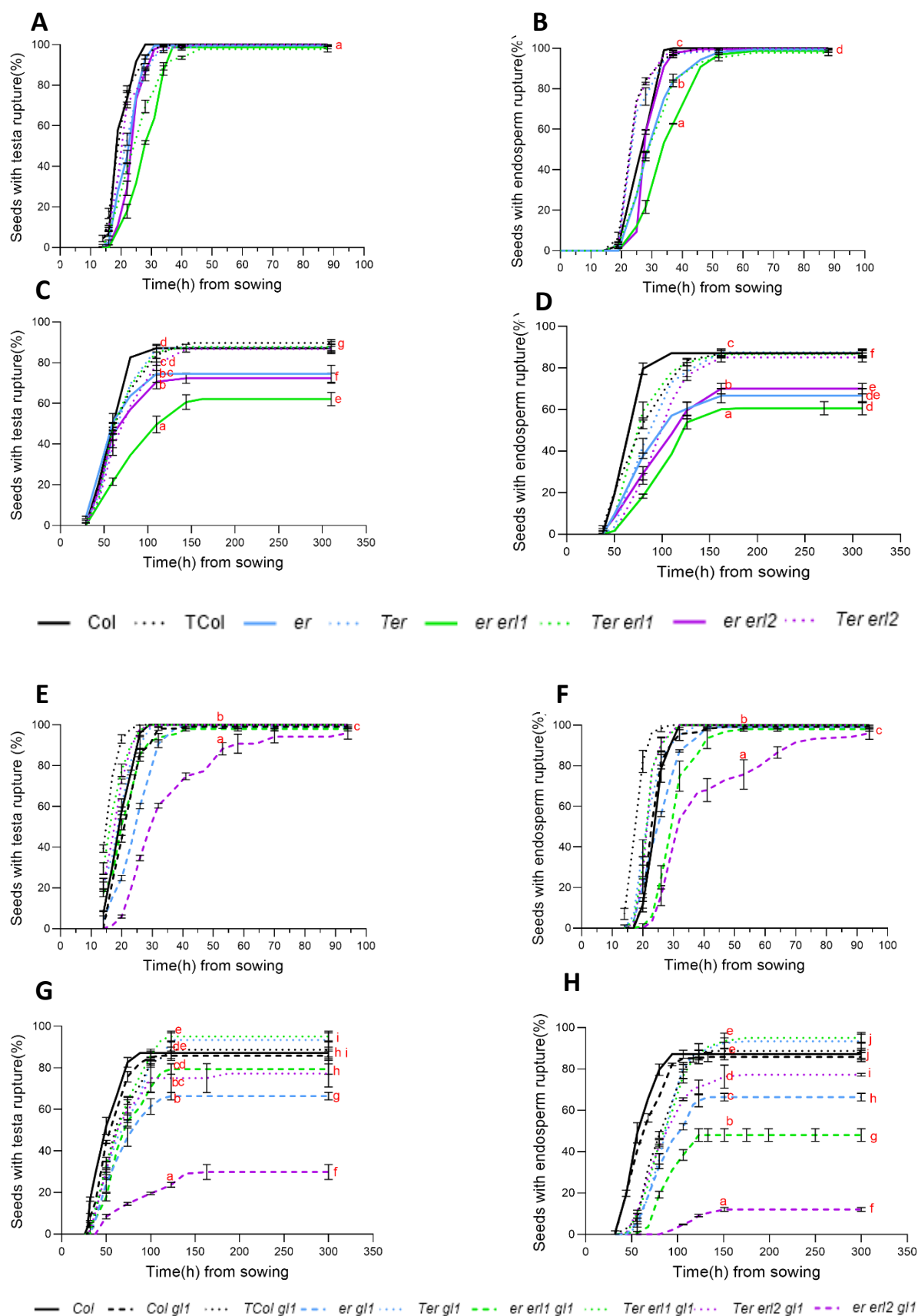


Figure 2-5: Expression of *ERg*: FLAG normalised to the geometric mean of reference genes. Four reference genes were used :AtbHLH, PDF2, Apt1 and TIP4. WT was used as the negative control, and the leaf materials obtained from the seeds that were successfully used in the study of ([Uchida et al., 2012](#)), were used as the positive control (n=4).

Four transgenic (T) lines, from two independent crosses for each line, in both *gl1* and *GL1* background, were used for germination assays under 0 and 150mM NaCl, where the timing of both testa rupture and endosperm rupture were recorded. On 0 mM NaCl, T_{50} values for testa and endosperm rupture in transgenic *GL1* lines recapitulated WT values. This was also the case in the *gl1* background, with even a trend for a slightly enhanced germination speed in the complemented lines compared to *gl1* or *erf* seeds, or even WT seeds (*ERf GL1*), especially for testa rupture (Figure 2-6 I&J). This trend was consistent across the two independent sets of transgenic lines tested (Supp figure 2-1).

On 150 mM NaCl media, complementation by AtML1: *ERg* dramatically reduced T_{50} values for both testa and endosperm rupture (47 and 53%, respectively-*Ter erf1*) in the highly salt-hypersensitive *er erf1* mutant, whether in the presence or absence of functional *GL1*. This was also the case in the *er erf2 gl1* background., while complementation of *er erf2 (GL1)* or *er (GL1)* with AtML1::*ERg* had no significant effect; hence still significantly higher than in WT (Figure 2-6 I&J)

However, in all transgenic lines on 0 mM NaCl, and all apart from *T erf2 gl1* on 150 mM NaCl, expression of *ERg* under the control of AtML1pro restored WT levels of the *final* percentages of seeds undergoing testa rupture and proceeding to endosperm rupture (Figure 2-6 A-H). In *T erf2 gl1*, final levels of testa and endosperm rupture were considerably raised, from 30% and 10-20%, respectively, to about 70-80%, instead of about 90% in WT and *gl1* (Figure 2-6 G &H).



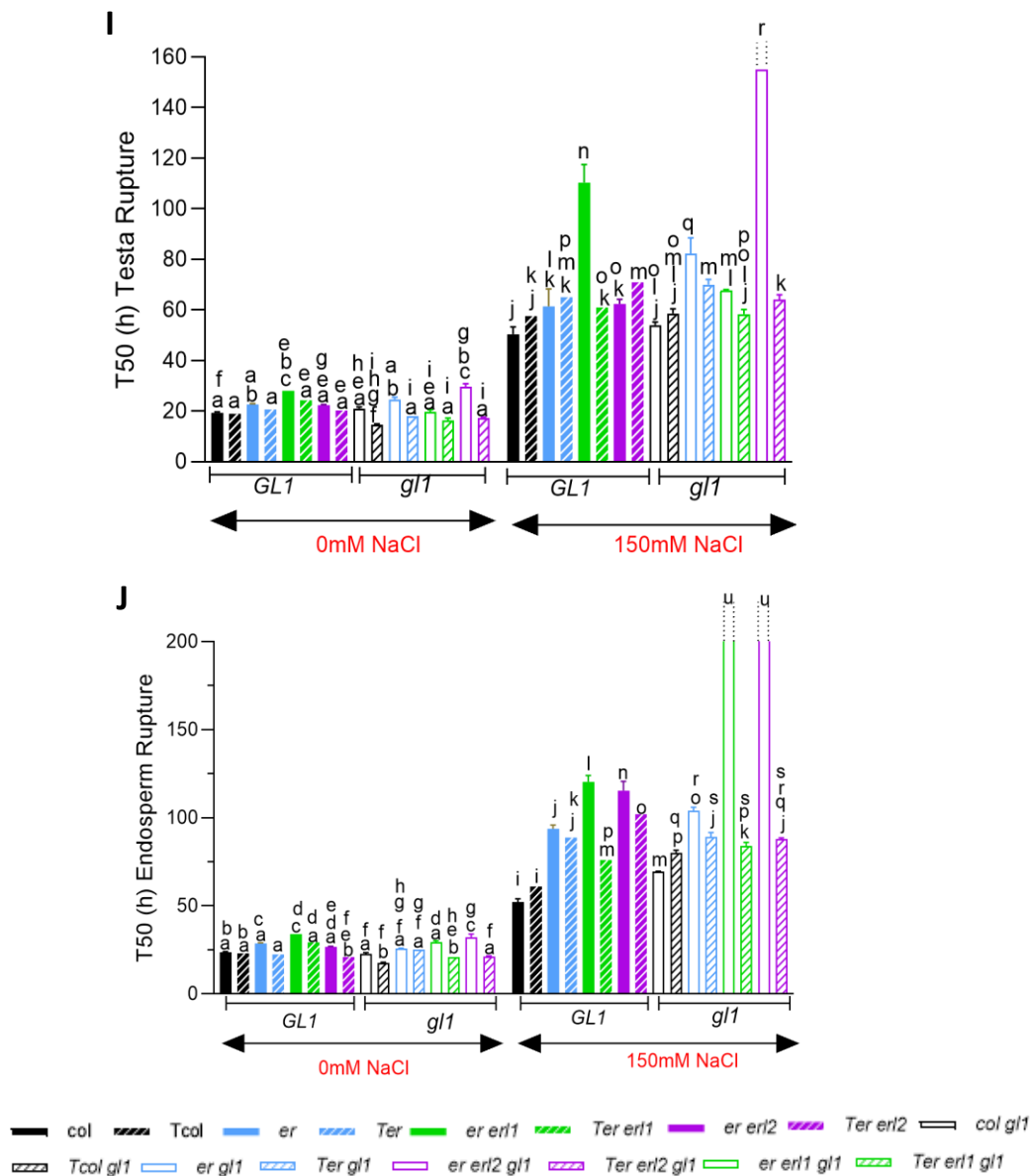


Figure 2-6: Expression of *ER* in epidermis under different combination of *ERf* +/-*GL1* rescued the *ERf* induced salt hypersensitive germination phenotype. A-D. Percentage of endosperm and testa ruptured seeds from Col, TCol, *erf GL1* and *Terf GL1* under 0mM (A&B) and under 150mM (C &D). **E-H.** Percentage of endosperm and testa ruptured seeds from Col, *gl1*, *Tgl1*, *erf gl1* and *Terf gl1* under 0mM (E&F) and under 150mM (G&H). Means and s.e.m. (n = 3 plates, 30 seeds per genotype in each plate; experiment repeated 3 times. Statistically significant germination kinetics were determined by one-way ANOVA and Fisher's LSD pairwise test and, denoted by different letters. **I&J.** Time(h) taken to reach 50% testa rupture (**I**) and endosperm rupture (**J**) Statistical significance was determined by a two-way ANOVA and Fisher's LSD pairwise test and denoted by different letters (p<0.05).

These results demonstrate the major role of the *ER* gene, and of its specific expression in epidermal cells, in the *erf* salt hypersensitivity germination phenotypes, and imply that their maternally inherited inhibition of germination is exerted via the seed coat alone, with a negligible role of the endosperm. They also show, however, that this is partially dependent on *GL1* signalling, with some specificity among *ERf* members, especially with respect to the *speed* of the germination process, which furthermore, contrary to final germination levels, was largely but not quite totally recapitulated in the transgenic lines (with 1-1.5 d delay in germination percentages reaching their plateau). That latter observation suggests a minor role of non-epidermal maternal cells on the *ERf-GL1* control of the kinetics of the germination process, contrary to its final success rate which appears to be controlled by *ERf-GL1* dependent epidermal properties of the seed coat.

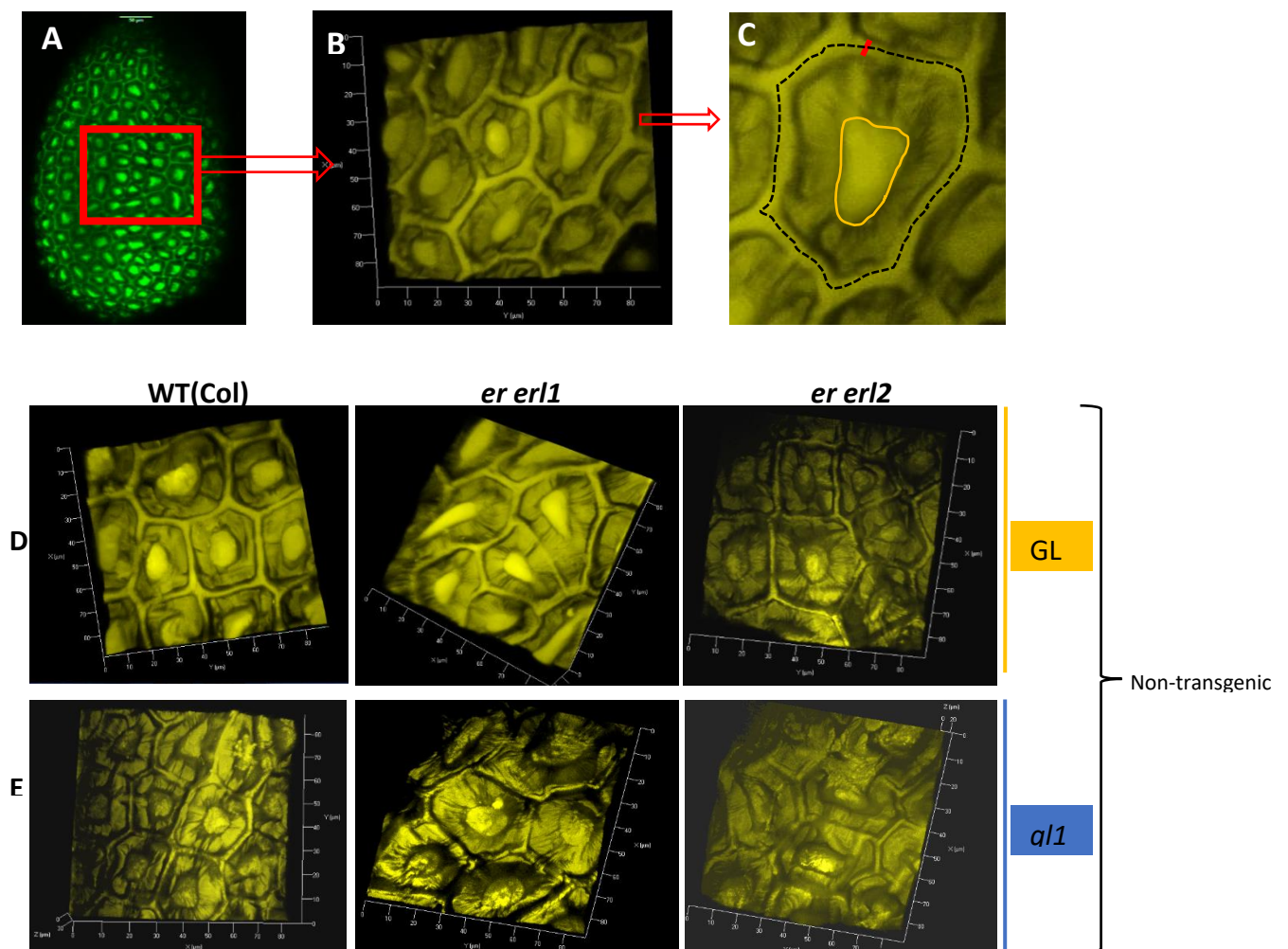
I then next asked: What properties of the mature seed coat do the *ERf* and *GL1* influence to promote seed germination and decrease its sensitivity to salinity?

2.3.5 Interactive functions of the *ERf* and *GL1* in the mature seed coat surface characteristics

To investigate that question, I first examined the patterning and autofluorescence spectrum of the mature seed coat epidermis, using confocal microscopy. Optical sections of the seed coat of WT (Col), *er*, *er erl1*, *er erl2* +/-*GL1* were captured (n=6 seeds; 2.84µm steps from the top of the columellae to the lower tangential wall of the outer *oi2* epidermal layer, typically 28-30µm depth in total), and used for 3-D reconstruction of the mature seed coat anatomy and morphology, and its surface characteristics at three positions along a median longitudinal transect, from the chalazal and micropylar end (C&M), to mid-way along the seed ("Middle" position), and to its distal end ("Distal" position) (Supp figure 2-2 A). Epidermal cell surface area (Figure 2-7 C), black dotted line), upper surface area of its central columella (Figure 2-7 C- yellow line), and cell wall thickness between the cell and adjoining cells (Figure 2-7 C red line) were compared among *erf* mutants and WT, in *gl1* and *GL1* background.

Cells were typically smaller at the chalazal and micropylar end of the seed than at the middle positions along the longitudinal transect. However, all measured parameters showed a similar genotypic ranking at all three locations (compare Figure 2-8 and Supp figure 2-2). For simplicity, results are therefore only shown in the main text for cells imaged mid-way along the seed. As shown in Figure 2-7 D; F, and in Figure 2-8 with quantitative data, *er erl1* seeds had larger *oi2* epidermal cells larger columella and thicker cell walls than WT. These differences were even more obvious in the triple mutant *er erl1/seg erl2* (Figure 2-9). *GL1* loss of function was neutral in *er erl1* background in cell sizes and cell wall thickness, but when combined with *er erl2* caused a significant increase in epidermal cell surface above WT levels, to similar levels as in *er erl1*, and of columella surface area and wall thickness too (Figure 2-7 G: Figure 2-8 A, B & C). Complementation with *TERg* reduced *ERf*-dependent differences, especially in epidermal cell surface area for which they were completely abolished (Figure 2-7 H&I and Figure 2-8 D-F). Intriguingly, the *er* single mutant seeds (*GL1* background) showed smaller epidermal cells than WT, in contrast to the two double mutants, but loss of *GL1* function or complementation by *TERg* induced changes in similar directions in epidermal anatomy (Figure 2-7 F-I Figure 2-8 A & D). Transgenic lines in *GL1* background, causes a general decrease in columella surface area, in all *erf* and in WT too, So In *gl1* background, hence, there were no more genetic differences among *erf* and *erf gl1*, *erf/erf gl1* and WT in columella surface area except for *er* (Figure 2-8 E). Complementation by *ERg* in *erf GL1* was not significantly affected on cell wall thickness, but in *gl1* background abolished the *er erl1* effect and trend to reversion of ranking among *erf* and WT (Figure 2-8 F).

Differences in columella and cell surface area indicate that the *er* and *er erl1* in *GL1* background cause an increase in the % columella surface area relative to cell (i.e., relatively greater increase in columella surface area than whole cell area), whereas *er erl2 GL1* has the similar relative increase as WT (Figure 2-10). In loss of function of *GL1*, same as above for *er erl1* and *er erl2*, but *er* shows relative decrease given the antagonistic effect on cell surface area and columella surface area compared to *er GL1*. (Figure 2-8 A-C). All the *erf+/-GL1* transgenic lines show significant differences in the columella proportion to the cell area compared to their parental lines. Complementation with *TERg +/-GL1* reduced columella to whole cell area ratio to WT level, while *TERg* in *er GL1* showed the highest significant reduction of the columella proportion to the cell area and *TERg* in *er gl1* showing a significant increase. (Figure 2-10).



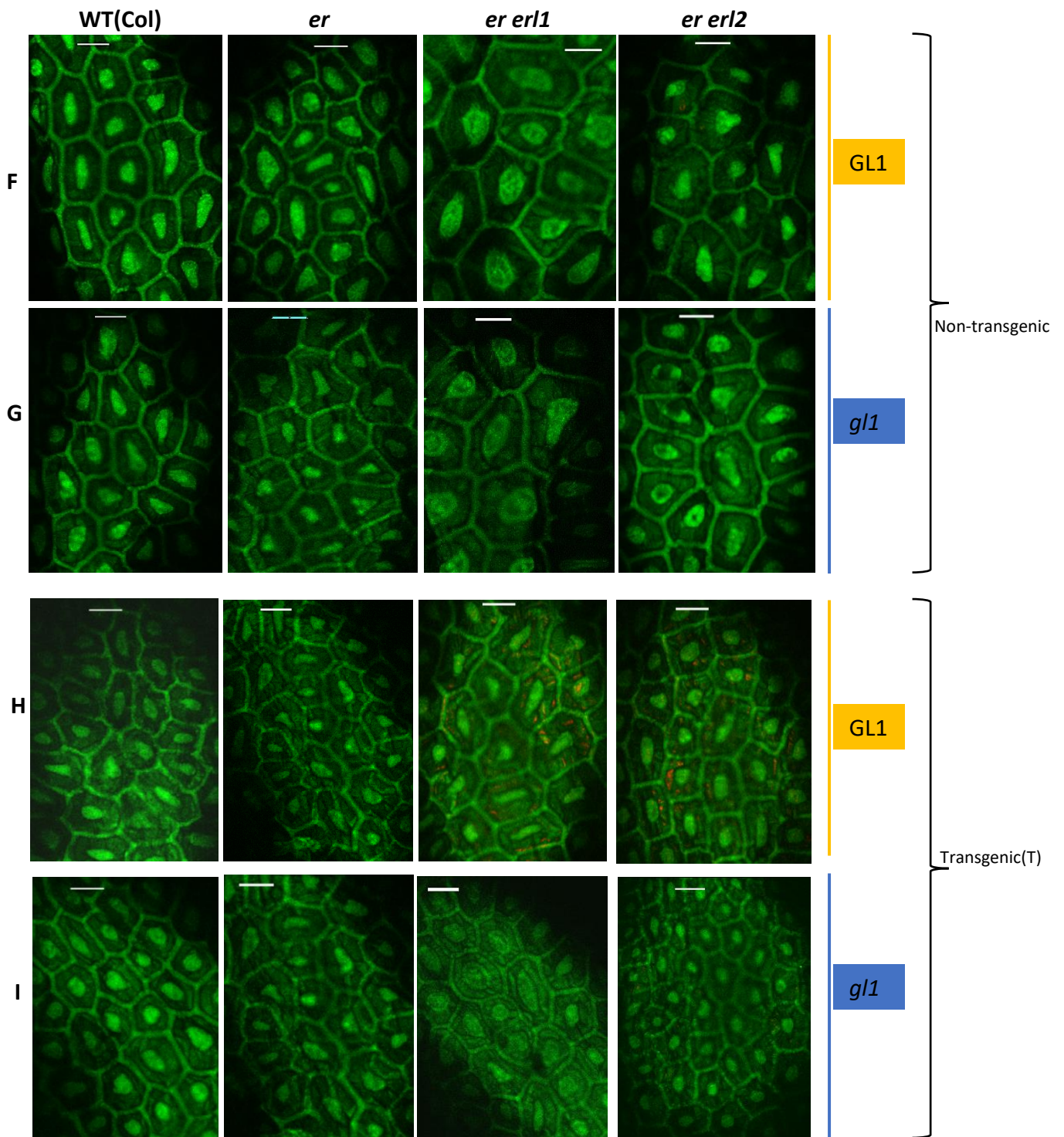


Figure 2-7: *ERF-GL1* influences surface characteristics of Arabidopsis mature seed coat. A-C. representative 3D reconstruction auto florescence spectrum of the mature seed coat (A), and enlarge view of the area of interest (B), and representation of the quantitative measurement obtained for the analysis (C) -black line represents the cell surface area, yellow line represents the columella surface area, and red line- cell wall width. D-I. 3D reconstruction of the mature seed coat auto florescence spectrum of non-transgenic (D&E-batch 1, F&G-batch 2) and transgenic (complementation with *ERg* in epidermis) (H& I-batch 2) WT, *er*, *er erl1*, and *er erl2* +/-*GL1*.

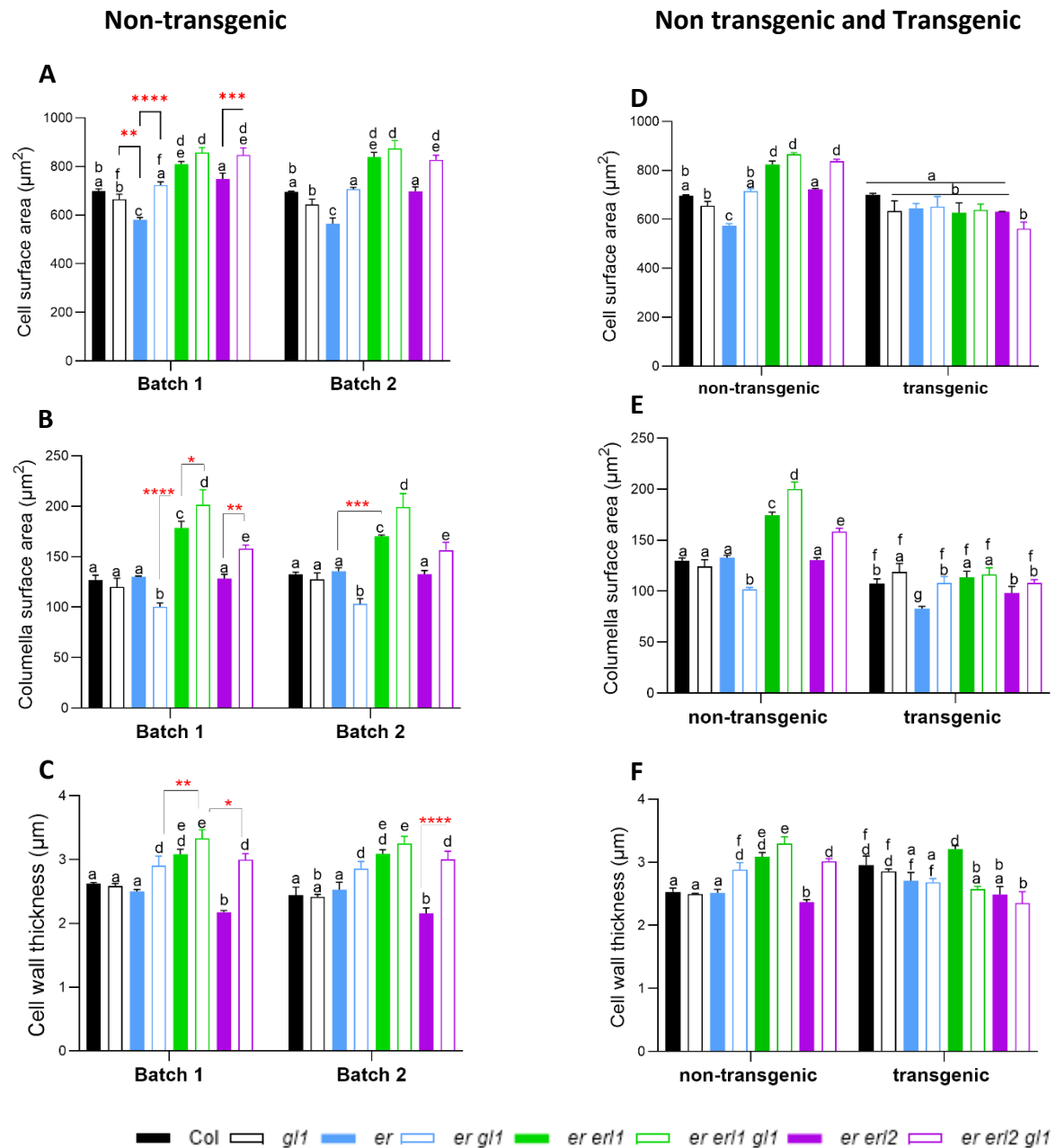


Figure 2-8: The loss of function of *ERF -GL1* influences the cell expansion and cell wall deposition of the *Arabidopsis* mature seed coat outer integument layer. A-C- Cell surface area (μm^2), columella surface area (μm^2) and wall width (μm) of (A, B, and C respectively) Col, *gl1*, *er*, *er gl1*, *er erl1*, *er erl1 gl1*, *er erl2*, and *er erl2 gl1* of mature seed outer epidermal layer (oi2). Experiment repeated with two separate seed batches- batch 1 and batch 2. D-F. Cell surface area (μm^2), columella surface area (μm^2) and wall width (μm) of (D, E, and F respectively) of mature seed outer epidermal layer (oi2) of transgenic *erf +/-GL1* expressing *ERg* in the epidermis. Results were shown comparatively with their parental lines. Mean and s.e.m (n=6) Statistically significant differences between samples were determined using two-way ANOVA and Fisher's LSD pair-wise test. bars labelled with different letters are significantly different from each other ($p < 0.05$).

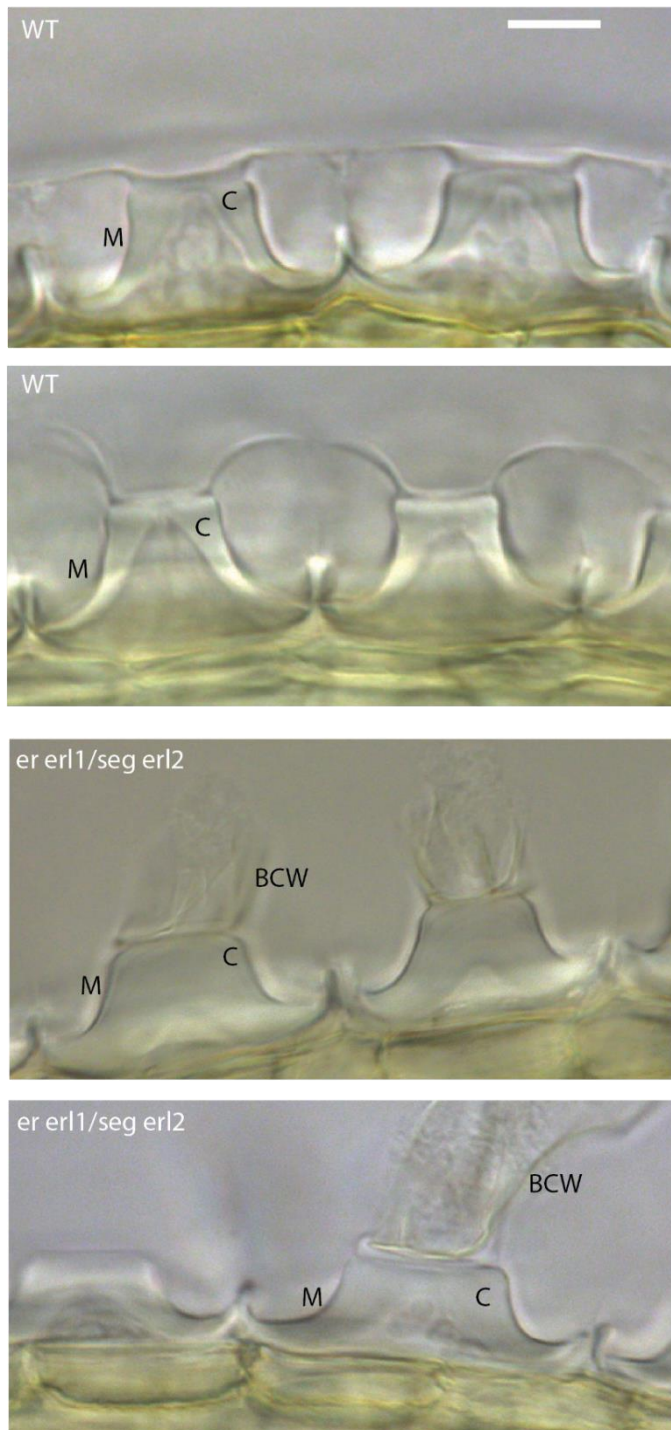
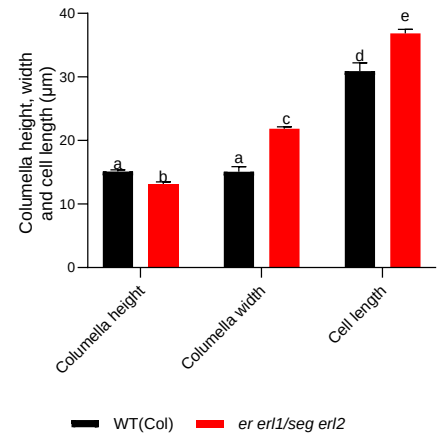
A**B**

Figure 2-9: Broader and shorter columella induced by *er erl1/seg erl2* mutant. **A.** Image panels of the outer integument oi2 layer bearing columella, mucilage pockets and broken primary cell walls of WT and *er erl1/seg erl2*. C-Columella, M-Mucilage pockets, BCW-Broken primary Cell Walls. Scale bar 10µm **B.** Columella height from the bottom of the cell to the top of the columella, width from the ½ of the height from the top of the columella, and the oi2 cell length (µm) of WT and *er erl1/ seg erl2*. Mean and s.e.m (n=6 seeds). Bars labelled with different letters are significantly different from each other (p<0.05).

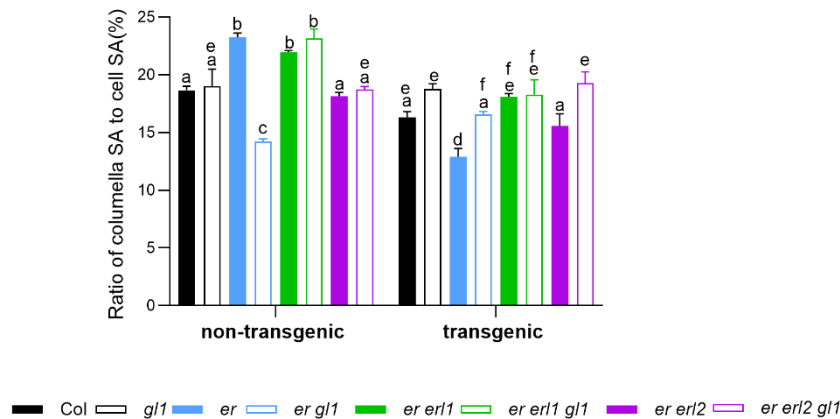


Figure 2-10: *ERf-GL1* induced secondary cell wall deposition alters the proportion of cell covers by columella. A&B- the proportion of columella covers the whole cell in non-transgenic and transgenic *ERf GL1*, *ERf gl1*, *erf GL1* and *erf gl1*. Mean, and s.e.m (n=6) Statistically significant differences between samples were determined using two-way ANOVA and Fisher's LSD pair-wise test. Bars labelled with different letters are significantly different from each other (p<0.05).SA-Surface area.

Columella height could not be accurately measured from Z-stack optical sections of the mature seed coat obtained by confocal microscopy due to a lack of sufficient depth of field with good resolution. However, whole mounts of cleared *er erl1/seg erl2* seeds collected 10 days post-fertilisation -when the columella had reached its final size ([Beeckman et al., 2000](#); [Western et al., 2000](#)) indicate a shorter and broader columella in the triple mutant compared to WT, and also consistently larger epidermal cells. They also showed a consistent trend for a smaller residual cytoplasm at the base of the columella than in WT (Figure 2-9 A). This could be due to a more advanced stage of development of the *erf* columella compared to WT at the same biological age. Those images also indicated more frequent primary cell wall rupture during seed clearing on the exposed surface of the mucilage pockets flanking columellae, than in WT seeds, suggesting perhaps weaker primary cell walls in the *erf*.

Altogether, these data demonstrate *ERf*-dependent control of the patterning and morphology of seed epidermis and of cell wall formation and suberisation during mucilage deposition and seed coat differentiation, with functional specificity among *ERf* members and, depending on the combination of *erf* mutations, interaction with *GL1*. Recapitulation of WT phenotypes in *er erl1* or *er erl2*, in both *GL1* and *gl1* backgrounds, by epidermal specific complementation of *er* loss of function by the *AtML1: ERg* transgene indicates predominantly autonomously controlled *ERf-GL1* interactive functions by the seed epidermis, and a preponderant role of the *ER* member in these functions.

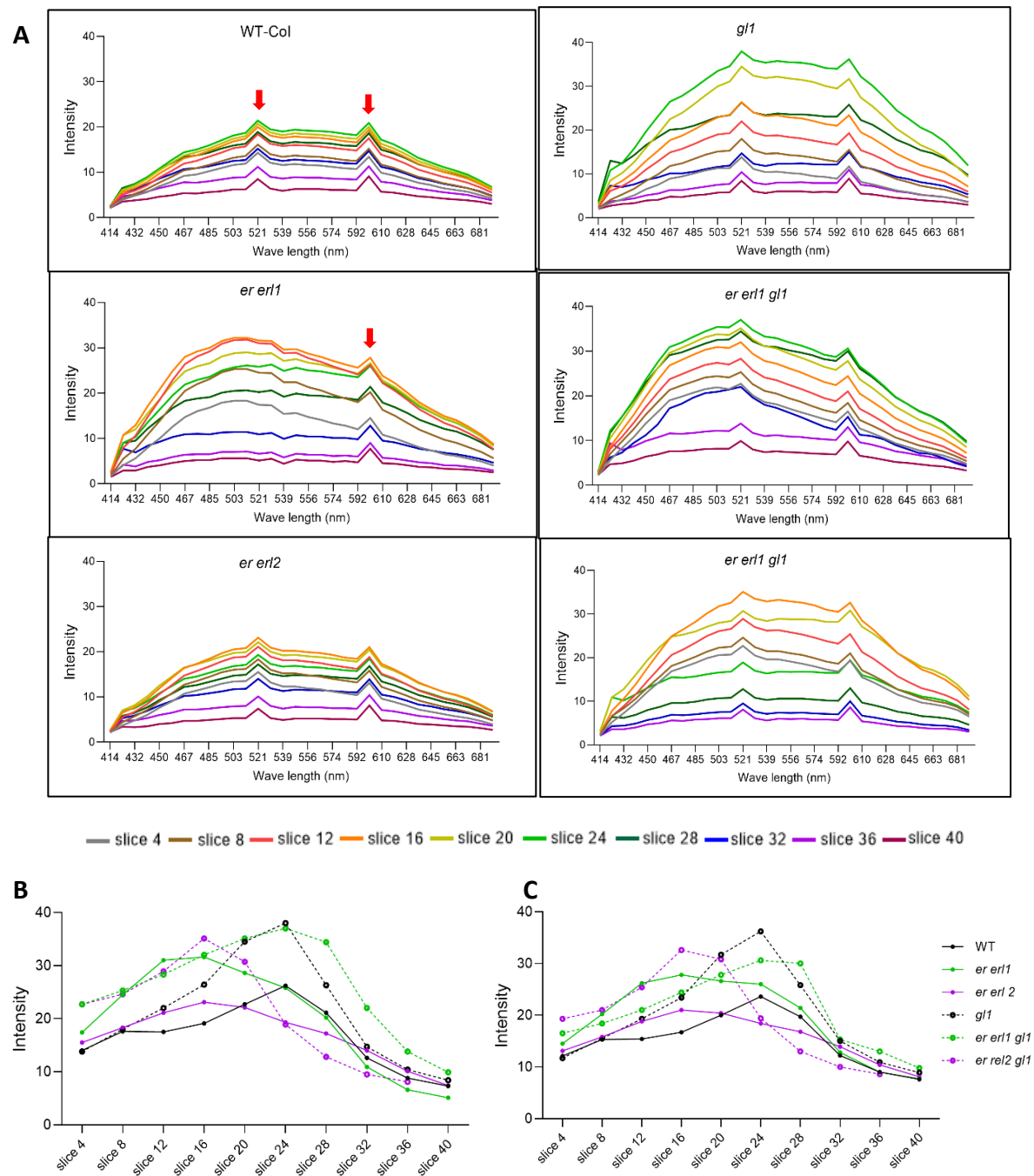


Figure 2-11: Loss of function of *erl1* lacks the autofluorescence peak at 521nm. A. Autofluorescence spectra starting from top of the columella to 28-30 μ m depth of the mature seed coat surface of WT-Col, *erl1*, *erl2* +/- *GL1*. First slice is at 2.13 μ m depth from top of the columella and 2.84 μ m depth between each slice, total depth 28-30 μ m. (n=3) **B & C.** the intensity at the 521nm (B) and at the 601nm (C) at each depth (slice) across the genotype.

For lack of time, the biochemistry of the seed epidermis and the composition of the columella and oi2 secondary cell wall material were not analysed. However, as an initial qualitative step, I compared the auto-fluorescence spectrum of the seed coat surface and underlying 28-30 μm towards the lower tangential wall of oi2, using confocal microscopy in WT, *er erl1* and *er erl2* seeds, in both *gl1* and *GL1* background (Figure 2-11 A). The autofluorescence spectra showed similar profiles across all Z slices in WT, with two clear conserved fluorescence peaks, one at 521nm and the other at 601 nm. In *er erl1 GL1*, the fluorescence intensity profiles were also similar across depths, but fluorescence intensities were generally elevated compared to WT in the range of high wave lengths and the 521 nm peak was furthermore absent. The 600 nm peak only was conserved. The combination of the *er erl1* and *gl1* mutations restored the WT autofluorescence profiles, and the first 521 nm intensity peak, but with the higher fluorescence intensities. In the *er erl2 GL1* had a similar autofluorescence spectrum to WT but when combined with *gl1*, induced generally higher fluorescence intensities than measured for *er erl2 GL1* and WT seeds. These increased intensities pattern has continued in single *gl1* mutant as well. In loss of *GL1* in loss of *erf* and WT background furthermore modified the spatial profiles of autofluorescence intensities with depth (Figure 2-11 B &C).

These data suggest a role of the *ERf* in the synthesis and deposition of secondary cell wall material in the columella and walls of the oi2 seed coat surface layer, and of the mucilage lining it. They also indicate an interactive role of *GL1* with that *ERf* function, with specificity among *ERf* members in that interaction.

2.3.6 *GL1* is involved in the importance of seed mucilage in the *ERf*-mediated regulation of seed germination sensitivity to salinity

During seed coat differentiation on the mother plant, the specialized outer epidermal cells start synthesizing pectin-rich mucilage polysaccharides and secreting them to the apoplast at the upper tangential corners of the cell. Starch granules (amyloplasts) accumulate in the centre of the cell starting from 3 DAFO and then starting from 6-7 DAFO to 9-10 DAFO to pectin rich polysaccharides increase the abundance. During this time secondary cell wall material forms a central columella in the cell, flanked by mucilage pockets bound by the outer periclinal primary cell wall. At maturity, the desiccated pectinous mucilage forms a thin lining at the surface of the Mucilage Secretary Cells (MSC) outer tangential wall. When the seed gets exposed to the soil solution, the highly hydrophilic pectinous matrix rapidly swells, rupturing the enclosing primary wall to form a loose gelatinous capsule around the seed, attached to the surface of the seed by cellulose microfibrils radiating from the columella, and forming the commonly so-called non-adherent outer mucilage, in contrast to the thin, denser layer of inner mucilage tightly bound to the cell through a cellulose rich-matrix ([Beeckman et al., 2000](#); [Western et al., 2000](#); [Windsor et al., 2000](#); [Western et al., 2001](#); [Voiniciuc, Yang, et al., 2015](#)).

Previous work in the Lab showed that the seed mucilage had a crucial role in mediating the *erf*-induced salt-hypersensitive germination phenotype ([Nanda et al., 2019](#)). Here I repeated these experiments, but including the *gl1*, *er gl1*, *er erl2 gl1* and *er erl1/seg erl2* seeds. Demucilaged *erf GL1* seeds germinated faster than intact seeds under 150mM, as reported earlier ([Nanda et al., 2019](#)). loss of *GL1* function enhanced that effect, and the percentage of seeds completing germination was raised by about 20 %. (Figure 2-12 A-C). The results suggest that *GL1* significantly contributes to the beneficial effect of the mucilage in delaying germination under salinity, especially in *er erl2* and *er erl1/seg erl2* backgrounds, and significantly enhances its “protection” of the embryo from breaking free through its surrounding tissues under adverse environmental conditions.

I next examined the amount of water-soluble mucilage expelled upon imbibition and its composition in *erf* salinity hypersensitive mutants, with and without functional *GL1*. We earlier reported that regardless of genotype at the *ERf* loci, seeds systematically expelled more water-soluble mucilage upon imbibition in a saline environment ([Nanda et al., 2019](#)). This experiment was repeated with *gl1* and *erf gl1* mutants and similar results were obtained. Similar amounts of water-soluble mucilage were recovered in *gl1* and *erf gl1* seeds than their *GL1* counterparts, after normalisation to seed size, with also a similar increase under imbibition with 150mM NaCl vs 0mM salt (i.e. *GL1* and *gl1* seeds fall on the same relationship on xy graph amount of mucilage per seed vs seed weight) (Figure 2-12 D). composition of the outer mucilage of *erf* seeds reported to be increased the ratios of the backbone

and side chain sugars GalUA/Gal especially in *er erl1*, *er erl2* and *er erl1/seg erl2* background compared to WT ([Nanda et al., 2019](#)). Extending this study, the loss of function of *GL1* in the *erf* background showed no difference to the compositions and GalUA/Gal ratio compared to its counterpart *GL1* except for the *er erl1/seg erl2 gl1* and *er erl2 gl1*. Both *er erl1/seg erl2 gl1* and *er erl2 gl1* mutants showed a significant reduction of the GalUA/Gal ratio compared to *er erl1/seg erl1* and *er erl2* respectively indicating that *GL1* interact with *ERf* in regulating the mucilage composition, especially the backbone and side chain sugars (Figure 2-12 E).

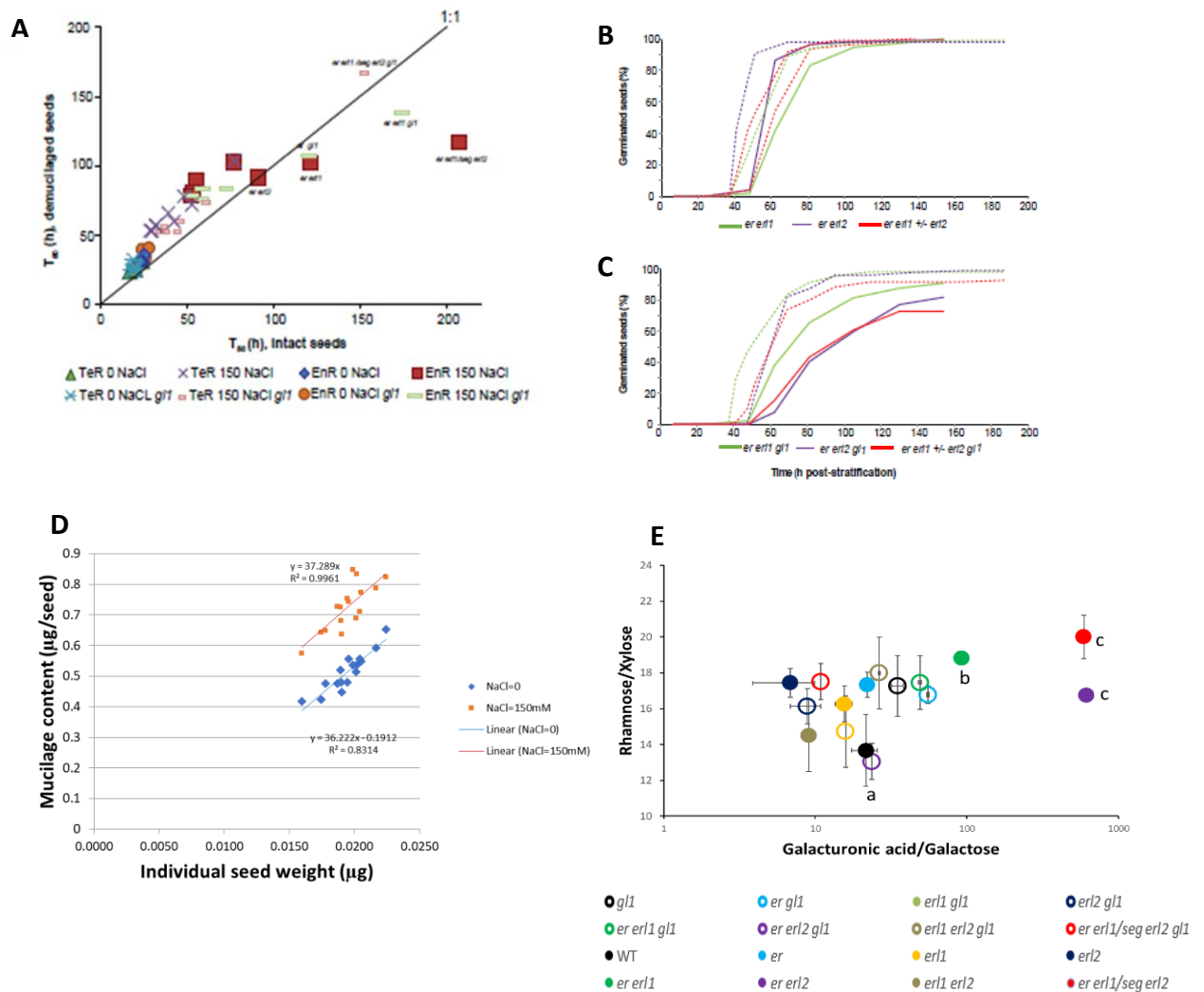
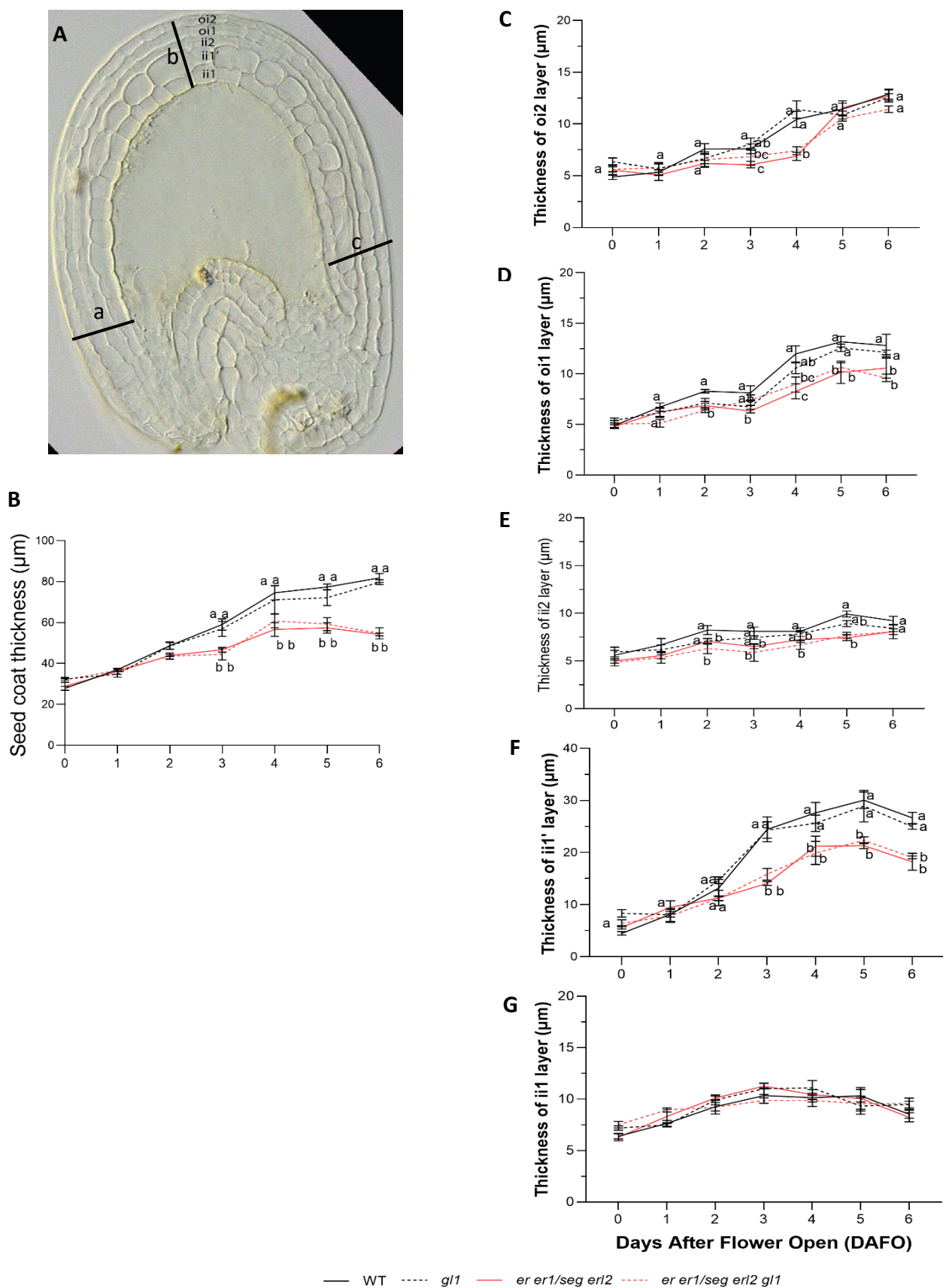


Figure 2-12: Loss of *GL1* function enhances the germination-promoting effect of mucilage removal in the salt-hypersensitive *erf* mutants. **A.** Comparison of testa rupture (TeR) and endosperm rupture (EnR) T_{50} values for intact seeds (x-axis) and “demucilaged” seeds (y-axis). Points denote mean values per genotype ($n = 4$ plates with 30 seeds per genotype per plate). Labels are on data points corresponding to genotypes where mucilage removal significantly advanced germination on 150 mM NaCl media. *er erf2 gl1* and *er erf1/seg erf2 gl1* data points are not shown as 50% germination was not reached during the timeframe of the assay (20 d incubation). **B&C.** Time-courses of endosperm rupture for *er erf1*, *er erf2* and *er erf1/seg erf2* seeds carrying functional *GL1* (**B**) or the *gl1* mutant allele (**C**) on 0 mM NaCl media, for a different seed batch. For sake of legibility only data for these most salt-sensitive lines are shown. Thick lines: intact seeds; thin lines: demucilaged seeds. $n=4$ plates with 30 seeds per genotype each. **D.** Correlation between amount of water-soluble mucilage as a function of seed weight in 0 and 150mM NaCl of WT, *gl1*, *erf +/-GL1* seeds. Means and s.e.m ($n = 4$ seed pools per genotype, experiment replicated 3 times). Regression lines: 0 mM NaCl, $y=36.2x-0.20$, $r^2=0.84$; 150 mM NaCl, $y=37.2x+0.002$, $r^2=0.99$. **E.** GalUA/Gal (x axis) and Rhm/Xyl (y axis) ratios of WT, *gl1* and *erf +/-GL1*. statistical significance of differences in GalUA/Gal ($P<0.05$) were indicated by letters by two-way ANOVA and Fisher’s LSD test, compared to all unlabelled data points. $P=0.08$ for differences in Rhm/Xyl between *er erf1/seg erf2* and WT.

2.3.7 The *ERf* controls cell expansion and proliferation and epidermal patterning in the developing seed coat, independently of *GL1*.

As a whole, the above experiments showed an interactive role of the *ERf* and *GL1* genes in the determination of the mature seed anatomy, morphology and differentiation. To further investigate the underlying processes, I next turned my attention to developing seeds and the unfolding of seed morphogenesis and maturation, from pollination (0 DAFO) to 20 DAFO, whose constitutive characteristics have been extensively documented. Developing siliques from individually tagged flowers at 0 DAFO were harvested daily over the next 20 days from the main inflorescence of WT(Col) and the most extreme salt-hypersensitive *erf* mutant, *er erl1/seg erl2*, in a *GL1* or *gl1* background, as well as Col-*gl1* as another control allowing for the examination of *ERf*-*GL1* interactions as well as main effects. Whole mounts of cleared siliques (see Methods) were slit open to expose the enclosed seeds and observed by a combination of light and confocal microscopy. In Arabidopsis, the rapid expansion of the seed cavity through active cell division and expansion in the seed integuments, is triggered by pollination and is completed by the embryo early heart stage, about 4-5 DAFO, at which point mucilage synthesis and columella formation begin ([Beeckman et al., 2000](#); [Western et al., 2000](#)). Given the results obtained above on *ERf*- and *GL1*-mediated differences in seed size, and mature seed coat anatomy and surface characteristics, I focused on the initial 6 days of seed development (0 – 6 DAFO in the first instance).

Consistent with the previous study of Nanda et al (2019), *er erl1/seg erl2* seeds were smaller and of a rounder shape compared to WT, with no significant influence of *GL1*, whether in WT or *er erl1/seg erl2* background (Figure 2-15 B). Cell shapes and tangential-sectional areas, and cell numbers within each of the 5 cellular layers of the seed coat – oi2, oi1, ii2, ii1 and ii'1 (Figure 2-13 A and see Figure 1-1) were compared through examination of optical sections through the longitudinal mid-axis of whole cleared seeds. Individual cell sectional area and total number of cells along the circumference of each of the 5 seed coat layers, as well as the thickness of each layer (i.e., anticlinal cell wall length), were recorded, at its distal end and opposite chalazal and micropylar end (Figure 2-13 A). Significantly thinner seed coats were observed in *er erl 1/seg erl2* ($\pm gl1$) seeds compared to WT (Figure 2-13 B), beginning between 2 and 3 DAFO, consistent with our earlier observations of a thinner seed coat in mature seeds ([Nanda et al., 2019](#)). This initially mostly reflected a reduced thickness of the innermost ii1' seed coat layer, but from 4 DAFO onwards, that reduction was compounded by an also thinner oi1 layer (Figure 2-13 B-H). Loss of *GL1* function alone, or in combination with the *ERf* had no significant effect on the thickness of the whole seed coat, nor of any of its individual layers.



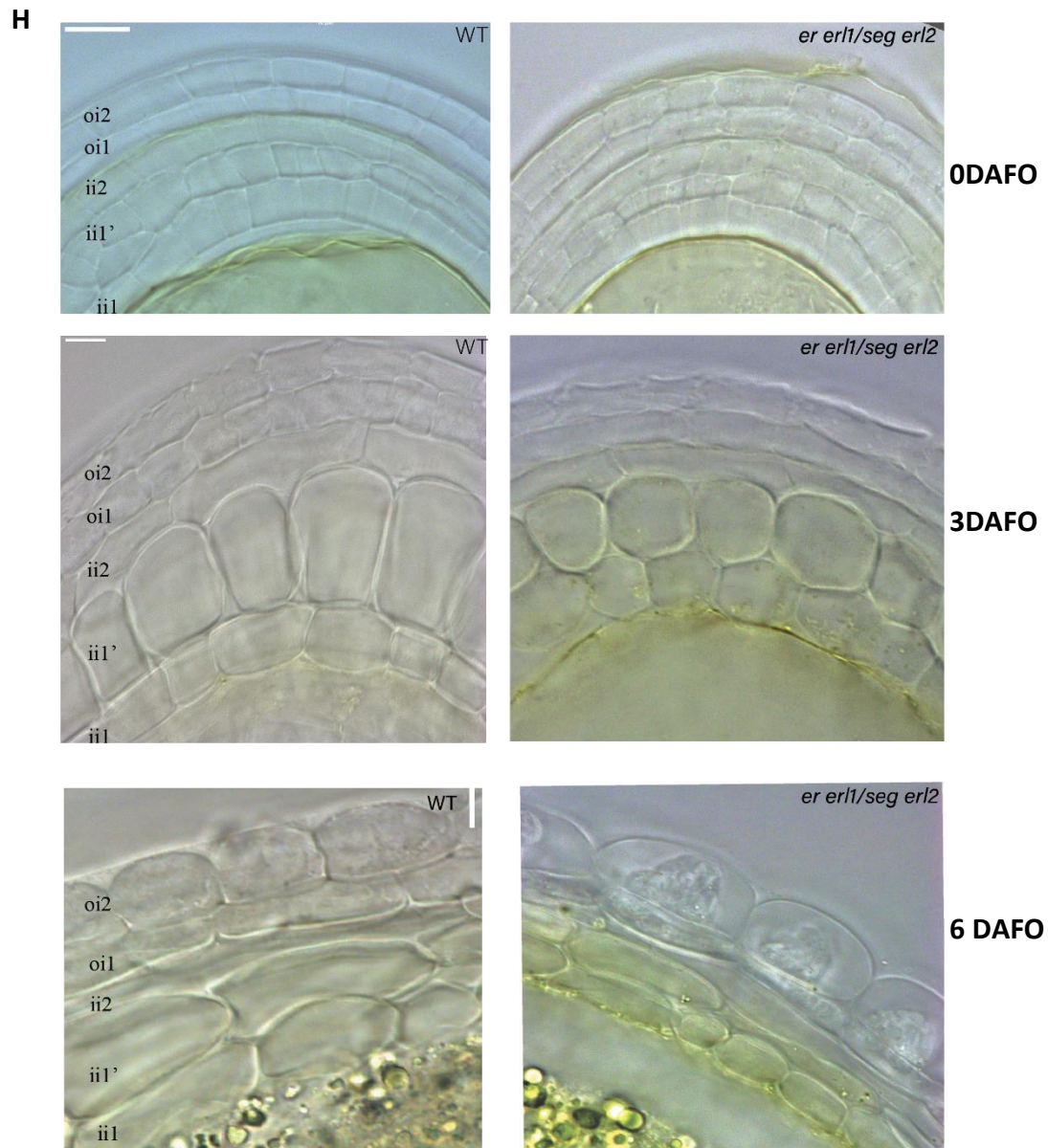
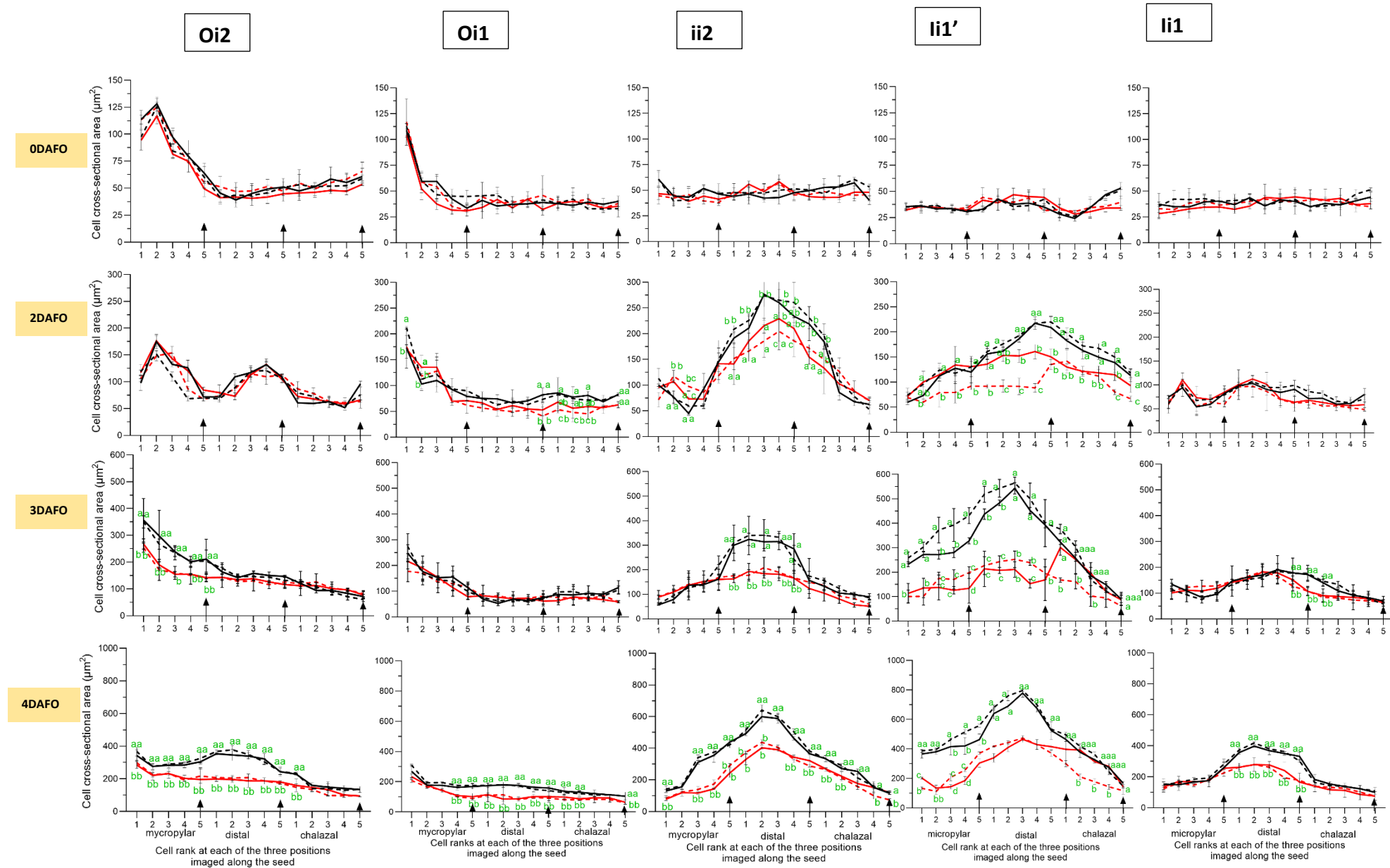


Figure 2-13: Inner integument ii1' layer majorly contributed to Arabidopsis's *Erf*-induced thinner seed coat. **A.** whole mount developing seed at 2 Days After Flower Open (DAFO) seed coat thicknesses were obtained at seed micropylar end(a), seed distal end (b-(opposite to chalazal and micropylar)) and seed chalazal end (c) of WT seed. **B-G.** total seed coat thickness (B) at distal end, Thickness of each integument layer oi2, oi1, ii2, ii1', ii1 (C,D,E,F,G respectively) of the WT, *gl1*, *erl1/seg erl2*, and *erl1/seg erl2 gl1* Arabidopsis seed from 0- 6 DAFO at the seed distal end Mean and s.e.m, n=5, experiment repeated two times with two independent seed batches. Significant genetic differences were tested at each stage using one-way ANOVA with Fisher's LSD pair-wise test and are denoted with different letters ($p < 0.05$). **H.** Fluorescence microscopic images of seed integument layers on 0, 3 (at the seed distal end) & 6 (at the chalazal end) DAFO of WT and *erl1/seg erl2*. Scale bar 10μm.

At 0 DAFO, there was no significant variation in cell cross-sectional areas along the circumference of the seed, except in the outer integument (oi1 and oi2 layers) where cells in the micropylar region were significantly enlarged compared to cells at the central or chalazal-end positions (Figure 2-14). However, within the next two days, a strong longitudinal gradient appeared along the inner integument (ii2 and ii1' layers) which showed a much-enhanced cellular enlargement in the central area of the seed compared to the flanking regions (increase by a factor of 2 to 3), and in WT compared to *er erl1/seg erl2*, whether in presence or absence of functional *GL1*. By 3 DAFO, these genetic effects were amplified, and significant along most of the seed circumference. They remained so over the following 3 days, during which a similar trend also appeared in ii1, but with much smaller genetic differences. Maximum cellular expansion was reached around 5 DAFO in WT, a bit later in the two mutants, at the micropylar end of oi1 and oi2. Comparison of genetic rankings of cell thickness and cross-sectional area (Figure 2-13 and Figure 2-14 14, respectively) suggests a role of the *ERf* on cell growth anisotropy in oi1 and ii2, with some specificity between the two layers – thicker but shorter oi1 cells in WT or *gl1* compared to *erf* (+/-*gl1*) seeds; no change thickness but longer ii2 cells in WT or *gl1* than in *erf* ± *gl1*.

The number of cells along the circumference of the same mid-longitudinal cross-section of the same 0-6 days-old developing seeds reached a maximum around 2-3 DAFO, except in ii2 where cells numbers remained close to their initial value at 0 DAFO (Figure 2-15 A). Cell numbers in the oi2 outer seed coat layer were consistently significantly reduced in *er erl1/seg erl2* ± *gl1* compared to WT and *gl1* (25-40% reduction), and also in ii'1, but to a much lower extent (20% at most in *er erl1/seg erl2*, and even less in *er erl1/seg erl2 gl1*). These data indicate a promoting role of the *ERf* on cell proliferation in the seed coat, in a layer-specific manner, mostly independent of *GL1* except for possibly in ii'1.

Altogether, these results reveal integument- and layer-specific *ERf* functions on cellular growth and cell proliferation during the expansion of the seed cavity during early embryo morphogenesis, mostly independent of *GL1* and mostly manifest during the first 2-3 days following fecundation, by which time the seed cavity was near its maximal expansion, in all lines.



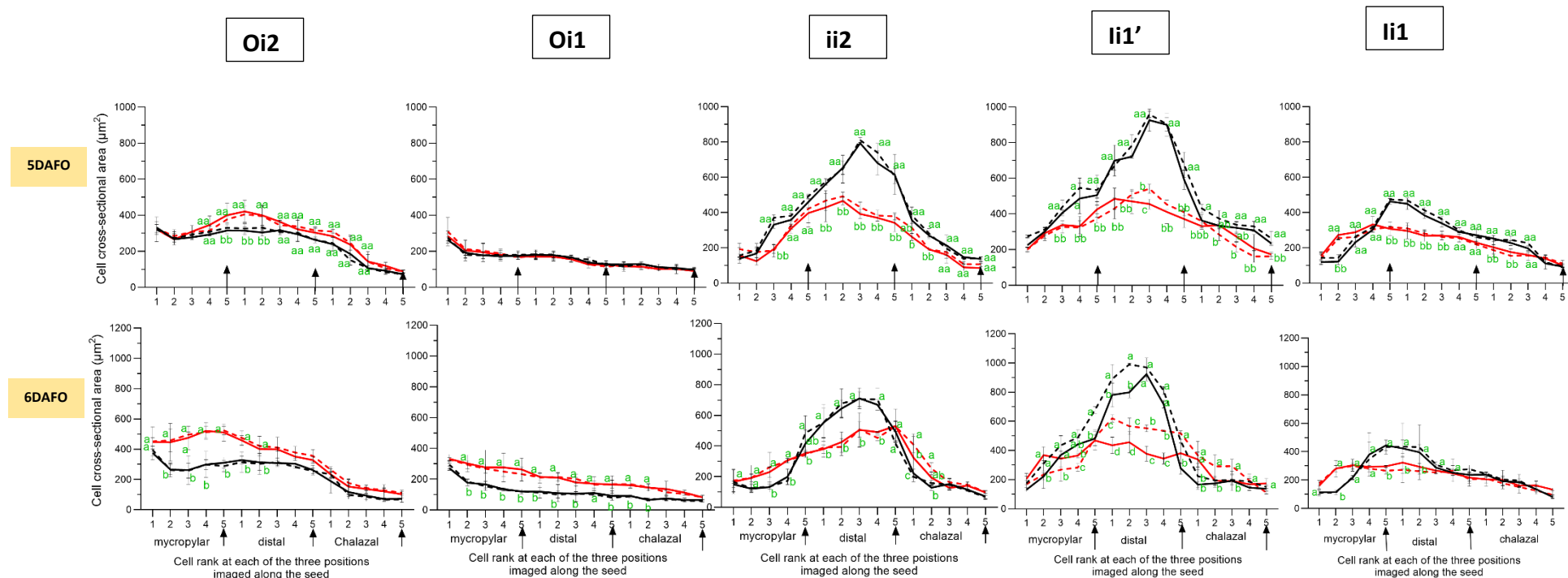


Figure 2-14: Development stage and location-specific *ERf*-induced cell expansion in the seed integument layer on seed development on the mother plant. cell cross-sectional area (μm^2) profile was developed for each integument layer, oi2, oi1, ii2, ii1', ii1 of WT, *gl1*, *er erl1/seg erl2* and *er erl1/seg erl2 gl1* from five cells in three different positions along the longitudinal axis of the seed: micropylar, distal and chalazal positions. The arrows point to the 5th cell at each location. Mean and s.e.m, n=5, repeated two times; statistically significant differences between samples in one location were determined using two-way ANOVA Fisher's LSD test and denoted with different letters.

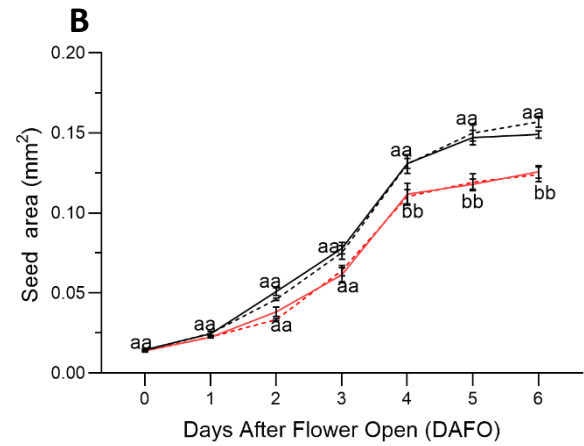
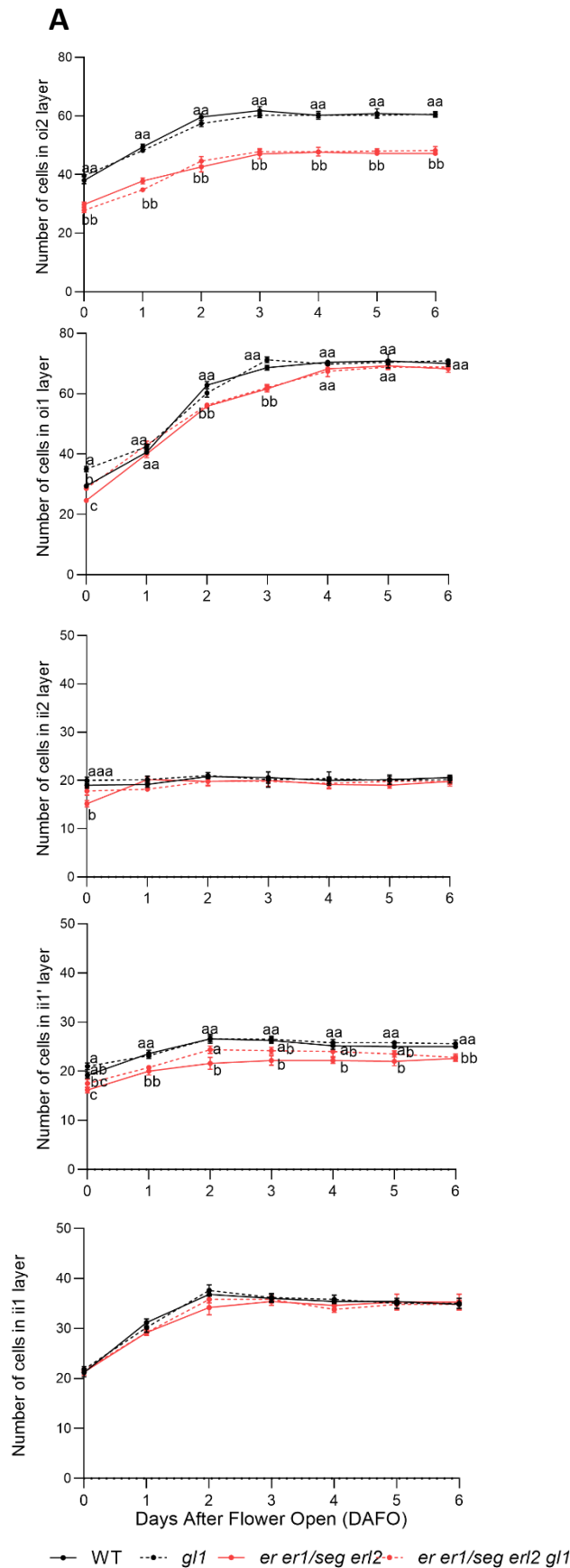


Figure 2-15: The ERF-induced, integument-specific cell proliferation and expansion results the smaller seed size of *erf* and *erf gl1* mutants at maturity. A. Number of cells along the circumferences of each seed integument layer oi2, oi1, ii2, ii1', ii1, respectively. B. Seed area (mm²) of WT, *gl1*, *er erl1/seg erl2* and *er erl1/seg erl2 gl1* from 0 to 6 DAFO. mean and s.e.m, n=5, repeated two times; statistically significant differences between samples were determined using two-way ANOVA Fisher's LSD test and denoted with different letters.

2.3.8 The *ERf* controls endosperm cuticle formation during early seed development, and interacts with *GL1* in that function

Recent studies have established the presence of a maternally derived endosperm-associated cuticle ([De Giorgi et al., 2015](#); [Ingram & Nawrath, 2017](#); [Loubery et al., 2018](#)) and also of an embryo-associated cuticle in the Arabidopsis seed ([Molina et al., 2008](#)). There is also an indication of an additional, external maternal cuticle, covering the seed coat together with waxes ([Ingram & Nawrath, 2017](#)). Notably, the endosperm-associated cuticle is derived from the ii1 seed coat layer and transferred to the endosperm at later stages of development ([Loubery et al., 2018](#)). Cuticles are fatty and waxy hydrophobic films ([Yeats & Rose, 2013](#)) that are thought to increase seed viability and dormancy in mature seeds ([De Giorgi et al., 2015](#)), through protection against oxidative stress. Given the demonstrated seed coat-mediated *ERf-GL1* control of seed germination (see above), and the overlap in the molecular pathways of seed dormancy and germination, I probed the possibility of *ERf*- and *GL1*-dependent properties of the endosperm-associated cuticle. Auramine O staining confirmed the presence of that cuticle in WT, as reported ([De Giorgi et al., 2015](#); [Loubery et al., 2018](#)) (Figure 2-16 WT photos) and showed that it was conserved in all mutants' seeds. A thin cuticle was visible in all lines already at 0 DAFO around the endosperm including in the chalazal region (Figure 2-16 a-c photos), with no detectable genetic differences at that early stage in cuticle thickness or staining intensity. However, over the next day, a clear trend appeared, indicating continued cuticle deposition in *erl1/seg erl2* and in the quadruple *er erl1/seg erl2 gl1* mutant seeds compared to WT and *gl1*. Daily observations over the next 5 days confirmed that trend and revealed early synergistic effects of *erf* and *gl1* mutations on cuticle formation between 1 and 2 DAFO, i.e., at the pre-globular and globular stages of embryo development. By 2 DAFO endosperm-associated cuticle thickness was significantly greater in *erf gl1* than *erf* seeds, and remained so over the following 3 days, when cuticle thickness reached a plateau, in all lines.

These data indicate a role of the *ERf* and then of *GL1* too in cuticle formation, and perhaps in cuticular composition. Increased cuticle thickness and compositional changes would have the potential to affect permeability between the seed coat and the seed internal compartments. Further high-resolution examination of the cuticle, and biochemical analysis of its composition are required to further investigate *ERf* members.

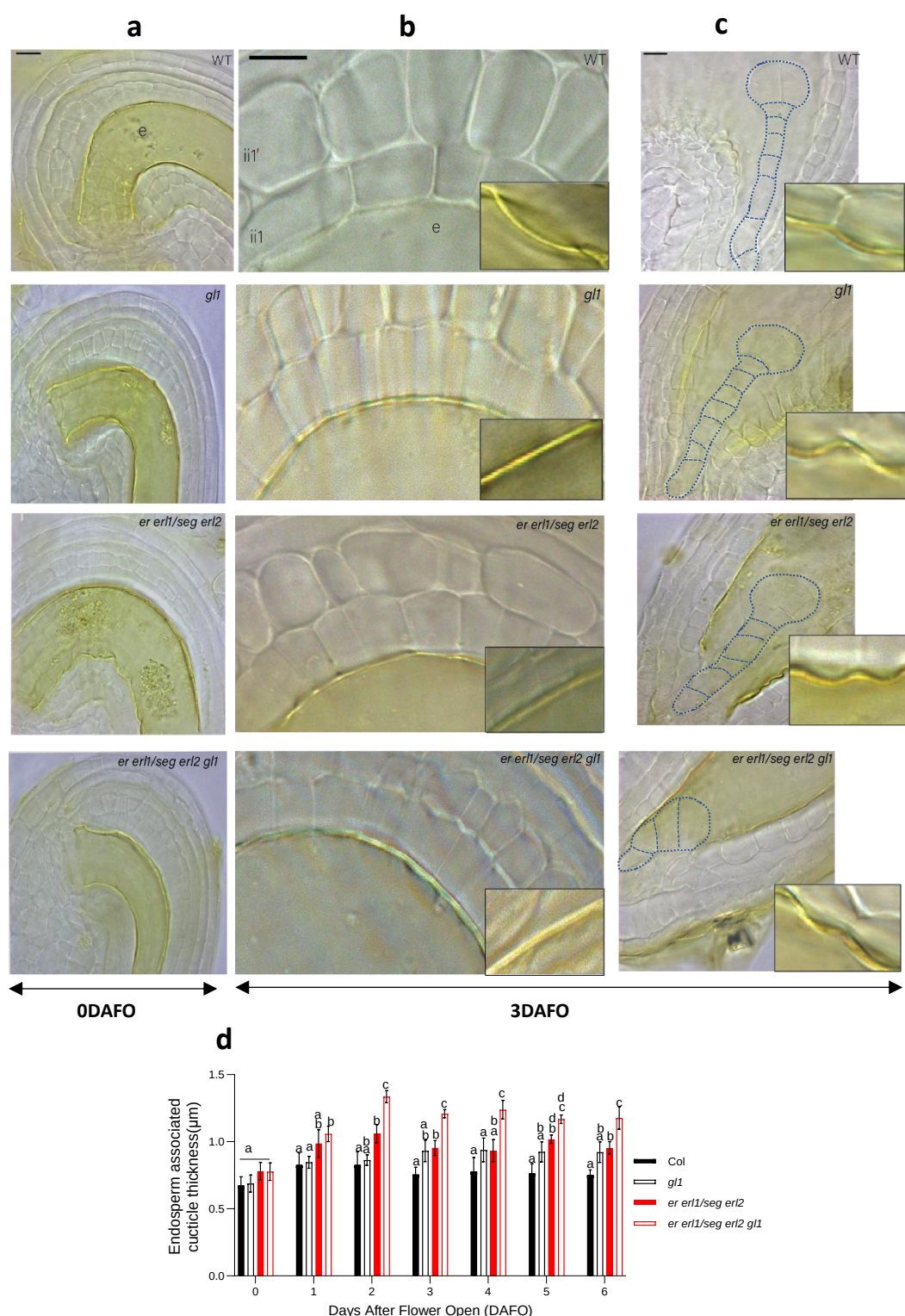


Figure 2-16: *ERF* and *GL1* synergistically interact in endosperm associated cuticular deposition. **a, b & c.** Microscopic images of the endosperm associated cuticle stained with Auramine O (30min) of 0 (**a**) and 3(**b** and **c**) DAFO of WT,*gl1*,*er erl1/seg erl2*, *er erl1/seg erl2 gl1*. **c-** illustrates the cuticular staining at the micropylar end at 3DAFO, where the growing embryo is attached to the seed via suspensor (marked with dotted blue lines). Enlarged views of the cuticle shown in the bottom right corner in **b** and **c**. staining time were increased for WT from 30min to overnight (enlarge view), for the quantitative analysis. Scale bars 10μm (**a** and **b**), 20μm in **c**. **d.** the thickness of the endosperm-associated cuticle (μm) of WT, *gl1*, *er erl1/ seg erl2* and *er erl1/ seg erl2 gl1*. Mean and s.e.m, n=5, repeated two times; statistically significant differences between samples were determined using two-way ANOVA Fisher's LSD test and denoted with different letters.

2.3.9 The *ERf* -*GL1* interacting pathways are involved in the development of the embryo suspensor

In the course of the microscopy examination of the time series of developing siliques sampled to investigate the *ERf* and *GL1* functions in the development of the seed maternal tissues, I noticed some defective or even degenerative embryo suspensors, also serendipitously noticed in some *erf* mutants in earlier experiments conducted in the Masle Lab (Kennedy, Nanda, Masle, unpublished data). To confirm the role of the *ERf* in the development of the embryo suspensor and investigate whether *GL1* was also implicated, I conducted a detailed systematic examination of cleared seeds excised from globular (2 DAFO, fully formed suspensor) WT, *gl1*, *er erl1/seg erl2* and *er erl1/seg erl2 gl1* siliques, using DIC light microscopy imaging. *erf* suspensors showed striking developmental defects (Figure 2-17). The Arabidopsis embryo suspensor developed from the basal cell during embryogenesis, undergoes transverse cell division and longitudinal cell expansion resulting in a stalk like structure ([Yeung & Meinke, 1993](#)) ((Figure 2-17 A & B-WT). Arabidopsis suspensor consists of a single cell file of 6-8 cells and is fully formed at the embryo globular stage ([Mansfield & Briarty, 1991](#)). The results showed that *erf* influences the shorter suspensor phenotype, through a reduced number of cells (Figure 2-17 A & B-*er erl1/seg erl2*). Loss of the function of *GL1* alone doesn't not induce a short suspensor phenotype; thus, *GL1* does not act upstream of the *ERf*, but enhances the *ERf*-induced short and fewer cell suspensor phenotype (Figure 2-17 A & B- *gl1*). However, *GL1* interacts with *ERf* to enhance the patterning defects of the embryo and suspensor induced by *ERf* loss of function in the *er erl1/seg erl2* mutant, with some seeds having two cell files in the suspensor instead of one in WT and *erf* mutants (Figure 2-17 A, B & C- *er erl1/seg erl2 gl1*). The time series of high resolution DIC images such as illustrated in Figure 2-17, will next be used to gain quantitative information on suspensor and embryo developmental defects, through anatomical and morphometric analysis that could not be done in the framework of this thesis for lack of time.

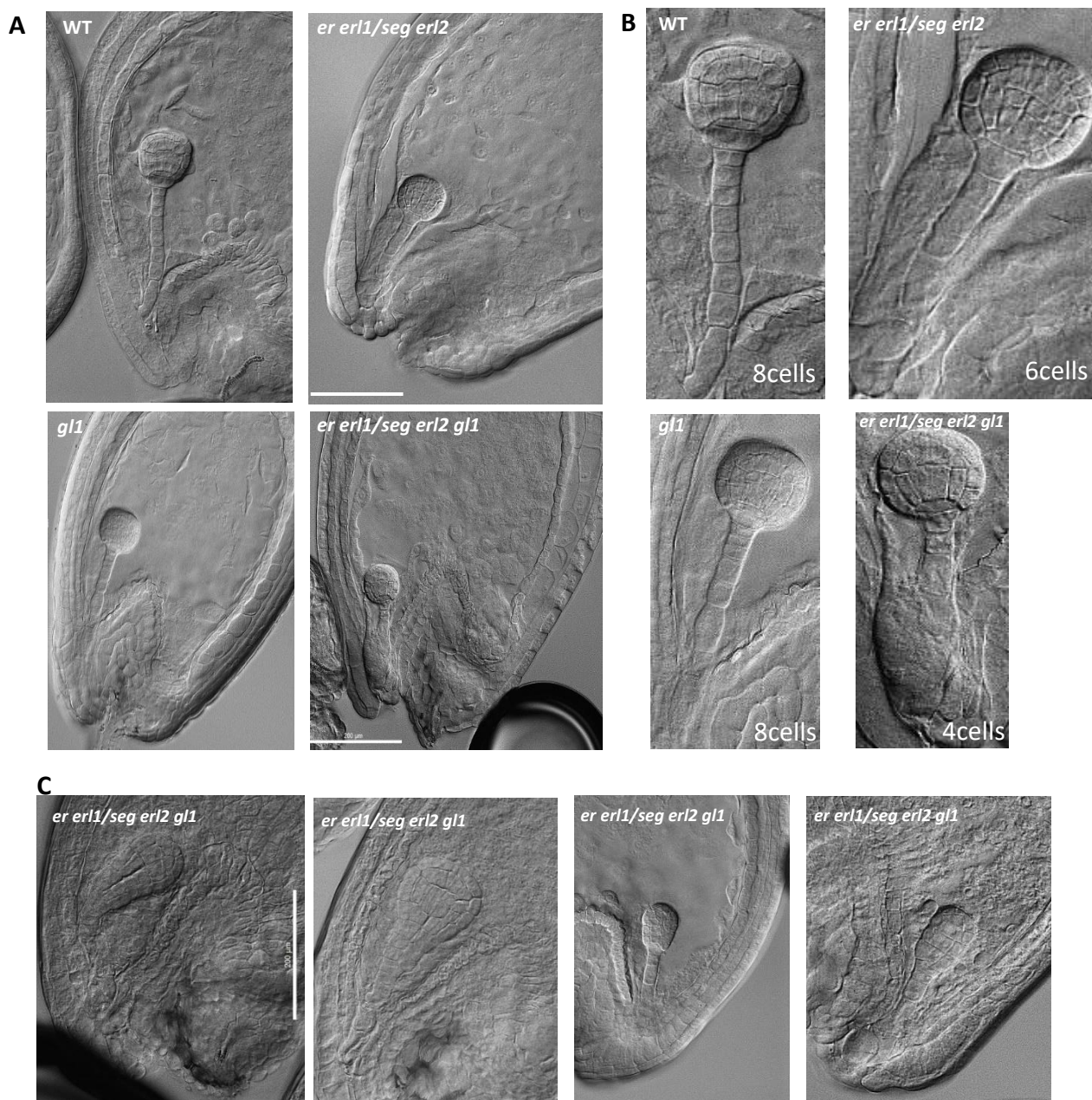


Figure 2-17: *Erf-GL1* synergistically interacts with cell growth and proliferation in the suspensor. A&B. the fully formed suspensor of the WT, *gl1*, *erl1/seg erl2* and *erl1/seg erl2 gl1* seeds at embryo globular stage (A) and the enlarged view of the suspensor (B) showing the decreased cell number and shortened suspensor phenotype in *erf* and *erf gl1* mutants. C. *erl1/seg erl2 gl1* shows the patterning defects of the embryo and the suspensor, embryo globular shape/development and suspensor one cell file has distorted. scale bar 200μm.

2.4 Discussion

2.4.1 The MYB transcription factor *GL1* synergistically interacts with the *ERf* in seeds in the regulation of germination

Cryptic genetic variation in a physiological attribute refers to variation that is released under specific conditions or exposure to novel environmental perturbations ([Hall et al., 2007](#); [Fu et al., 2009](#); [Lempe et al., 2013](#)). While long documented and having far-reaching evolutionary consequences ([Masel, 2006](#)), the underlying mechanisms are little known. Large cryptic genetic variation in the germination speed of non-dormant *Arabidopsis* seeds under salinity was recently reported and shown to be controlled by several QTLs, most of them distinct from QTLs for germination under optimal conditions ([Wei Yuan et al., 2016](#)). Causal genes remain mostly unknown.

In an earlier study, we discovered that the *Arabidopsis* *ERECTA* family of LRR-RLKs, known for its roles in aerial plant development, epidermal patterning, and immune responses, is a key player in seeds and acts to cue and pace germination according to environmental conditions ([Nanda et al., 2019](#)). Here, I report the discovery of another unsuspected regulator of seed germination and its modulation under adverse conditions. The data presented in this chapter provide evidence that loss of *GL1* function enhances seed germination sensitivity to salt stress in *erf* mutants lacking *ER* signalling, alone, or in combination with the loss of one or both of its two paralogues, *ERL1* and *ERL2*. Lack of functional *GL1* further increases the time required for the salt hypersensitive *erf* seeds to initiate and complete germination, and also the spread of germination completion within seed cohorts (Figure 2-1). The R2R3 MYB transcription factor *GL1/MYB0* is known as an indispensable component of the trichome-differentiation activator complex, specifically required for the initiation of trichomes ([Oppenheimer et al., 1991](#); [Payne et al., 2000](#); [Hülkamp, 2004](#); [Morohashi & Grotewold, 2009](#)). It has never been implicated in seed germination nor in any aspect of seed biology, nor in the *ERf* signalling pathways documented in multiple aspects of aerial development, or in the regulation of responses to biotic or abiotic stresses.

The different magnitudes of the germination-sensitising effect on salinity of the *gl1* knock-out mutation in the *er*, *er erl1*, *er erl2*, and triple *erf* mutant backgrounds indicate functional specificity between the *ERL1* and *ERL2* paralogues in their interactions with *ER* and *GL1*. The fact that *gl1* caused a small but significant shift in the kinetics of testa rupture, in opposite directions in the *er erl1* and *er erl2* backgrounds (Figure 2-1) provides additional evidence for specificity among *ERf* members and indicates antagonistic and *GL1*-dependent interactions of *ERL1* and *ERL2* with *ER*. Furthermore, the slower germination induced by loss of *GL1* function in *er erl2* but not *er erl1* in the absence of salinity

(Figure 2-1) shows that beyond exerting a strong control on cryptic genetic variation in germination speed under salinity, the *ERf* and *GL1* together also exert some control on constitutive germination, with again functional specificity between *ERL1* and *ERL2*.

The *ERf*-mediated regulation of seed germination was previously shown to be primarily maternally controlled through germination assays on F1 seeds derived from reciprocal crosses of WT and salt hypersensitive *er erl1* double mutant ([Nanda et al., 2019](#)). I extended that approach here and carried out germination assays of independent F1 seed batches from crosses between either *er erl1* or *er erl2*, furthermore carrying either functional *GL1* or *gl1* loss-of-function alleles with WT or *gl1* controls. These crosses enabled the comparison of the germination kinetics of seeds that all shared a heterozygous embryo of the same genotype at the *ERf* and *GL1* loci -since the *ERf* and *GL1* genes are all dominant genes- but were enclosed in either predominantly WT or predominantly *erf/gl1* mutant maternal tissues. The results a) confirm the maternal inheritance of *ER*, *ERL1* and *ERL2* functions in seed germination and b) demonstrate that the synergistic role of *GL1* is also primarily maternally determined.

As pointed out earlier, given the Arabidopsis seed structure, the above approach did not, however, allow to separate the contributions of the seed coat and of the underlying endosperm which, while being extremely reduced and not a reserved compartment to fuel embryo expansion in germinating seeds contrary to other species, plays important signalling roles during germination, and also the control of seed size, in interaction with the other two compartments ([Papi et al., 2000](#); [Garcia et al., 2003](#); [Luo et al., 2005](#); [Zhou et al., 2009](#); [Fang et al., 2012](#); [Li & Li, 2015, 2016](#); [Nanda et al., 2019](#); [Iwasaki et al., 2022](#)) . To bypass this issue, I here used a transgenic approach to restore *ERECTA* expression under the control of the well-characterised AtML1 epidermal-specific promoter in mutants carrying a constitutive *er* knock-out mutation, alone or in combination with constitutive knock-out mutations of *gl1* and *ERL1* or *ERL2*, the results show that this is sufficient to recapitulate WT germination level, in all *erf ±gl1*, or nearly so in the individual case of *er erl2 gl1*. They thus provide unambiguous evidence that the maternally-derived *ERf* and *GL1* function on the capacity of seeds to germinate under salinity and are both mediated cells with epidermal identity ([P. Z. Lu et al., 1996](#)). Moreover, the fact that overexpression of the *ERECTA* member alone in the seed epidermis was sufficient to compensate for the loss of both native *ER* and *ERL1* genes, or *ER* and *ERL2*, indicates a preponderant role of *ER* in the *ERf*-mediated control of seed germination, and also in the interactions between the *ERf* and *GL1* in that process.

The nature of the *ERf-GL1* dependent properties of the mature seed coat, and underlying mechanisms of action during seed germination are unknown. However, the experiments reported here provide important leads. They demonstrate novel roles of both the *ERf* and *GL1* on the patterning, anatomy,

morphological and spectral characteristics of the seed coat and, importantly, associations between *ERf*- and *GL1*-mediated differences in these attributes and in seed germination phenotypes. The salt-hypersensitive *erf* seeds during germination, *er erf1* and *er erf2*, show thicker anticlinal secondary cell walls in the seed coat oi2 surface layer, as well as broader columellae. These differences were amplified in the even more salt-sensitive *er erf2 gl1* seeds lacking *GL1* function, but remained unchanged in *er erf1 gl1* seeds as did the salt-sensitivity of germination.

These results indicate promoting interactive roles of loss of *ERf* and *GL1* functions in the deposition of the secondary cell wall material in the seed coat epidermis and its surface columellae. Together, these have the potential to modify the mechanical and permeability properties of the seed coat so as to slow down the reactivation of metabolism within *er erf2 ±gl1* germinating seeds, and to limit the capacity of the embryo to break through the seed coat. Future studies are required to test that proposition, but consistent with the literature, earlier experiments showed an increased induction of the mechano-sensitive *TCH3* gene preceding testa rupture in *er erf1/seg erf2* germinating seeds compared to WT ([Nanda et al., 2019](#)). That observation is suggestive of enhanced mechanical constraints imposed on *er105 erf1.2/seg erf2.1* embryos before endosperm rupture compared with the WT, in line with the anatomical and morphological *ERf*- and *GL1*- dependent modifications of the mature seed coat identified in the present study.

The thicker oi2 cell walls in the salt hypersensitive *erf* seed coat and collapsed underlying oi1, ii2 and ii1 cells are constitutively highly suberised. The columellae are so too, and they are mechanically protective of the seed interior. They are also the main anchoring points to the seed epidermis of the pectinous mucilage that gets expelled from the seed specialised epidermal cells upon contact with the soil solution, through an array of cellulose microfibrils that radiates from their top. That mucilage shell constitutes the first line of contact between the seed and its environment, and therefore of environmental change signalling to the seed coat and its interior. There is mounting evidence of the importance of cell wall sensing and signalling in biotic and abiotic stress responses. We previously reported the instrumental role of the seed mucilage in enforcing the germination delay observed in the salt hypersensitive *er erf1*, *er erf2* and *er erf1/seg erf2* seeds under exposure to salinity. The data presented here reveal the implication of the *GL1* transcription factor, indicating synergistic *ERf* and *GL1* roles on the germination modulatory role of the mucilage under salinity. The mechanisms remained unknown. They might involve *GL1-ERf* interactions in the synthesis and structure of the mucilage. We earlier reported different xylose to rhamnose ratios in WT and *er erf1/seg erf2* seed

water soluble mucilage fractions ([Nanda et al., 2019](#)), indicative of increased pectin branching and cross-linking, mostly by calcium bonds, in the mutant. And here, I report differences in the auto-fluorescence spectra of WT and salinity hypersensitive *erf* $\pm$ *gl1* mature seed coat, that suggest differences in the *ERf*- and *GL1*-dependent differences in the biochemistry and deposition patterns of wall material and mucilage. Together with the more spacious mucilage pockets exhibited by *er erf1/seg erf2 oi2* 10 days after pollination; the thicker secondary epidermal *oi2* cell walls and broader columella at maturity; the modified cellulose content of cell walls of *er* mutants as well as changes in uronic acid and neutral sugars reported in leaves ([Sanchez-Rodriguez et al., 2009](#)), these data all concur to indicate a direct role of the *ERf* in regulating the formation and architecture of both the mucilage and the cell walls in the seed coat. and thereby their biophysical modifications during seed imbibition and degradation by cell-wall-degrading proteins, and so the signalling of changes in the seed environment to the inner endosperm and embryo.

The implication of *GL1* in the germination promoting role of the mucilage in salt-hypersensitive *erf* backgrounds is fascinating. In aboveground organs, *GL1* controls trichome patterning through the formation of an activator complex with *GL3/EGL3* and *TTG1*, and induction of *GL2* and *TTG2* expression ([Ishida et al., 2007](#); [Morohashi et al., 2007](#); [Zhao et al., 2008](#)). Remarkably, these same molecular components are also expressed in developing seeds and known to act in epidermal cell differentiation, mucilage pectin synthesis and stabilization during embryo morphogenesis and seed maturation on the mother plant, through a pathway where *EGL3* similarly interacts with *MYB5* and *TTG1*, to activate a set of downstream transcription factors among which also *TTG2* and *GL2* (reviewed by ([North et al., 2014](#)));([Leonkloosterziel et al., 1994](#); [Rerie et al., 1994](#); [Debeaujon & Koornneef, 2000](#); [Nesi et al., 2000](#); [Penfield et al., 2001](#); [Johnson et al., 2002](#); [Zhang et al., 2003](#); [Western et al., 2004](#)). The present study strongly suggests that a) *GL1* may be an upstream component of that transcriptional network; b) that this network intersects with the *ERf*-mediated germination pathway. This constitutes an exciting area of future investigation.

2.4.2 *ERf* and *GL1* synergistically increase *Arabidopsis* endosperm-associated cuticle thickness

Cuticles are vital waxy and fatty hydrophobic protective barriers of epidermal origin, which line the surface of aerial organs ([Kolattukudy, 1980a, 1980b](#); [Kolattukudy & Espelie, 1985](#)), and protect the plant against water loss, biotic and abiotic stresses, and are essential to the control of gas exchange ([Riederer & Schreiber, 2001](#)). Interestingly, a thick cuticle, tightly bound to the outer walls of the endosperm was recently identified in mature *Arabidopsis* seeds ([De Giorgi et al., 2015](#)). That cuticle is produced by the inner integument layer 1 (ii1) early in seed development, hence is of maternal origin, and is subsequently transferred to the endosperm, around the torpedo and walking stick stage of embryogenesis ([Loubery et al., 2018](#)). It is 10 times thicker than the leaf cuticles ([De Giorgi et al., 2015](#)), and in the mature seed is fused to the endosperm on one side, and to the tannic cell walls of ii1 on the other side ([L. Demonsais et al., 2020](#)). These walls are impregnated with mostly proanthocyanidin derived from the flavonoid pathway, and are fused to the overlaying so-called “brown pigment layer (bpl)” ([Beeckman et al., 2000](#); [Debeaujon et al., 2003](#); [Kitamura et al., 2004](#); [Pourcel et al., 2007](#)) formed by the periclinal walls of the collapsed ii'1 and ii2 cells, and are also impregnated with the same tannic oligomeric flavonoids through diffusion from the ii1 layer during seed maturation.

Notably here, in the context of seed germination under saline conditions, the tannic periclinal ii1 cell walls were recently shown to in fact remain attached to the endosperm, together with the cuticle, after testa rupture ([Lara Demonsais et al., 2020](#)). From then on, the bi-layered structure formed by the cuticle and tannic ii1 periclinal walls therefore constitutes the physical barrier that directly separates the endosperm from the environment. That structure is thought to play important biophysical roles, with respect to permeability between the seed interior and the environment, and mechanical protection of seed inner compartments ([Loubery et al., 2018](#); [L. Demonsais et al., 2020](#)).

My observations of the seed coat in young developing seeds over the reported time span of endosperm cuticle formation, uncover promoting roles of both *ERf* loss of function, and *GL1* loss of function on endosperm cuticle formation (Figure 2-16). Consistent with the literature, the cuticle appeared fully formed by 3-4 DAFO, around the embryo heart stage, showing no subsequent thickening over the next 3 days. Cuticular compounds are highly hydrophobic ([Yeats & Rose, 2013](#)), and endosperm cuticles are thought to play a role in the maintenance of seed viability and dormancy. The formation of a thicker endosperm cuticle could be one of the mechanisms involved in the *erf-gl1* synergy in delaying germination under salinity stress, and maintaining seed viability in the salt hypersensitive during prolonged exposure to a saline environment.

It will be intriguing in further experiments to examine the biophysical properties of the fused endosperm cuticle and ii1 tannic walls above it gets exposed to the soil solution upon testa rupture. *GL1* and other transcription factors of the MYB-bHLH-WD40 complex have well-established roles not only in trichome initiation but also anthocyanin accumulation (Qi et al., 2011; Wei et al., 2019). PAs are known to reinforce seed dormancy, and in non-dormant seeds to regulate seed coat permeability and water uptake (Li et al., 1993; Debeaujon & Koornneef, 2000; Lepiniec et al., 2006; Debeaujon et al., 2007), and analysis of the *tt8* transparent testa mutant affected in PAs biosynthesis in the seed coat suggested a protective role of PAs against oxidative stress (Jia et al., 2012) and their involvement in ROS and ABA signalling during germination. Overall, this raises the possibility that *GL1* and *ERf*, separately and together, may interact with the biosynthesis and deposition of PAs in the seed coat during seed development, as they do for endosperm-associated surface cuticular compounds (Figure 2-16), their absence promoting the production and possibly the composition and structure of both the tannic and cuticular layers of the seed coat, and thereby delaying the initiation of germination through testa rupture and slowing down its progression in *erf ± gl1*, or even causing a temporary arrest after testa rupture, via a decreased permeability and enhanced antioxidative capacity of the combined mucilage, tannic ii1 and cuticle covering the endosperm (Tanaka et al., 2004; Dominguez et al., 2009; Espana et al., 2014; De Giorgi et al., 2015). As discussed in the (L. Demonsais et al., 2020) study, seeds can temporality arrest germination after testa rupture, releasing their tannin content to the environment and together with the cuticle, making an impermeable barrier to the water and oxygen.

2.4.3 A direct signalling role of the *ERf* and *GL1* during germination

The regulation of seed germination involves complex interactions of exogenous and endogenous factors. Among the latter, the major phytohormones ABA and GA play prominent and extensively documented antagonistic roles, through a negative feedback loop whereby ABA inhibits germination while GA promotes it. That hormonal signalling loop involves interactions with ethylene and brassinosteroids, as well as ROS. The induction of *RGL2* expression, a negative regulator of GA signalling, and of the ABA signalling transcription factors *ABI3* and *ABI5* during germination under salt stress have been reported in WT *Arabidopsis* seeds (Yuan, 2011). That induction was enhanced in *erl1/seg erl2* seeds which also showed an increased sensitivity to exogenous ABA application during germination (Nanda et al., 2019); Figure 2-3) Furthermore, *RGL2*, *ABI3* and *ABI5* were upregulated during seed imbibition and testa rupture as occurs in dormant seeds (Koornneef, 1982; Giraudat et al., 1992; Leon-Kloosterziel et al., 1994; Finch-Savage & Leubner-Metzger, 2006; Lefebvre et al., 2006; Weitbrecht et al., 2011a). Elevated *RGL2* often leads to repress testa rupture (Piskurewicz et al., 2008).

The experiments presented here confirm these findings and provide evidence that the *ERF-GL1* synergy in reducing germination sensitivity to salinity is also partially ABA-dependent (Figure 2-3). This is the first time that *GL1* is shown to be involved in an ABA-GA signalling loop. Interestingly, however, DELLA proteins GAI, RGA1 and RGL2 have all been demonstrated to physically interact with *GL1* to repress its transcriptional function in trichome initiation in aerial organs ([Perazza et al., 1998](#); [Maes et al., 2008](#); [Qi et al., 2014](#)). All act to repress GA responses and are involved in gibberellic acid mediated signalling during seed germination ([Lee et al., 2002](#); [Tyler et al., 2004](#); [Cao et al., 2005](#)). Furthermore, the GA responsive GIS transcription factors function upstream of the trichome development MYB-bHLH-WD40 complex - GL1/MYb23-GL3/EGL3/TTG1 -, consistent with the reported upregulation of *GL1* by GA. It will be interesting in future studies, to explore whether similar interactions between the germination-promoting GA signalling pathway and the MBW complex are also involved in effecting the *GL1*-mediated promotion of seed germination under salinity uncovered in the present study.

Additionally, of most interest here, is the recent report of a novel non-canonical function of *ERF* proteins ([Sarnowska et al., 2023](#)) where the *ERF* kinases undergo endocytosis and promote phosphorylation of the SWIB3 component of the chromatin modelling complex in the nucleus and its binding to the *GID1* GA receptor, as well *GD1a* transcription, thereby promoting the degradation of DELLA proteins and thus the derepression of GA response necessary to germination. *er erl1 erl2* seeds show reduced *RGL2*, *ABI5*, *ABI3* and *GD1a* expression ([Nanda et al., 2019](#); [Sarnowska et al., 2023](#)). It is therefore tantalising to speculate that besides interacting in seed development on the mother plant, the *ERF* and *GL1* might also interact during germination itself through interactive roles in the transcriptional control of GA receptors and DELLA proteins, hence GA signalling and thereby MBW formation SWIB3 phosphorylation and binding to *GD1a*, and reduced *GD1a* transcription, to facilitate the release of *GL1* from DELLAs and hence the formation of MBW as well as GA-mediated cell wall degradation in the endosperm ([Nambara et al., 1991](#); [Jacobsen & Olszewski, 1993](#); [Ogawa et al., 2003](#)) and GA-ABA homeostasis to promote germination. The central role of ABA, and antagonism with GAs in the regulation of germination of both dormant and non-dormant have been extensively documented ([Kucera et al., 2005](#); [Muller et al., 2006](#); [Holdsworth et al., 2008](#); [Graeber et al., 2012](#); [Nonogaki, 2014](#)). How they are affected by salinity is not entirely clear. The current model is that salinity and drought promote ABA synthesis in the endosperm and release to the embryo, thereby activating the transcription of embryo growth repressors, such as the *ABI3* and *ABI5* transcription factors, and also triggering an auto-feedback loop that maintains *RGL2* mRNA levels high and represses cell wall modifying enzymes ([Giraudat et al., 1992](#); [Finkelstein & Lynch, 2000](#); [Lopez-Molina et al., 2001](#); [Lee et](#)

[al., 2002](#); [Lopez-Molina et al., 2002](#); [Piskurewicz et al., 2008](#); [Piskurewicz et al., 2009](#); [Graeber et al., 2014](#); [D. Yan et al., 2014](#)).

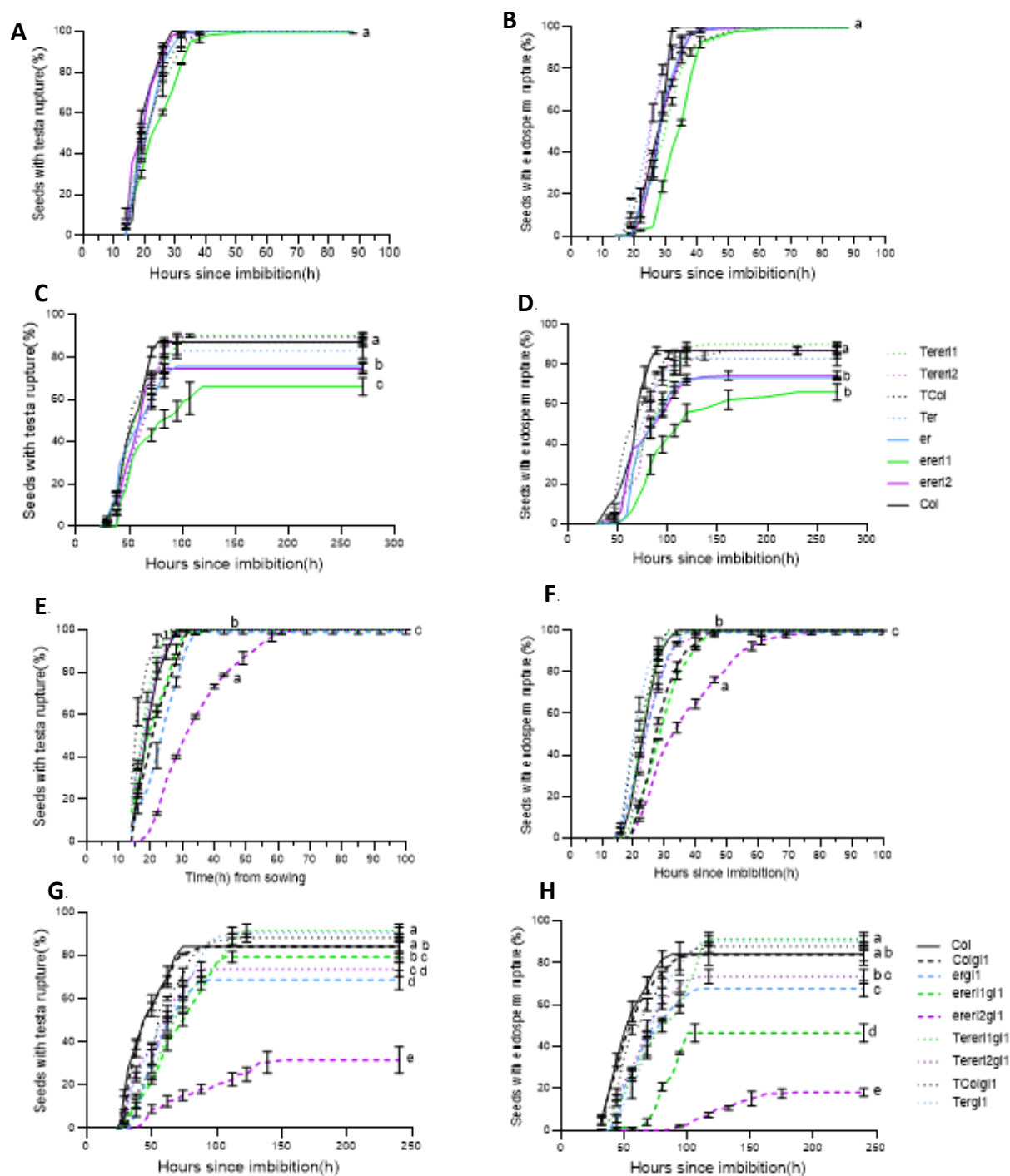
A broader *ERf-GL1* role of *ERf* and *GL1* in the hormonal regulation of germination is likely, in the first instance through auxin and jasmonic acid. Numerous developmental functions of the *ERf* involve auxin. Moreover, a recent study in tomato reported a role of *S/ER* in seed germination, which beyond extending our earlier finding in *Arabidopsis* to another species, suggests that this function is mediated via auxin signalling in interaction with ABA ([Chen et al., 2024](#)). In addition, Jasmonate Zim-Domain (JAZ) proteins which act as JA co-receptors and transcriptional repressors in JA signalling have been shown to interact with *GL1* and other transcription factors of the MYB-bHLH-WD40 complex in aerial organs.

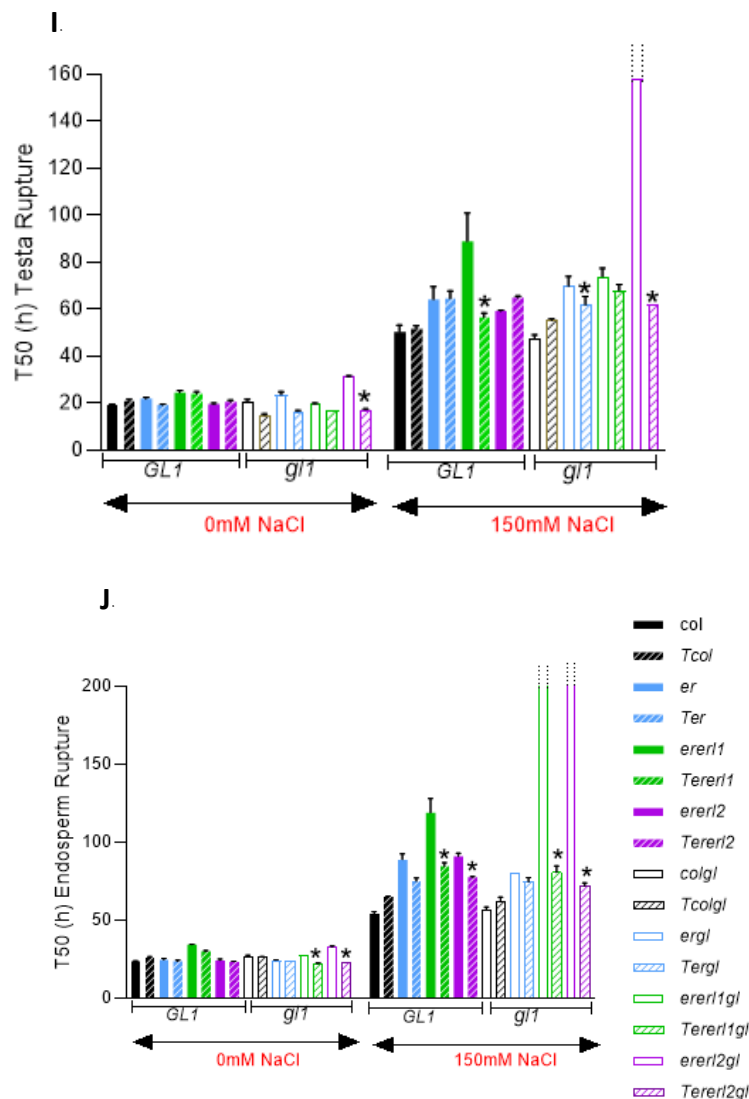
GL1 loss-of-function has no influence on its own, but dramatically enhances the germination delay and spread of all four *erf* salt-hypersensitive tested demonstrates that *GL1* is not integral to the *ERf* signalling pathway in seed germination; *ER* promotes seed size, in a combinatorial dependent manner with *ERL1* and *ERL2* ([Nanda et al., 2019](#); [Wu et al., 2022](#)), and the salt-hypersensitive mutants are characterised by smaller seeds than WT or WT-like *erl1*, *erl2*, *erl1 erl2*, suggesting partly overlapping mechanisms in the *ERf*-mediated functions on seed germination and seed size, this is not the case of *GL1*. *GL1* loss of function had no detectable influence on seed size, whether in a WT or in the *erf* salt hyper-sensitive background. Furthermore, while the *ERf* had a strong promoting role on cell proliferation in the oi2 surface layer of young developing seeds compared to *er erl1/seg erl2 erf* mutant seeds, and to some extent in the ii1' layer of their inner integument, the presence or absence of functional *GL1* was neutral. Altogether, these observations indicate that *GL1* acts through an overlapping but distinct pathway, intersecting downstream of the *ERf*.

The next Chapter reports on a transcriptomic analysis of *erf* and WT developing seeds, through the time course of the critical developmental window 0-6 DAFO identified in the above experiments, as a first step to investigate the interacting *ERf-GL1* molecular pathways in seed development, and their maternal control of seed germination.

2.5 Supplementary Figures

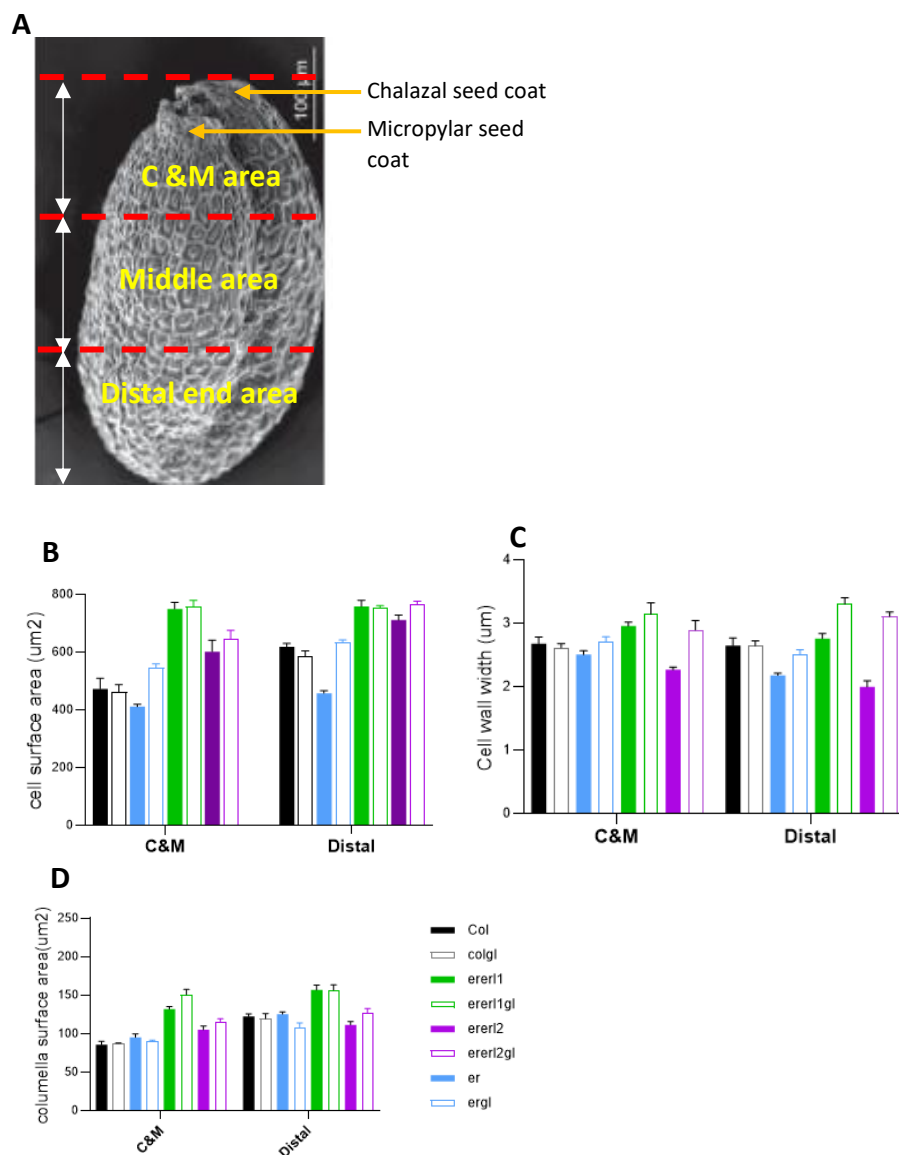
2.5.1 Supplementary Figure 1





Supp figure 2-1. Expression of *ER* in epidermis under different combination of *ERf* +/-*GL1* rescued the *ERf* induced salt hypersensitive germination phenotype. A-D. Percentage of endosperm and testa ruptured seeds from Col, TCol, *erf GL1* and *Terf GL1* under 0mM (A&B) and under 150mM (C &D). E-H. Percentage of endosperm and testa ruptured seeds from Col, *gl1*, *Tgl1*, *erf gl1* and *Terf gl1* under 0mM (E&F) and under 150mM (G&H). Means and s.e.m. (n = 3 plates, 30 seeds per genotype in each plate; experiment repeated 3 times. Statistically significant germination kinetics were determined by one-way ANOVA and Fisher's LSD pairwise test and, denoted by different letters. **I&J. Time(h) taken to reach 50% testa rupture (**I**) and endosperm rupture (**J**) Statistical significance was determined by a two-way ANOVA and Fisher's LSD pair-wise test and denoted by different letters (p<0.05).**

2.5.2 Supplementary Figure 2



Supp figure 2-2. The loss of function of *ERf -GL1* influences the cell expansion and cell wall deposition of the *Arabidopsis* mature seed coat outer integument layer. A. Three locations of the seed considered for quantitative analysis. B-D. Cell surface area (μm^2) (B), wall width (μm)(C) and columella surface area (μm^2) (D) of Col, *gl1*, *er*, *er gl1*, *er erl1*, *er erl1 gl1*, *er erl2*, and *er erl2 gl1* of mature seed outer epidermal layer (oi2). Experiment repeated with two separate seed batches- batch 1 and batch 2.

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Chapter 3

The *ERf-GL1* regulatory molecular network during early seed development

3.1 Introduction

It was recently established that the Arabidopsis *ERECTA* gene family (*ERf*) promotes germination under salinity and osmotic stress ([Nanda et al., 2019](#)). My results in the previous Chapter confirmed that finding, and further, identified the *MYB R1-R3 GL1* transcription factor, previously known for its role in trichome development as another novel player in the control of seed development and seed germination, and a novel interactor of the *ERf*. The function of both genes in seed germination is maternally derived, and essentially mediated by the seed coat only, and is mostly conditional in its severity on salinity level in the seed environment. Some of the seed coat properties and mechanisms likely involved were discussed in the previous chapter, and are primarily determined during seed development on the mother plant.

Seed coat development and differentiation is a critical process that ensures seed protection, viability, and successful germination. It is tightly regulated by a complex gene network that controls cell division, expansion, differentiation, and biochemistry and also the storage in the mature seed coat of sets of mRNA transcripts and proteins which, upon seed imbibition are instrumental in metabolism reactivation and fuelling the expansion of the embryo, and in the hormonal signalling network between embryo-endosperm and seed coat.

Microscopy observations of the developing and mature seed coat showed that the *ERf* exerts a layer-specific control over several key processes, including cell proliferation, cell expansion, epidermal cell wall formation and cuticle formation, during early seed development. Notably, loss of *ER* function alone or in combination with its paralogues *ERf* and *GL1* induced reduced cell proliferation in the seed coat and altered expansion patterns, along with a thicker endosperm cuticle compared to wild-type (WT) seeds, which is essential for intra-seed and seed-environment exchanges. Additionally, the *GL1* transcription factor synergistically enhances the effect of *ERf* secondary cell wall formation during seed maturation. Furthermore, the role of mucilage, which starts to be secreted by the outermost seed coat layer from around 4 DAFO, plays a key role in the *ERf* and *GL1* germination-promoting function ([Nanda et al., 2019](#)) (Chapter 2, Figure 2-2). The endosperm cuticle and mucilage play a crucial role during germination, facilitating the permeability and hydrophilic nature around the seed, especially under stressful environments. In addition, *ERf*-induced sensitivity to salinity during germination shows partial

overlap with a function of *ERf* on seed size ([Nanda et al., 2019](#)) which is determined during the first five days of seed development ([Beeckman et al., 2000](#); [Western et al., 2000](#)). As a whole, these results suggested a complex role of the *ERf* and *GL1* in regulating seed development and modifying the characteristics of the mature seed epidermis, and they identified an early developmental critical window between 1 and 6 DAFO. Here, I report the results of a genome-wide transcriptomic analysis of developing seeds and enclosing siliques during that critical period in WT and the most hypersensitive *er erl1/seg el2* mutant, in the presence or absence of functional *GL1*.

Previous studies have identified a vast number of genes involved in the regulation of early seed development. Among those, and as examples, the gene INNER NO OUTER (INO) is specifically expressed in the outer integument and is essential for its growth and symmetry ([Villanueva et al., 1999](#); [Voiniciuc, Yang, et al., 2015](#)); ABERRANT TESTA SHAPE (ATS) regulates the growth and curvature of the integuments ([Leon-Kloosterziel et al., 1994](#); [Debeaujon & Koornneef, 2000](#); [McAbee et al., 2006](#)); KANADI (KAN) genes are involved in the polarity and differentiation of integumental tissues ([McAbee et al., 2006](#); [Voiniciuc, Yang, et al., 2015](#)), NAC family transcription factors (TFs) NARS1 and NARS2, regulate seed coat and embryo development ([Kunieda et al., 2008b](#)); TRANSPARENT TESTA (TT) genes, such as TT2, TT8, and TTG1, regulate the biosynthesis of PAs and other flavonoids in the seed coat ([Debeaujon & Koornneef, 2000](#); [Johnson et al., 2002](#); [Zhang et al., 2003](#); [Western et al., 2004](#); [Gonzalez et al., 2009](#)); MUCILAGE MODIFIED (MUM) genes, including MUM2 and MUM4, are essential for mucilage biosynthesis and secretion ([Western et al., 2001](#); [A Macquet et al., 2007](#); [Dean et al., 2017](#)); SHINE (SHN), a member of the AP2/ERF family of transcription factors, regulate cuticle formation by controlling the expression of cutin biosynthetic genes ([Aharoni et al., 2004](#); [Kannangara et al., 2007](#)); FIDDLEHEAD (FDH) and BODYGUARD (BDG) genes are involved in cutin and wax biosynthesis, contributing to the integrity and functionality of the seed coat cuticle ([Pruitt et al., 2000](#); [Kunst & Samuels, 2003](#); [Kurdyukov et al., 2006](#); [Jakobson et al., 2016](#)).

In addition to these genetic regulators, phytohormones, or plant hormones, are crucial regulators of seed development and germination. It is well established that the ABA and GA strictly seed dormancy and germination. In a previous study ([Nanda et al., 2019](#)) and in chapter 2, we established that the *ERf*-*GL1* interact with the ABA signalling loop during seed imbibition, and the two-step germination process. ABA is a key regulator of seed dormancy and maturation. It accumulates during seed development, promoting the synthesis of storage proteins and lipids while inhibiting premature germination. ABA's role in maintaining dormancy is well-documented; ABA induces dormancy by

repressing GA biosynthesis and promoting the dormancy-related genes ([Nambara et al., 1991](#); [Nambara & Marion-Poll, 2005](#)); (Reviewed in ([Kucera et al., 2005](#))) ([Finkelstein et al., 2008](#)). The ABA signalling pathway involves several components, including PYR/PYL/RCAR receptors, PP2C phosphatases, and SnRK2 kinases which regulate ABA-responsive gene expression ([Fujii et al., 2007](#)); (Reviewed in ([Cutler et al., 2010](#))). GA antagonises the effects of ABA and promotes seed germination by degrading DELLA proteins, which are negative regulators of GA signalling. During germination, GA stimulates the synthesis of hydrolytic enzymes that degrade the endosperm cell walls, providing nutrients to the growing embryo. GA also promotes the elongation of embryonic cells, facilitating radicle protrusion through the seed coat ([Sun & Gubler, 2004](#)); (Reviewed in ([Holdsworth et al., 2008](#))) ;([Yamaguchi, 2008](#)). Auxin and cytokinin (CK) also play a crucial role in cell division and expansion during seed development. CKs also interact with ABA and auxins, influencing the hormonal crosstalk that determines seed dormancy and germination ([Khan, 1971](#); [Black et al., 1974](#); [Thomas et al., 1997](#); [Werner & Schmülling, 2009](#); [Mok, 2019](#)) whereas ethylene promotes seed maturation and desiccation and facilitates seed germination by enhancing the breakdown of ABA and promoting GA biosynthesis ([Achard et al., 2003](#); [Linkies et al., 2009](#)) This well-studied interplay between these major phytohormones ensures proper seed development and modulates germination.

My primary aim in this chapter was to gain insights into molecular components of the newly identified *ERf-GL1* maternally controlled pathway in Arabidopsis seeds, with a focus on early seed development, and to establish a baseline for the future identification of salinity-sensitive candidate genes in the regulation of seed germination, through genome-wide RNA sequencing (RNA seq). RNA seq has been widely used in plant biology research for genome-wide transcriptomic study and analysis in relation to growth stage, tissue, or environmental conditions. The transcriptome is a complete set of RNA molecules, including messenger RNAs (mRNAs), non-coding RNAs, and other RNA species produced by the cell in an organism or a specific tissue. Whole-genome RNA sequencing allows the identification and quantification of various RNA species and variants at a particular developmental stage of a particular organ, tissue, or now even cell, in a given environment. Given the novelty of evidence showing *ERf* and *GL1* functions in seeds, I undertook a genome-wide, unbiased investigation of the transcriptomes associated with knock out mutations of *ERf* members and *GL1* over a time point series spanning early embryogenesis and expansion of the seed envelopes to their final size.

3.2 Material and method

3.2.1 Sample collection for RNA extraction

The Arabidopsis accessions Col-0 (CS1093) and Col *gl1* (CS3079) were used as wild-types (WTs) in this study. As the *er105 erl1-2 erl2-1* triple mutant is sterile, the segregating progeny of *er105 erl1-2/+ erl2-1* and *er105 erl1-2/+ erl2-1 gl1* plants were used and will be referred to as *er erl1/seg erl2* (or *erf*) and *er erl1/seg erl2 gl1*(or *erf gl1*). A fresh batch of seeds was developed from the seeds used in our earlier publication ([Nanda et al., 2019](#)). Plants of all genotypes were grown in soil simultaneously, with an ample water and nutrient supply, within a growth chamber providing a constant 21°C temperature, 16-hour day length and a light intensity of 120-130 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The biological age of the siliques was marked daily on the flower pistil using water-soluble colour pens, over a 6-day period from flower opening (0 DAFO). The developing siliques (20 siliques for 0 DAFO, and 10 siliques for 3, 5 and 6 DAFO per one biological replicate) were collected daily and placed on the dry ice immediately after being severed from the inflorescence, and then snap-frozen in liquid nitrogen, and stored at -80°C freezer until RNA extraction.

3.2.2 Germination assays

Mature seeds were collected from the same plants as used for RNA seq analysis of developing siliques, and stored together before use, and for a minimum of one month to release dormancy. For germination assays, seeds were surface-sterilised using a 1:9 solution of 1.25% sodium hypochlorite: 100% ethanol, rinsed twice with 100% ethanol and left to air dry before being plated on modified Hoagland's media (2 mM KNO₃, 5 mM Ca[NO₃]₂ .4H₂O, 2 mM MgSO₄ .7H₂O, 2 mM KH₂PO₄, 0.09 mM Fe-EDTA and micronutrients), at pH 5.8, with 0.7% Type M Agar (Sigma-Aldrich A4800) containing 0mM or 150mM NaCl. Seeds were stratified at 4°C, in the dark for two days, directly on plates, before being transferred to a growth chamber and exposed to continuous light of 100-115 $\mu\text{mol m}^{-2}\text{s}^{-1}$ intensity and a constant temperature of 21°C. Germination was scored daily over a 10-day period, and the data were represented as % seeds showing testa or endosperm rupture., to check for reproducibility of results and eliminate the possibility of confounding seed batch effects in the interpretation of RNA seq analysis done on the same plants (Similar results as reported previously ([Nanda et al., 2019](#)) were obtained (Figure 3-1).

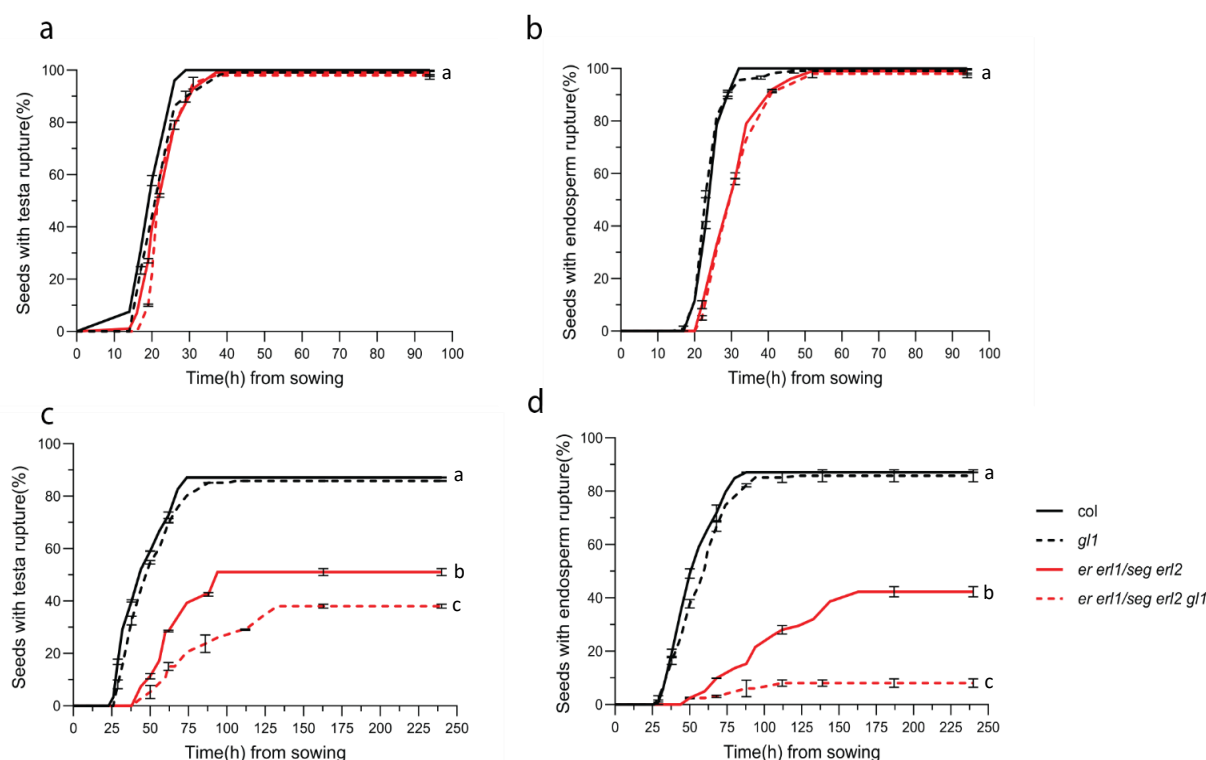


Figure 3-1: Germination of the loss of function of *ERf* (*er erl1/seg erl2*), *GL1* (*gl1*) and *ERf GL1* (*er erl1/seg erl2 gl1*) seeds under control-0mM NaCl (a & b) and salt stress-150mM NaCl (c & d). Germination was scored daily over a 10-day period, and the data were represented as % seeds showing testa (a & c) or endosperm rupture (b & d). Data are means (+_SE) n = 3 plates, 30 seeds per genotype in each plate; Experiment repeated 3 times. Statistical significance was determined by a one-way ANOVA and Fisher's LSD pair-wise test and denoted by different letters (p<0.05).

3.2.3 RNA extraction and quality control

The Qiagen RNeasy Plant Mini Kit (cat. nos. 74903 and 74904) was used for total RNA extraction with buffer RLT per the manufacturer's instructions. In the present experiment, the early critical development window from 0 to 6 DAFO, reported in Chapter 2, was selected as the most likely to yield information on the *primary* molecular agents of the *ERf* and *GL1* interactive maternal control of seed germination (Nanda et al., 2019; Chapter 2 herein). Total RNA was extracted from five biological replicates of 10 pooled 0 to 6 days-old developing siliques sampled 0 DAFO, 3 DAFO, 5 DAFO, and 6 DAFO. Total RNA quality was verified and approved using the Agilent Bioanalyzer (2100 expert) Plant RNA Nanochip (Figure 3-3., Supp figure 3-2; and Supp table 3-1). Only samples with RNA integrity number (RIN number) ≥ 7 were retained for further analysis; 4ug of RNA was dried in an RNA

stabilisation matrix and sent to GENEWIZ Azenta Life Sciences, China (<https://www.genewiz.com/>) following instructions. for whole transcriptome sequencing.

3.2.4 Transcriptome sequencing and Sequencing data quality assessment

Library construction, purification, quality control and quantification, sequencing cluster generation and high throughput Illumina sequencing were carried out by GENEWIZ from Azenta Life Sciences, China (<https://www.genewiz.com/>). cDNA libraries were prepared from poly(A) mRNA isolated using Oligo(dT) beads. The libraries were sequenced on an Illumina platform using a 2x150bp paired-end configuration, with a target depth of 20M pair reads per sample (40M total reads) ensure comprehensive coverage and accurate differential expression analysis. The various steps of the analysis I conducted on the returned raw data are summarised in the Flow Chart depicted in Figure 3-2. Bcl2fastq (v2.20.0.422) was used to process the original image data for base calling and preliminary quality analysis. The raw data (Pass Filter Data) obtained from this step was stored in FASTQ format, which contains the base sequence and the corresponding sequencing quality information. The quality of the sequencing data was evaluated using FASTQC (V0.10.1). We ensured high data quality with a Phred score of Q30 or higher, meaning that 95% of the bases had an error rate of less than 0.1%. ($Q = -10 \log_{10} P$, where P is the probability of an incorrect base call. A higher Phred score indicates a lower likelihood of an error). Per base quality score, overall sequence quality score distribution, and base composition (A/T and G/C content to avoid reading GC -rich regions and AT-rich regions) was assessed by FastQC (v0.10.1).

3.2.5 Mapping and generation of count tables with featureCount

Before data analysis, low-quality reads, contaminations and adaptor sequences were removed using Cutadapt software (version 1.9.1). In that step, adapter sequences, any bases at the 5' or 3' ends of the read that are marked as "N" or of quality score <20, and reads that were less than 75bp long after trimming, were eliminated. The filtered data were then aligned to the reference genome using Hisat2(v2.0.1) with default parameters ([Kim et al., 2015](#)), Species: *Arabidopsis thaliana*, Origin: Ensembl, Version: TAIR10). However, raw alignment output is not sufficient for biological interpretation. Mapping results should be analysed with respect to genome coverage for the genomic features of interest, such as genes, exons, introns, promoters, and other regulatory regions. Assessing coverage in these areas helps to determine whether the RNA-seq data provides adequate

representation of the transcriptome and allows for a meaningful biological interpretation. Many next-generation-sequencing pipelines use counts of the number of reads that overlap with predetermined genomic features of interest. In this experiment, the counts were summarised at gene levels by counting all the reads that overlap any exon, for each gene, using featureCount (reference annotation-GFF file format, TAIR10). These read counts were then used for count-based statistical analysis for differential gene expression analysis (DEGs) ([Oshlack et al., 2010](#); [Liao et al., 2013](#); [Liao et al., 2014](#)).

3.2.6 Identification of Differentially Expressed Genes (DEGs) using edgeR, Glimma, and limma-voom.

The bioinformatics pipeline introduced by ([Law et al., 2014](#); [Law et al., 2016](#); [Law et al., 2020](#)) was used for the identification of DEGs, with some modifications tailored to the experiment.

Data pre-processing

The count data generated by featureCount were imported into R and organized using the edgeR Bioconductor package for pre-processing and quantification of transcripts. Pre-processing filtering of data was carried out to remove rRNA contaminations. Raw counts were then converted to Counts per Million reads (CPM), and log2-counts per Million (log-CPM) values using the *cpm* function in *edgeR* to normalise for differences in library sizes. The genes that were lowly expressed were then filtered out using the *filterByExpr* function. By removing the genes with count=0 for all samples and then by removing the genes that did not have a significant number of reads in any sample, using the default function that keeps genes with a minimum of 10 read counts., The actual filtering uses the CPM values; the median library size in our experiment is `20M and therefore $10/20=0.5$; the function keeps the genes that have CPM 0.5 or more in at least five samples. Gene expression distribution was then normalised by the *trimmed mean of M-values* (TMM) method using the *calcNormFactors* function in *edgeR* to ensure that each sample had a similar range and distribution of expression value. Then, unsupervised clustering of samples was performed using *glimmaMDS* made in package Glimma.

Differential expression analysis-limma (negative binomial distribution)

Differential expression analysis was done in *limma* ([Ritchie et al., 2015](#)) with *voom transformation-limma-voom* ([Law et al., 2014](#)). Limma uses the TMM normalised log CPM values and performs the mean-variance relationship using precision weights calculated by the Voom function. Contrasts were

set as *gl1* vs *Col*, *erf* vs *Col*, *erfgl1* vs *erf* and *erfgl1* vs *gl1* to identify the differentially expressed genes between genotypes at each of the four selected developmental stages between 0 to 6 DAFO. Genes with adj P values ≤ 0.05 (Benjamini-Hochberg false discovery rate/adjusted p-value) were considered significant.

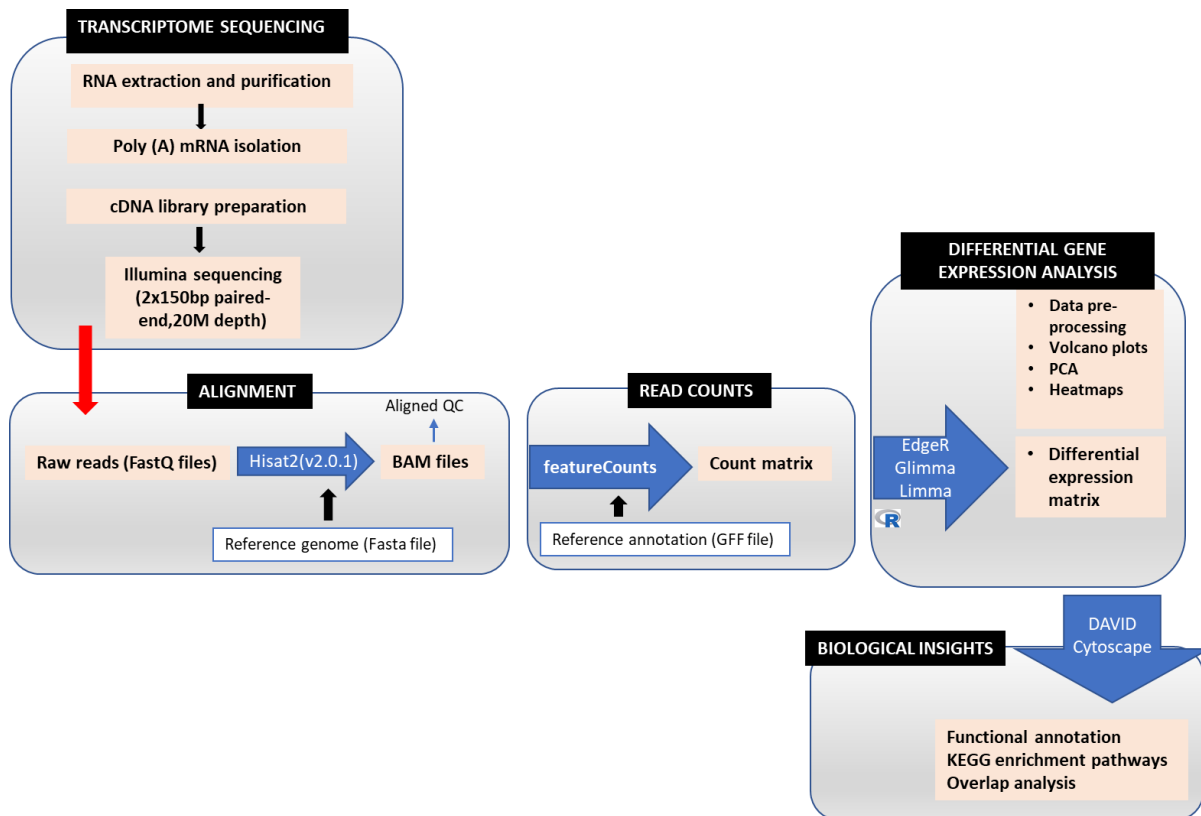


Figure 3-2: The RNA sequencing and subsequent data analysis workflow.

The high-quality RNA was used to isolate the mRNA using Oligo(dT) beads. The isolated mRNA is then converted into complementary DNA (cDNA) libraries, sequenced using the Illumina platform with 2x150 base pairs (bp) paired-end reads at a depth of 20 million reads. The raw sequencing reads in FASTQ format are aligned to a reference genome (FastaQ files) using Hisat2 (v2.0.1), resulting in Binary Alignment Map (BAM) files that undergo quality control (QC). The read counts are quantified using featureCount, producing a count matrix. Differential gene expression analysis is performed using EdgeR, Glimma, and Limma R packages, which includes data pre-processing, generating volcano plots, Principal Component Analysis (PCA), and heatmaps. Finally, the functional enrichment analysis of gene ontology terms (GOs) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were carried out using Database for Annotation, Visualization, and Integrated Discovery (DAVID), and then the overlap analysis of the functional enriched terms and pathways between pairwise comparisons were carried out in Cytoscape.

3.2.7 Gene functional enrichment analysis

Gene ontology (GO) enrichment analysis was performed using DAVID (<https://david.ncifcrf.gov/>) (Huang et al., 2009; Sherman et al., 2022) to classify the functions of identified DEGs in different pairwise comparisons. GO analysis was performed on 0, 3, 5, and 6 DAFO data between the *er erl1/seg erl2, gl1, er erl1/seg erl2gl1* vs WT and between the *er erl1/seg erl2gl1* vs *gl1*, to examine the main pathways affected by loss of function of *ERf* or *GL1* alone, compared to WT or in combination in *er erl1/seg erl2gl1* compared to WT and *gl1* (WT). For a more in-depth analysis of the enrichment pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed for each pairwise comparison. Significantly enriched GO terms were identified for each expression category using the Enrichment Analysis (Fisher's Exact Test) module in *DAVID* with the FDR set at 0.05.

3.2.8 Functional enrichment overlaps (GO terms and KEGG pathway enrichment) analysis between the pairwise comparisons

The functional annotations obtained from *DAVID* (GOs and KEGG pathway analysis) were imported to *EnrichmentMAP* plugin in Cytoscape (<https://cytoscape.org/>) to examine the overlap of functional enrichment terms and pathways between pairwise comparisons. The network-based overlap analysis among pairwise comparisons was then performed. The network-based overlap analysis identified common and unique biological themes across the multiple pairwise comparisons.

3.3 Results

3.3.1 Assessment of sequencing quality, mapping and data pre-processing before DEGs analysis

Total RNA quality

Total RNA was extracted from five biological pools of developing siliques of four genotype - Col (WT), loss of function of *gl1* (in WT background), and loss of function of *er105 erl1-2/+ erl2-1* and *er105 erl1-2/+ erl2-1 gl1* hereafter referred to as Col, *gl1*, *er erl1/seg erl2* (or *erf*) and *er erl1/seg erl2 gl1* (or *erf gl1*). Total RNA quality was examined using the Qubit reader and Agilent bio analyser (2100 expert) (Supp table 3-1)

Figure 3-3 shows a representative example of total RNA quality assessment data. All RNA extracts were of similarly high quality (Supp table 3-1): a) RIN numbers were all greater than 7, a value commonly considered as a quality threshold for RNA sequencing ([Schroeder et al., 2006](#); [Sigurgeirsson et al., 2014](#)); b) they shared distinct 18S and 25S ribosomal peaks, with an absence of smaller peaks between those two peaks, and a mostly flat baseline ahead of the 18S ribosomal peak as illustrated by the gel image and enlarged electropherogram shown in Figure 3-3, indicating RNA integrity and purity ([Technologies, 2003](#); [Schroeder et al., 2006](#)). All results for all 60 samples included in Supp figure 3-1 and Supp table 3-1. As shown in the enlarged electropherogram and gel image in Figure 3-3, the high-quality RNA samples, the peaks of the electropherograms and the sharpness of the RNA bands on the gels indicate the good quality of my starting material. In an intact RNA sample, the ratio between 25S and 18S should be 2:1, and there should be a sharp peak. Less defined peaks will be generated when the RNA has somewhat degraded, and smaller and thus faster migrating fragments are generated. Noise above the baseline indicates a possible contamination of the cDNA.

Assessment of sequencing and mapping quality

RNA libraries with approximately 20M -25M pair reads per sample were generated (Supp table 3-2). The percentage of bases with a quality score of 30 or higher (Q30%) ranged from 94 to 96% across samples (corresponding to an error probability of 0.1%) (Figure 3-4a, and Supp table 3-2).

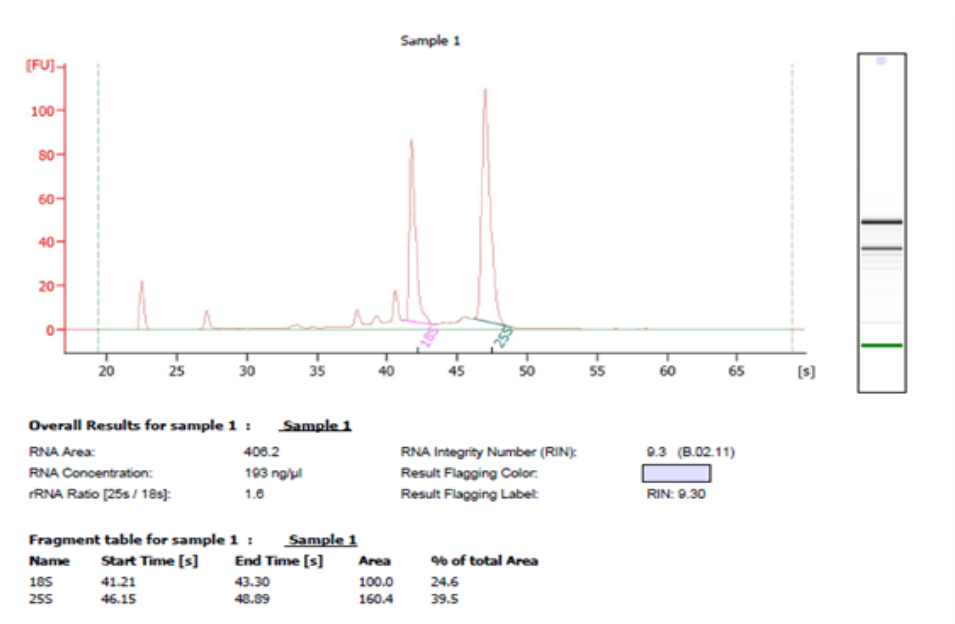


Figure 3-3: Representative Agilent bioanalyzer electropherogram and a gel image of the total RNA, of one sample used in this study. Two distinct sharp rRNA peaks corresponding to 18S RNA (small) and 25S RNA (large) preceded by a flat base indicate the low noise level and tight integrity of RNA used in this study. Sharp corresponding bands are visible on the gel image shown right to the electropherogram (RIN =9.3)

The average quality score over the full length of all reads was plotted for each sample (Figure 3-4b, with Col_d0 sample shown as a representative example). Mean quality score values were 37 for all the samples used in this study. Per base sequence content showed little variation in the first eight positions and the proportion of the four nucleotides remained relatively stable over the length of the read with %A=%T and %G=%C, and A/T and G/C lines running parallel to each other (Example in Figure 3-4c). Following sequence quality assessment, the data were filtered to remove any adapter sequence, as well as the 5' or 3' end-bases labelled as N or with of quality value lower than 20, and reads that were less than 75 bp long after trimming. The filtered data were then mapped to the reference genome (*Arabidopsis thaliana*, TAIR 10). The number of reads per sample and genotype was fairly consistent around 40-50M, and the percentage of reads mapped to the reference genome averaged $\approx 97\%$ for all genotypes across the four time points (Table 3-1). The proportion of those which were multiply mapped averaged 16.9%, and uniquely mapped reads constituted about 76.5% of the total number of mapped reads (Supp table 3-3). 78.9% to 97% of reads mapped to an exon (**Error! Reference source not found.**; Col_d0 as an example of). Reads mapped in proper pairs, i.e., paired-end reads where both Read1 and Read2 aligned to the reference genome in the expected orientation and at an appropriate

distance from each other (Supp table 3-3), gradually decreased from 0 DAFO to 6 DAFO, for all the genotypes. (Table 3-1).

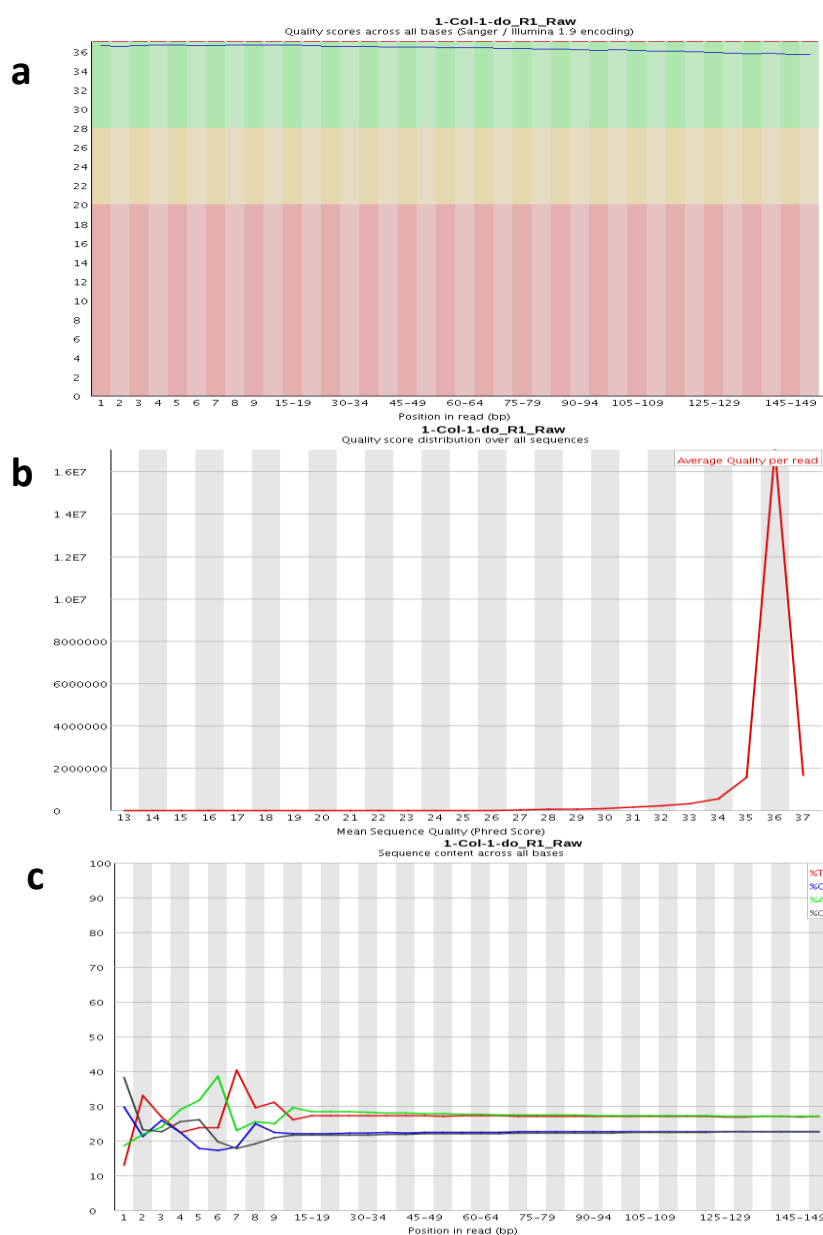


Figure 3-4: Assessment of sequencing quality of raw data **a.** A representative graph showing the quality scores across all bases (Sample Col_d0_replicate1), X axis: base position in reads, Y axis: quality score. The higher the score the more reliable the base calling is. If the quality value is 30, the error rate is 0.1%. **b.** A representative curve of quality score distribution in overall sequences. (Sample Col_d0_replicate1) X axis: the average value of the quality score of the corresponding base. Y axis: the number of sequences. An average quality value greater than 30 (such as in the example shown) is an indication of high quality. **c.** A representative curve showing sequence ATCG content distributions across all bases. This is used to determine whether there is a difference in the percentage of A/T and G/C. X-axis: base position in the read. Y axis: percentage of base presence (A,T, C,G) at each position. The data were then filtered to remove the adapter sequences and the 5'- or 3'- ends containing bases labelled N.

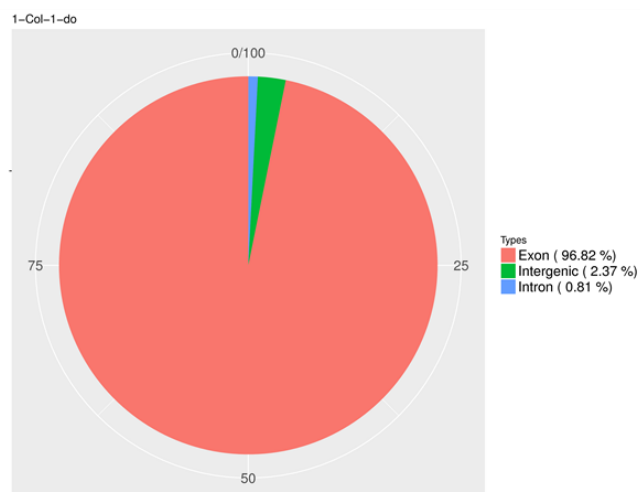


Figure 3-5: Example (Col-d3 -replicate 1) pie chart showing the distribution of reads in different genomic regions. Mapped reads were assigned to genomic features – exons, introns and intergenic regions. Sequences located in the intron region may be due to immature mRNA contamination or incomplete genomic annotation, whereas sequences mapped to intergenic region may be due to incomplete genomic annotations and background noise.

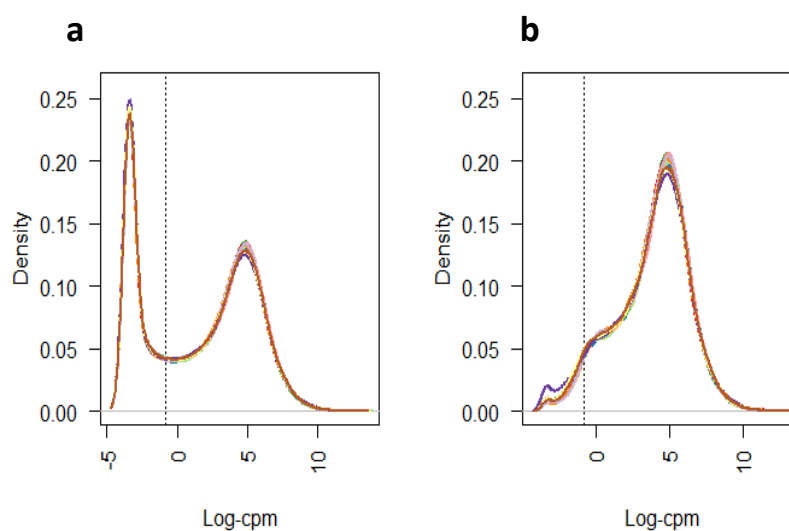


Figure 3-6: Example (ODAFO) of log-CPM density plot of before (a) and after (b) filtering out unexpressed or lowly expressed genes. Default function used the filtering of keeping at least the 10 gene count in 5 samples (sample number is defined according to the biological replicates used in the experiment), actual filtering used the CPM values -hence, the cut off equivalent to a CPM value of about 0.5 ($\log\text{CPM} \approx -1$) i.e., $10/20$ (library size) ≈ 0.5 . Dotted vertical lines mark the log-CPM threshold used in the filtering step.

A read count Table was generated from read alignments; BAM files were fed to *featureCount*, which computed counts at the gene level by counting all the reads that overlapped with any exon within each gene (reference annotation-GFF file format, TAIR10). Raw count numbers were first transformed to log (Counts Per Million bases; logCPM), to account for differences in actual library sizes among samples, as per common practice, with a median library size across samples of about 20 million (Supp table 3-3). To remove too lowly expressed genes, hence unreliable count numbers, I filtered out the genes with count=0 for all samples and then removed the genes with too low counts, using the default function that keeps genes with a minimum of 10 read counts. The actual filtering uses the CPM values. As the median library size in my experiment was about 20M reads, only genes with a CPM of 0.5 (10/20) i.e., $\log\text{CPM} > -1$ in at least 5 samples (i.e., 5 biological replicates used in the experiment) were included in further data analysis (Figure 3-6) ([Law et al., 2016](#)).

As this experiment aimed to analyse changes in gene expression across genotypes and time points, no further normalisation to account for gene length differences was deemed necessary as would be the case for comparisons of absolute expression levels between gene sets ([Law et al., 2016](#)). The number of genes kept represented 63% on average of the pre-filtering numbers at all the time points of numbers pre-filtering. The log CPM values followed an unimodal normal distribution for each sample (Figure 3-6b and Supp figure 3-2). Nevertheless, normalisation by the method of Trimmed Mean of M-values (TMM) was next performed to ensure that the expression distributions for each of the 80 individual samples were similar (see example at 0 DAFO in Figure 3-7b, and all other time points in Supp figure 3-3), and thus to minimise any bias or hidden confounding factors in the downstream analysis of DEGs. The scaling factors were close to 1 showing that the normalisation was mild, as also apparent from the comparison of panels a and b in Figure 3-7.

Table 3-1: Average values of total number of reads, number of paired reads and alignment rates across all five replicates at the four silique developmental stages. Reads mapped in proper pairs represent Paired-end reads in which both Read1 and Read2 align to the reference genome with the expected orientation and within an appropriate distance from each other. The alignment rate represents the total mapped read % to the reference genome.

Samples	0DAFO	3DAFO	5DAFO	6DAFO
Col				
total number of reads	47655342.4	47824802.8	44465144.4	45344509.2
reads mapping in proper pairs	41091858(86%)	38276762(80%)	33623977.2(76%)	33585158(74%)
alignment rate (%)	97-98%	95-98%	97-98%	98%
gl1				
total reads	47445119.2	45469732.4	45055013.6	44568932.4
reads mapped in proper pairs	42219985.2(89%)	35356627.6(78%)	33162551.2(74%)	29038088.8(65%)
alignment rate (%)	98%	97-98%	97%	98%
erf				
total reads	45251017.6	47745986	44182665.6	44498672
reads mapped in proper pairs	39263079.2(86.76%)	37433905.2(78%)	29038088.8(66%)	24864936.8(56%)
alignment rate (%)	97-98%	96-98%	96-98%	96-98%
erf gl1				
total reads	51677328	46383692	44677309.2	44914614.8
reads mapped in proper pairs	45845583.2(89%)	37933165.6(82%)	30351050.8(68%)	29580762.4(66%)
alignment rate (%)	96-97%	96-98%	96-97%	96-97%

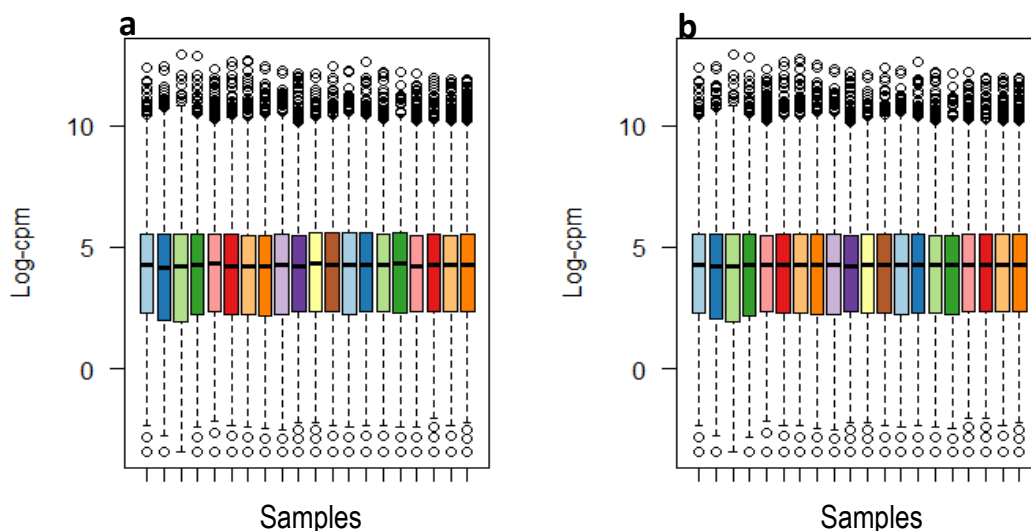


Figure 3-7: Example boxplots (0 DAFO) of log-CPM values showing expression distributions for unnormalized data (a) and normalised data after applying TMM normalisation (b). X axis of the box plot; samples and y axis; the log CPM value distribution. mild normalisation across all the samples as the scaling factor close to 1.

Principal Component Analysis (PCA) was then carried out on the normalised data, for an initial overview of global similarities and dissimilarities of global gene expression levels across samples. Multi-dimensional (MDS) plots were generated using the *glMDSPlot* function in the Glimma package with normalised log CPM values as input in an unsupervised PCA (Figure 3-8). PCA analysis with all 80 sequenced RNA extracts included, showed a clear separation of samples according to developmental stage, with 3 well-separated clusters corresponding to data at 0 DAFO; 3 DAFO; and 5-6 DAFO together which showed a broad overlap (Figure 3-8a). Separate PCA analysis at each of the 4 developmental stages considered individually was then conducted in order to more closely examine inter-sample variance. The two first PCA components explained 25 to 38% and about 20% of the total inter-sample variance, respectively, depending on the time point (Figure 3-8b), i.e., together accounted for about 55-60% of the variance. The next two components, PC3 and PC4, in the next two orthogonal dimensions, together accounted for an about additional 20% of total variance. At each of the four developmental stages, samples separated according to genotype, but in different ways. *er erl1/seg erl gl1* expression data were consistently the most distant from those of the other three genotypes, while the clustering of those varied in time, with a clear separation of *er erl1/seg erl2* from Col (WT) and *gl1* at 0 DAFO but not 5 DAFO, or of *gl1* from the other two at 6 DAFO. There were also several instances of significant variability among biological replicates (e.g., *er erl1/seg erl2 gl1* at 0 DAFO, or *er erl1/seg erl2* and Col at 6 DAFO, or Col at 5 DAFO). These results implied that all factors (genotype, time point and replicate) should be included in the downstream linear modelling of differential gene expression.

At this point, it was impossible to declare any particular replicate as an outlier due to hidden inadvertent confounding factors. All 10 pooled siliques within each replicate, and across all lines, were from flowers that were all tagged on the day of the opening to carefully standardise actual silique biological age at sampling, and were all from the main inflorescence, from plants all grown together and visually similar. All replicates were therefore included in DEG analysis, and data filtering was made at the subsequent step of gene ontology analysis, where only DEGs with low inter-replicate variability were included for maximum stringency in biological interpretation. For the identification of Differentially Expressed Genes (DEGs), linear modelling was carried out in limma, on the log-CPM values. The mean-variance relationships showed a clear interdependency (e.g., 0 DAFO data in Figure 3-9a), as typically observed in RNAseq experiments. The voom function was used to remove heteroscedasticity from the data (e.g., 0 DAFO in Figure 3-9b, and see Supp figure 3-4 for all the other time points), a pre-condition for reliable multilinear modelling of logCPMs, which has the embedded assumption that data are normally distributed.

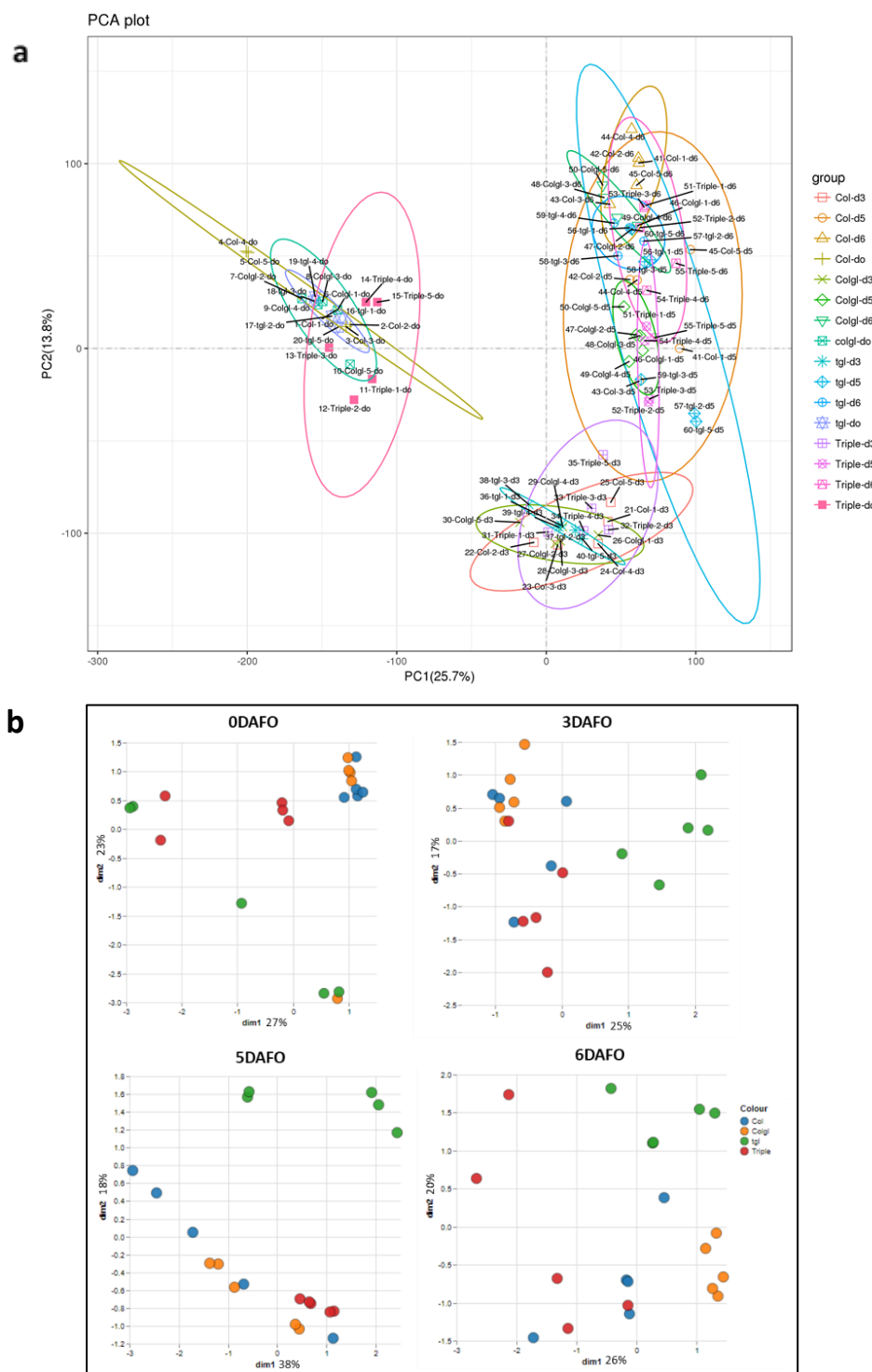


Figure 3-8: Principal component analysis all the samples together(a) and separately for each time points(b).
 a. Each point represents an individual sample, and the points are color-coded according to their respective groups, as indicated in the legend. The two principal components, PC1 and PC2, explain 25.7% and 13.8% of the total variance, respectively. b. MDS plots for each time points, representing the log-CPM values two dimensions. samples were coloured by genotype. Distance of any two points (samples) corresponds to the to the leading fold-change. Note; the labelling of genotypes is as Col (WT), *Colgl- gl1*, *Triple- er erl1/seg erl2*, *tgl- er erl1/seg erl2 gl1*.

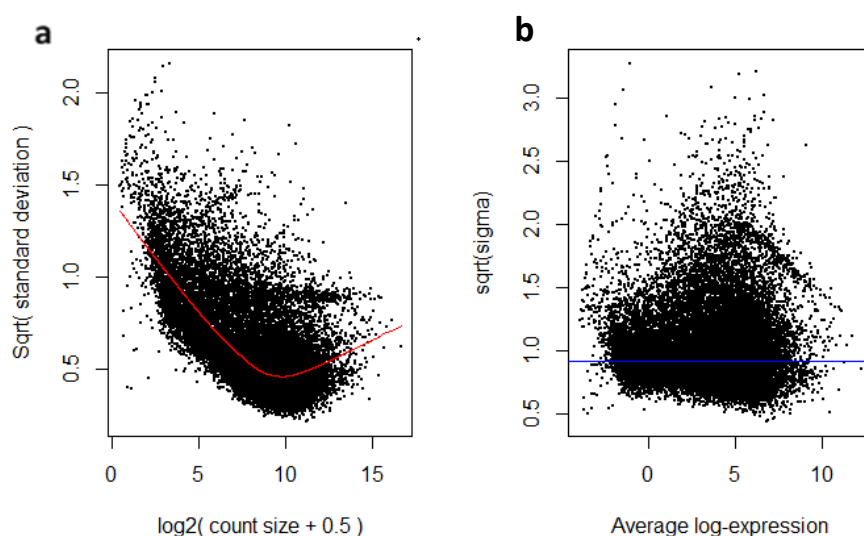


Figure 3-9: Removing the heteroscedasticity of the data using the voom function.

The mean (x-axis) -variance (y-axis) plot of each gene in time point 0 DAFO were plotted before (a) and after (b) applying the voom precision weights. The voom function estimates the mean-variance relationship for the log-CPM values, and then the estimated variance is used to calculate the precision weights for each gene. **a.** created within the voom function and displays the mean expression level of each gene (log2-transformed mean-counts) with an offset of 0.5 to handle zero counts (x-axis) against the square root of the standard deviation (y-axis). **b.** the average log-transformed expression levels of genes (x-axis) against the log 2 square root of the residual standard deviation from the linear model fit (y-axis). For both plots, each dot represents a gene, and a fitted red curve (a) shows the overall trend of the mean-variance relationship and blue line (b) average log 2 residual standard deviation.

Table 3-2: significantly up and down-regulated genes in each time points with three different cut-offs.

	efit -FDR < 0.05, non-zero log2FC					treat- FDR <0.05, log2FC=1 (FC=2)					treat- FDR <0.05, log2FC=0.5 (FC=1.4)				
	erf vs col	gl1 vs col	erfgl1 vs col	erfgl1 vs erf	erfgl1 vs gl1	erf vs col	gl1 vs col	erfgl1 vs col	erfgl1 vs erf	erfgl1 vs gl1	erf vs col	gl1 vs col	erfgl1 vs col	erfgl1 vs erf	erfgl1 vs gl1
0DAFO															
down	1216	58	681	905	803	14	1	104	75	99	14	1	104	75	99
Not sig	19777	21968	21065	20416	20645	22043	22058	22043	21943	21917	22043	22058	21918	21943	21917
UP	1067	34	314	739	612	3	1	3	42	44	3	1	3	43	44
3DAFO															
down	72	346	600	325	1092	10	1	88	72	93	16	1	154	110	165
Not sig	21045	20572	20167	20605	19103	21131	21142	21026	21043	21021	21124	21139	20929	20989	20909
UP	26	225	376	213	948	2	0	29	28	29	3	3	60	44	69
5DAFO															
down	121	44	645	403	1076	10	0	110	85	130	25	1	215	162	232
Not sig	20745	20811	19966	20368	19317	20934	20945	20802	20834	20786	20917	20944	20661	20727	20649
UP	79	90	334	174	552	1	0	33	26	29	3	0	69	56	64
6DAFO															
down	930	1272	1384	822	663	6	1	90	62	77	30	48	201	144	171
Not sig	18436	17604	17464	18279	18858	20096	20207	20072	20124	20096	20150	20097	19850	20007	19965
UP	842	1332	1360	1107	687	4	0	46	22	35	28	63	157	57	72

Linear modelling was then carried out using the limma *limFit* function and with various combinations of paired genotypes as contrasts. The *efit* of Limma uses adjusted p-values (obtained from applying the Benjamini-Hochberg (BH) method ([Benjamini & Hochberg, 1995](#)) to correct for False Discovery Rate (FDR) for multiple hypotheses testing, with FDR set at 0.05 to ensure that the expected proportion of false positives in the sets of significant DE genes would be at most 5%. I first extracted the DEGs for further gene set analysis by imposing an FDR < 5% on any non-zero log₂Fold Change (log₂FC). However, for a more stringent filtering of true DEGs, an alternative requirement of a minimum log-fold change threshold was applied using the “*treat*” function, where FC refers to the fold change in the number of transcripts between the two members of the pair compared in each contrast. Table 3-2 shows the number of differentially expressed genes obtained for the various contrasts under the requirement of FDR < 5% and non-zero log₂FC, or the stricter combined requirements of an FDR no greater than 5% and a log₂FC value of at least 1 or 0.5, corresponding to minimum fold changes in gene expression of at least 2 or 1.4, respectively. These more stringent requirements drastically reduced the number of up- or down-regulated genes, from a few hundred down to fewer than 100 or even 10, depending on time point and contrast, and 200-231 at most at 5 DAFO in *er erl1/seg erl2 gl1(erf gl1)* compared to Col, or *er erl1/seg erl2 gl1(erf gl1)* compared to *gl1* (Table 3-2). The three global data sets of DEGs obtained using the three above variants of thresholding rules were all included in further data analysis.

The magnitude of differential gene expression between *er erl1/seg erl2 gl1* compared to WT or *gl1* was relatively stable over the 0-6 DAFO developmental window considered in the study, with DEG numbers in the range of 600 to 900. This was more than the sum of DEG numbers associated with either *er erl1/seg erl2* or *gl1* separately at 3 DAFO and 5 DAFO, but in between at 0 DAFO; and at 6 DAFO, similar to the differential expression induced by *gl1* alone. In other contrasts, such as *gl1* or *er erl1/seg erl2* alone in WT background there was a sharp increase of DEGs between 5 DAFO and 6 DAFO compared to WT (Figure 3-10a & b); loss of either *ERf* function or *GL1* function induced similar temporal profiles of DEG numbers, except at 0 DAFO when only *er erl1/seg erl2* seeds showed differential expression compared to WT; *gl1* did not (Figure 3-10c). They indicate a dominant early transcriptional role of the *ERf* at the time of pollination and the inception of seed development, and then, around 3 to 5 DAFO i.e., after the completion of seed cavity expansion and as the seed coat epidermis started to differentiate, *gl1* transcriptional effects kicked in, amplifying those induced by loss of *ERf* function. These results denote interactive, developmentally regulated roles of the *ERECTA* family and *GL1* on gene transcription during early seed development. They also confirm that *GL1* is not integral to the *ERf* signalling pathway in the seed but rather part of an intersecting pathway.

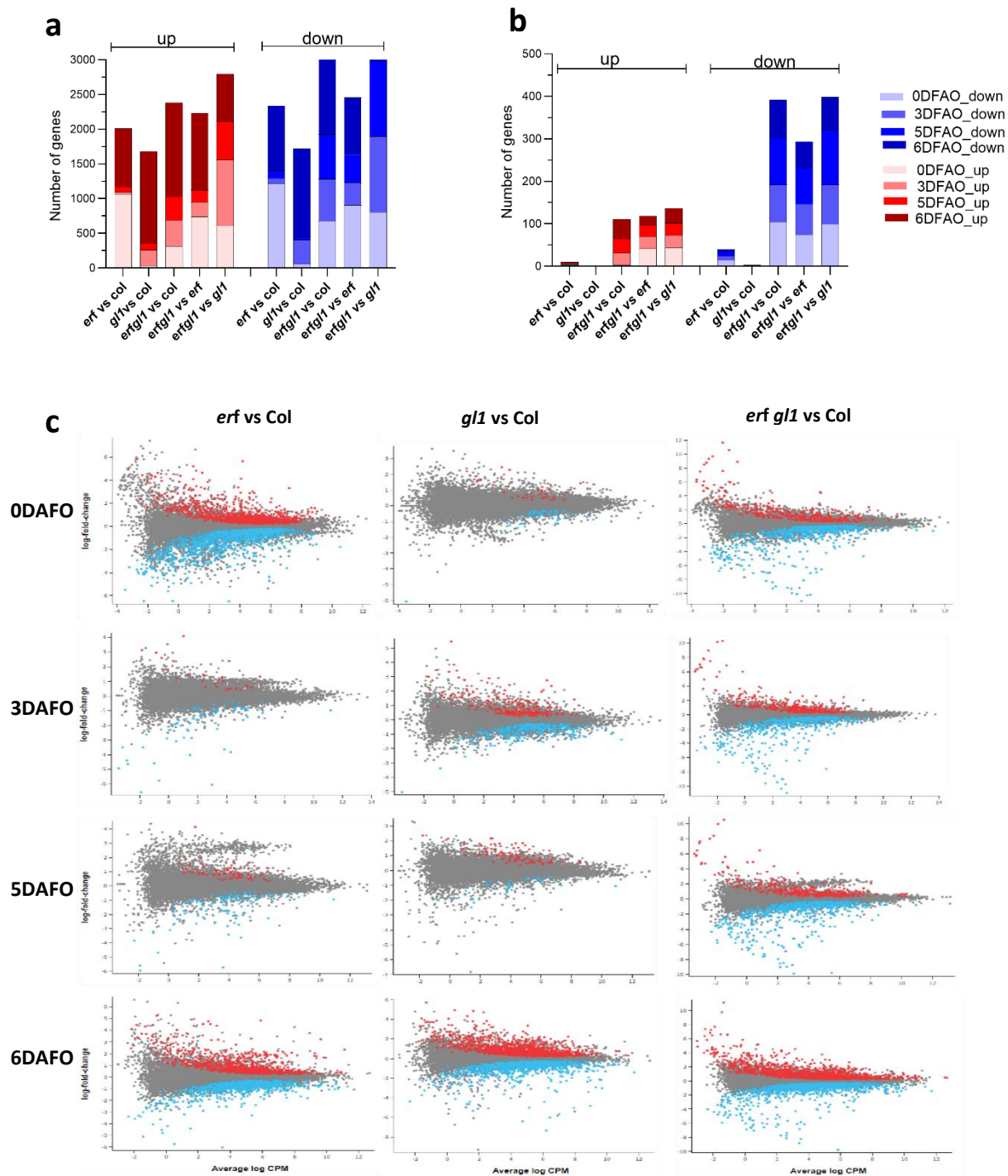


Figure 3-10: Temporal variation of differentially expressed up- and down-regulated genes between pair-wise comparisons. a & b. number of differentially expressed genes between *erl1/seg erl2* (*erf*), *gl1*, *erl1/seg erl2 gl1* (*erf gl1*) vs Col (WT), *erl1/seg erl2 gl1* vs *erl1/seg erl2* and *erl1/seg erl2 gl1* vs *gl1*, with significance thresholds set at FDR < 0.05 and non-zero log2FC (a) and FDR < 0.05 and log2FC = 1 (b). c. Mean-difference plot for the significantly (FDR < 0.05 and non-zero log2 FC) up- and down-regulated genes (red dots and blue dots, respectively) in the various pairwise comparisons - *erf*, *gl1* or *erf gl1* vs Col- generated from Glimma using the glMDPlot function.

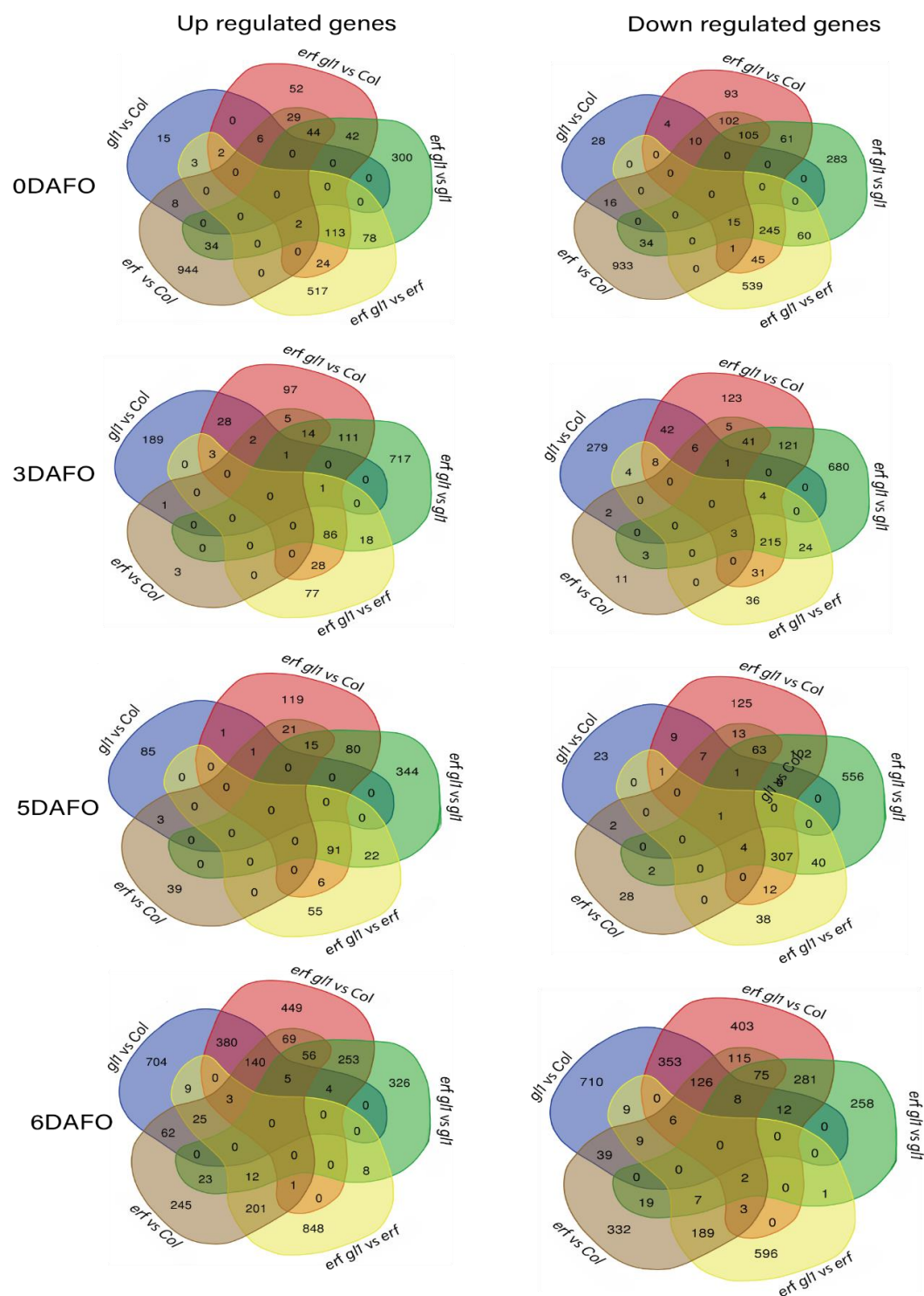


Figure 3-11: Venn diagrams showing the number of commonly up- (left) and down-regulated (right) genes in the five pairwise comparisons (*erl1/seg erl2* (*erf*), *gl1*, *erl1/seg erl2 gl1* (*erf gl1*) vs Col (WT), *erl1/seg erl2 gl1* vs *erl1/seg erl2* and *erl1/seg erl2 gl1* vs *gl1*) across the 4-time points 0, 3, 5 and 6DAFO. Significant DEGs-FDR<0.05 and non-zero log2FC.

Venn diagram analysis was carried out to visualise the number of significantly upregulated and downregulated DEGs that were common to the various contrasts (FDR <0.05, non-zero log2FoldChange) (Figure 3-11). In the first instance, I was interested in the common up and down-regulated genes in the two loss of function mutants, *erf* and *gl1*, compared to background wild type Col. At pollination (0 DAFO), the two lines shared 8 and 16 up- and down-regulated genes, respectively, but only 1 and 2, respectively, at 3 DAFO; 3-and 2-at 5 DAFO, but increasing to 62 and 39, respectively, at 6 DAFO, i.e., within only 1 day when differential gene expression was much more extensive than at earlier time points. The differential gene expression induced by loss of *GL1* function in WT background (*gl1*) and in the *er erl1/seg erl2* background (*erf gl1*) (comparison of contrasts *gl1* vs WT and *gl1* vs *er erl1/seg erl2*) showed little overlap, with only 3 common upregulated genes and none among the down-regulated genes at 0 DAFO, 0 and 4, respectively, at 3 DAFO, none at 5 DAFO and 9 and 0, respectively at 6 DAFO. These data indicate that the *GL1* and *ERf* molecular networks during seed development substantially differ, again consistent with the germination phenotypes and the conclusion from data in Chapter 2, that *GL1* does not act upstream of the *ERf* and is not an integral component of the *ERf* network. The much greater number of DEGs in the *er erl1/seg erl2 gl1* (*erf gl1*) mutant compared to either of the two single mutants indicates interacting pathways in the transcriptional control of early seed development.

3.3.2 Gene Ontology (GO) and KEGG pathway enrichment analysis of differential gene expression induced by loss of *ERf* or/and *GL1* functions.

To examine the functional significance of the differential gene expression associated with knock-out mutations of the *erf* or *gl1*, separately or combined, the gene sets that were up- or down-regulated (FDR< 0.05, non-zero log2FC and logFC 1) in each of the three mutants - *erf*, *erf gl1*, and *gl1* compared to WT, were analysed, using the online Gene Ontology (GO) enrichment analysis tool DAVID (Database for Annotation, Visualization and Integrated Discovery) <https://david.ncifcrf.gov/> (Huang et al., 2007; Huang et al., 2009), with Arabidopsis genome draft version -Araport11 as a reference. GO terms, Biological Processes-BP(GO_BP), Molecular Functions-MF (GO_MF) and Cellular Components-CC(GO_CC) for up and down-regulated genes were ranked in decreasing levels of significance according to *p*-value and FDR-corrected *p*-values from Fisher's exact test. The expression profiles of the most stringent DEGs obtained using FDR< 0.05, logFC 1 filtering rule, were analysed first, before analysis of the whole gene set obtained using FDR< 0.05, non-zero log2FC as filtering thresholds for significant gene differential expression (Figure 3-12a and b).

Strict filtering of DEGs gives insights into the roles of ERf and GL1 in seed development and stress response.

Significantly down-regulated genes in *erf* and *erf gl1* seeds compared to control related to stress responses and to development: stress responses (AT4G32208, AT5G05750); plant defence responses (AT5G05400); AT2G13810 (ALD1, AGD2-Like Defense Response Protein 1); touch and mechanosensitive responses (AT2G41100, *TCH3* encoding the calmodulin-like TOUCH3 protein, which was found to be induced in germinating *erf* seeds under salinity, (Nanda et al., 2019); light spectrum (AT5G02200, *FHL*, *Far-Red-elongated Hypocotyl-Like 1*); development processes, such as AT2G01280 (*MEE65*, *Maternal Effect Embryo Arrest*) involved in embryo development (Figure 3-12a). Five genes were significantly upregulated in both *erf* and *erf gl1* compared to control WT, including AT5G12420 at 6 DAFO, a gene encoding WSD7 involved in wax biosynthesis, and which has been implicated in responses to salt stress and water deprivation (C. Zhang et al., 2022) (Patwari et al., 2019) (Figure 3-12a).

In contrast, *gl1* seeds lacking only *GL1* function showed only three significantly down-regulated genes compare to WT, two of which were also differentially expressed in *erf* and/or *erf gl1*, AT2G01280 (*MEE65*) and AT1G30250 of unknown function. The third gene, AT3G29644 (Short Open Reading Frame (soRF) 29) was up regulated in *gl1* at 0 DAFO but down-regulated in *erf gl1* at all time points, thus under interactive transcriptional *ERf* and *GL1* controls (Figure 3-12a). Nothing is known of that *soRF29*, but interestingly, it is among the genes that were found to be downregulated after miRNA65 induction in the shoot apical meristem (Sinha et al., 2023) where both the *ERf* and *GL1* have well-documented roles.

A number of genes were only differentially expressed when *GL1* and *ERf* functions were both abolished (Figure 3-12b) i.e., showed no differential expression in *erf* or *gl1* single mutants. These correspond to a set of genes under transcriptional control of both the *ERf* and *GL1*, which are individually necessary and sufficient to maintain their normal expression, but when both lost alter their expression, for most of them causing their inhibition. The majority of those genes related to defence responses and signal transduction; two smaller groups corresponded to genes involved in amino acid biosynthesis, and notably, plant cell wall organization (three genes). These 3 genes, *AT5G39280*, *AT5G39300*, and *AT5G39260* all belong to the *EXPANSIN* gene superfamily (*EXPA21*, *EXPA23*, and *EXPA25*, respectively, and were highly up-regulated at 6 DAFO (Figure 3-12b), i.e., around the time the seed coat starts to undergo massive differentiation. EXPAs promote cell expansion through pH-dependent cell-wall-

loosening and extension, endosperm development, and also in GA-mediated germination ([Chen & Bradford, 2000](#); [Chen et al., 2001](#); [A. Yan et al., 2014](#)).

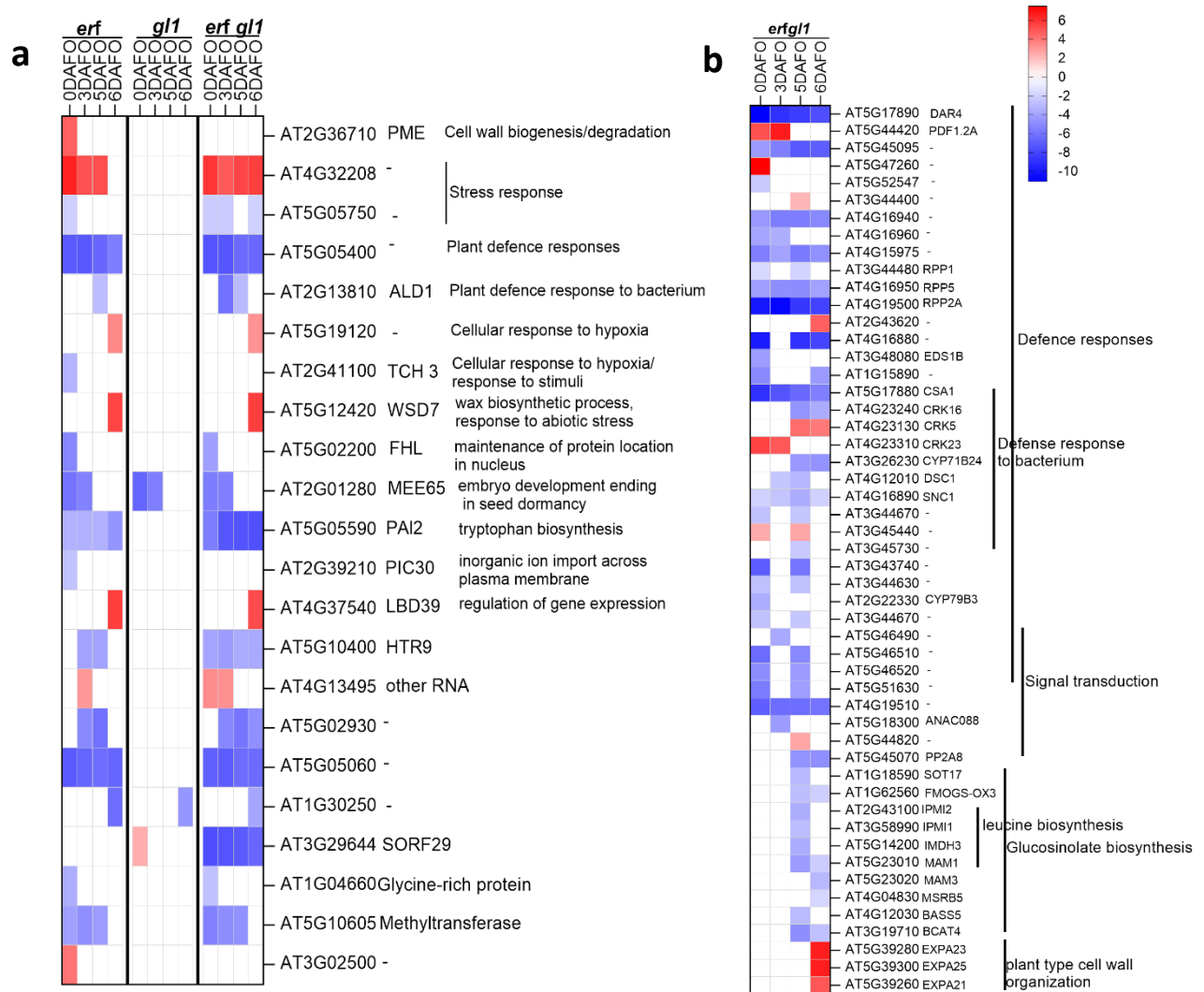


Figure 3-12: Highly expressed up and down-regulated genes from stricter DEGs filtering ($FDR < 0.05$, $\log_2 FC = 1$) in *er erl1/seg erl2* (*erf*), *gl1*, and *er erl1/seg erl2 gl1* compared to Col (WT) across 4 time points. The rows represent individual genes, and the columns represent different time points for each mutant. Gene expression levels are displayed as $\log_2$ fold changes relative to the wild type (WT), with up-regulated genes in red and down-regulated genes in blue, as indicated by the scale bar. **a.** Differentially expressed genes common to 2 or all three mutants- *erf*, *gl1* and *erf gl1* showing significant downregulation of stress and defence response genes and upregulation of wax biosynthesis gene in *erf* and *erf gl1*, **b.** Genes differentially expressed only in *erf gl1* across four-time points mainly represent the genes belongs to the functional group of defence responses, signal transduction, amino acid biosynthesis and plant type cell wall organisation. Genes were assigned to the functional group using an online tool DAVID (Database for Annotation, Visualization and Integrated Discovery).

Overall, the annotations of the most highly differentially expressed DEGs in the three mutants show that the *ERf* and *GL1* pathways are implicated in a range of biological processes in the developing seeds, including cell wall biogenesis, stress responses, and developmental processes during early seed development. The differential expression patterns across genotypes and developmental stages suggest a complex regulatory network where *ERf* and *GL1* interact to modulate gene expression.

I next examined the annotations of the larger sets of DEGs yielded by the less stringent filtering, $FDR < 0.05$ and non-zero $\log_2FC$, using the DAVID bioinformatics tool. Significantly enriched GO terms ($p < 0.01$ and $FDR < 0.05$) were represented using EnrichmentMAP-Cytoscape, which generates “EnrichmentMAP” where enriched GO terms are depicted as “nodes” (pink/grey circles in Figure 3-13 and Supp figure 3-5) whose size is proportional to the number of included genes. Documented or putative genetic links between nodes (i.e., functional groups) according to the literature, are represented by a line which, when interrogated online, gives the accession numbers of the genes linking the nodes. Related GO_BP terms were then clustered manually in broader biological functional groups at each time-point. For the easy comparison between the mutants an illustration of the broader BP groups of assigned BP terms were shown in **Error! Reference source not found.** for 0 and 6DAFO (For other development stages and for more detailed figures see supp Fig 5 for GO_BP, supp Fig 6 for GO_CC and supp Fig 7 for GO_MF)

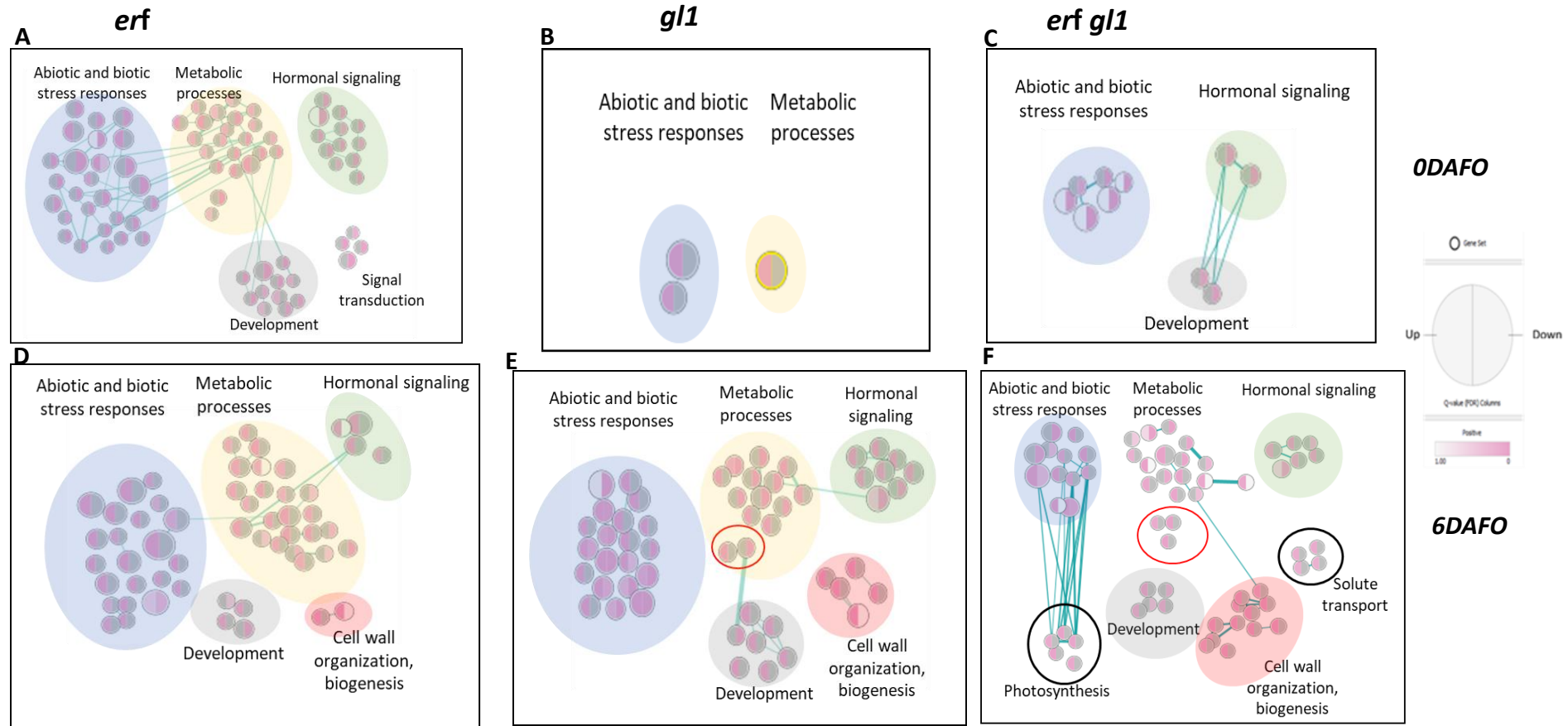
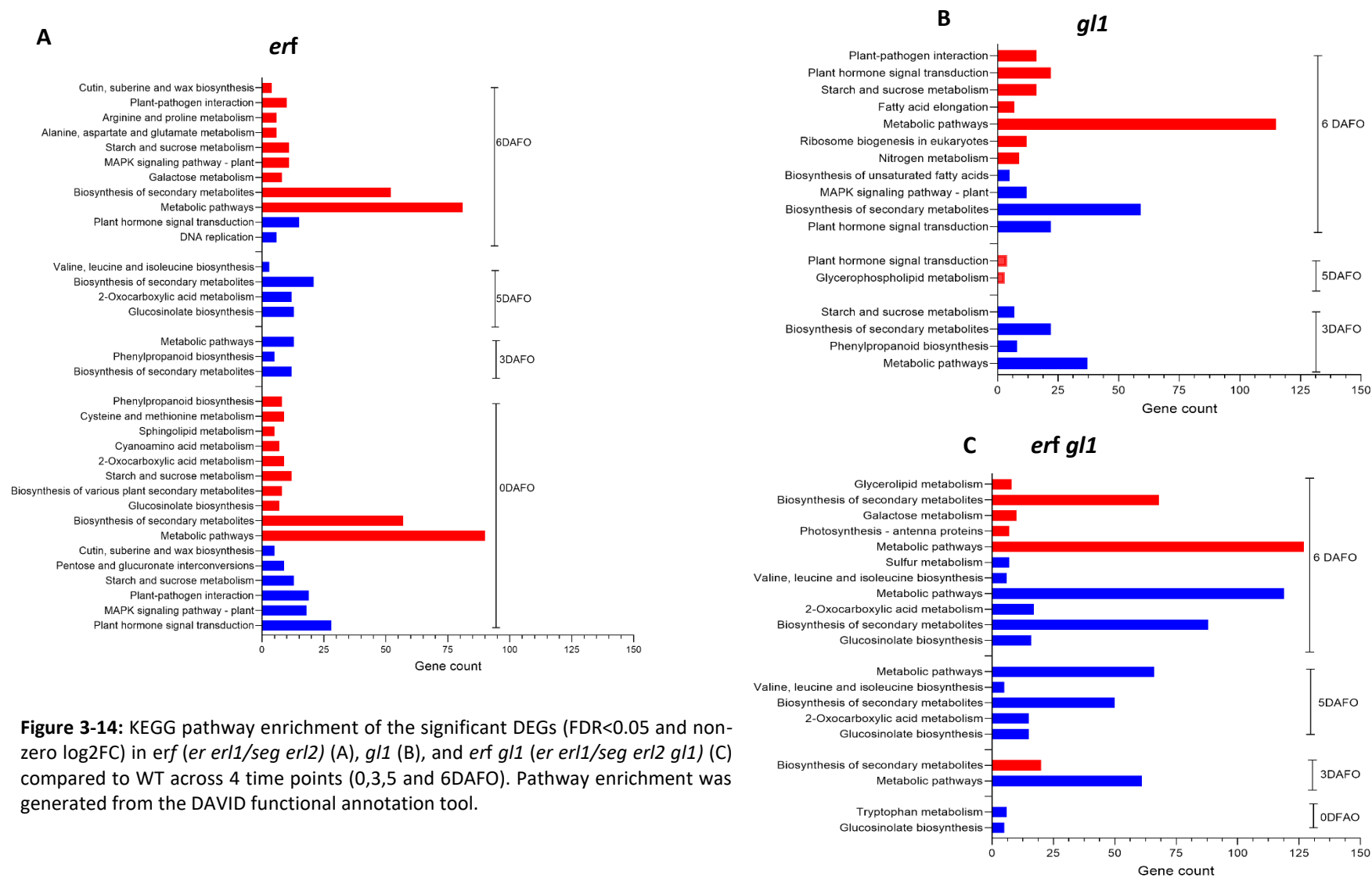


Figure 3-13. Gene ontology terms (Biological Process (GO_BP)) enrichment analysis of significant DEGs (FDR<0.05 and non-zero log2FC) in *erf* (*er erl1/seg erl2*), *gl1* (*erl1/seg erl2 gl1*) compared to Col (WT) across 0, and 6 DAFO (**A,B,C** and **D,E,F** respectively). The functional annotation from DAVID was uploaded to Cytoscape, and the enrichment map of significant ($p < 0.01$ and FDR<0.05) GO_BP terms were grouped using the -EnrichmentMAP plugin. Nodes (pink/grey circles) in the map represent the up (left) and down (right) regulated genes set, and the size of the circle is proportional to the number of genes associated with the term. See supp Fig 5 for more extended details of the BP terms associated in each of the group. Genetic links between nodes were represented by the green line. Red circled terms in E and F are belong to the wax and cutin biosynthesis.



3.3.3 Distinct and overlapping transcriptional networks of *ERf* and *GL1* in *Arabidopsis* early seed development: Implications for abiotic stress response, metabolic processes, and cell wall biosynthesis

Loss of function of *ERf*

At 0 DAFO GO_BP in *erf* seeds showed an enrichment compared to WT in genes involved in signal transduction and protein phosphorylation; hormonal signalling –in particular salicylic acid and ethylene signalling, ABA and auxin; defence responses against bacterium or fungus, or unspecified; a range of abiotic stress response pathways, such as to water deprivation, cold, hypoxia, wounding, or light spectrum (far-red/red ratio in particular), and for a smaller group, to metabolic and catabolic processes, and development such as flowering and shoot system development. Globally, these functions are consistent with the reported roles of the *ERf* in immune responses and disease resistance and the regulation of organ growth (Figure 3-13A; Supp figure 3-5) ([Masle et al., 2005](#); [Shpak et al., 2005](#); [Sanchez-Rodriguez et al., 2009](#); [Uchida et al., 2012](#); [Bemis, 2013](#); [Jorda et al., 2016](#))

Three days later, at the 3 DAFO time-point, as the number of DEGs between *erf* seeds and WT had drastically decreased, down-regulated genes showed only an overrepresentation of genes involved in hydrogen peroxide catabolism (Supp figure 3-5; 4 genes, $p < 0.05$, $FDR < 0.05$), while the few up-regulated genes (< 10) were associated with stamen development (2 genes) and the regulation of cell proliferation (2 genes), two processes in which the *ERf* signalling has central well-established roles in aerial organs ([Shpak et al., 2003](#); [Shpak et al., 2004](#); [Woodward et al., 2005](#); [Hord et al., 2008](#)). However, the level of enrichment did not reach statistical significance for any of these BP groups.

At the 5 DAFO, as the seed cavity was near its final size (Reviewed in ([Sun et al., 2010](#))) 4 BP terms were significantly enriched, all within the set of down-regulated genes: response to wounding; glucosinolate biosynthesis, and amino acid metabolism (Supp figure 3-5). Within 1 day, at 6 DAFO, the number of significantly up- and down-regulated genes had dramatically risen (Figure 3-10), 34 and 2 GO_BP terms were declared as significantly enriched ($p < 0.05$, $FDR < 0.05$) within the set of up-regulated genes and down-regulated genes, respectively (Figure 3-13D; Supp figure 3-5). Enriched terms belonged to abiotic and biotic stress responses, metabolic processes, and hormonal signalling as at 0 DAFO; genes of the ABA and jasmonic acid pathways were the only types to be overrepresented among hormonal signalling genes, along with genes involved in ion transport, cell wall organisation, tissue development. The bulk of enriched GO_MF (molecular function) terms in *erf* seeds corresponded to “protein kinase activity” (serine/threonine/tyrosine - and Ca-dependent protein kinase activity), as well as calmodulin binding, ATP binding, and protein binding (Supp figure 3-7). The most represented GO_CC (cellular

compartment/component) terms within the set of up-regulated genes were “membrane integral component, cytosol; chloroplast, chloroplast stroma, chloroplast membrane and thylakoid”. In contrast, the most enriched GO_CC terms within the set of down-regulated genes by *ERf* loss of function were the nucleus, the plasma membrane and extra cellular region, while “membrane integral component” was a minor term (Supp figure 3-6).

KEGG enrichment pathway analysis was then carried out in order to get insights into post-transcriptional pathways associated with the most prevalent functional groups of DEGs based on *p* value and *FDR* ($p < 0.05$, $FDR < 0.2$) (Figure 3-14). At 0 DAFO, this analysis yielded 10 and 7 KEGG enriched pathways for the up- and down regulated gene sets, respectively (Figure 3-14A). “Unspecified” metabolic pathways and “secondary metabolites” biosynthesis pathways were the most represented for upregulated pathways, with 90 and 57 corresponding genes. Furthermore, the loss of *ERf* function also induced an upregulation of starch and sucrose metabolism pathways (12 genes). Downregulated pathways showed enrichment in pathways relating to MAPK signalling, consistent with *ER* and *ERL2* kinases knock-out, and reduced *ERL1* signalling, and to hormonal signalling, plant-pathogen interactions, starch and sucrose metabolism, pentose and glucuronate interconversions, the synthesis of glucosinolate which have a well-established role in plant defence, and interestingly the synthesis of complex polyesters, cutin, suberin and wax esters, as well as of phenylpropanoid compounds ([Schlaeppe et al., 2008](#); [Bednarek et al., 2009](#); [Clay et al., 2009](#); [Müller et al., 2010](#); [Stotz et al., 2015](#); [Nakazaki et al., 2019](#)).

At 5 DAFO, an enrichment of four KEGG pathways was identified for the set of downregulated genes ($p < 0.05$ and $FDR < 0.2$), relating to glucosinolate biosynthesis, 2- oxocarboxylic acid metabolism, biosynthesis of secondary metabolites, but also to amino-acid synthesis -valine, leucine and isoleucine (Figure 3-14A), while at 6 DAFO, KEGG enrichment pathway analysis yielded two pathways for down-regulated genes, namely plant hormone signal transduction and DNA replication (Figure 3-14A), but nine pathways for the set of up-regulated genes, with 81 and 52 genes, respectively, associated with unspecified metabolic pathways and biosynthesis of secondary metabolites, respectively. Remarkably, cutin, suberin and wax biosynthesis, starch and sucrose metabolism, plant-pathogen interaction and MAPK signalling, which were all enriched within the set of down-regulated genes at 0 DAFO, were in contrast, now significantly up-regulated at 6 DAFO.

Taken together, these results are consistent with the extensively documented functions of *AtER* and *AtERL* paralogs as membrane Leucine-Rich-Repeat Receptor-like kinases, and their numerous reported biological functions in immune and defence response, and hormonal signalling such as by auxin.

However, they also indicate broader roles beyond those previously reported, particularly in inter-hormone signalling and abiotic stress responses, and synthesis of secondary metabolites, notably cutin, suberin and waxes.

Loss of function of *GL1*

gl1 seeds showed remarkably limited differential gene expression compared to WT (Figure 3-10), that preferentially affected genes with GO_BP terms “response to organic cyclic compounds, response to water deprivation and response to lipids at 0 DAFO (Figure 3-13 B and Supp figure 3-5), biotic and abiotic responses and to cell wall organization, xylan catabolism and macromolecule biosynthetic process within the up and down regulated genes, respectively, at 3 DAFO (Supp figure 3-5), but quite different biological processes two days later, by 5 DAFO, namely: auxin signalling pathway; development and growth, plant epidermis development, anatomical structure maturation and root morphogenesis; all BP_terms that were not enriched within DEGs detected in the *erf* seeds (Supp figure 3-5).

Within the next 24h interval, when the transcriptional deregulation associated with *gl1* suddenly became much more extensive, there was a significant enrichment in BP-terms relating to: abiotic and biotic stress responses, similar to *erf* DEGs at the same stage; response to stimuli among which response to mechanical stimuli, red and far red light, response to lipids, were not terms detected in *erf* seeds; metabolic processes, and more specifically secondary metabolism; hormonal signalling (including more diverse hormonal signalling pathways than in *erf* counterparts -auxin, jasmonic acid, ABA, and brassinosteroids,- and still “ cell wall organisation and biogenesis, plant organ/tissue development”, in contrast to *erf* seeds, as observed 5 DAFO (Figure 3-13E, Supp figure 3-5).

Enriched GO_MF included terms transferase activity, transferring glycosyl groups at 3 DAFO; and ion-ion binding, heme binding, monooxygenase activity (associated with all the up regulated genes at 5 DAFO), ATPase activity, xyloglucosyl transferase activity, polysaccharide binding at 6 DAFO (Supp figure 3-7). Four KEGG pathways were correspondingly significantly enriched ($p < 0.05$, FDR < 0.2) unspecified metabolic pathways, secondary metabolites biosynthesis pathways, starch and sucrose metabolism and phenylpropanoid biosynthesis, at 3 DAFO; plant hormone signal transduction and glycerophospholipid metabolism at 5 DAFO; and at 6DAFO, a significant up regulation of unspecified metabolic pathways with more than 100 genes, and also of nitrogen metabolism, fatty acid elongation,

starch and sucrose metabolism, plant hormone signal transduction and plant pathogen interaction. Interestingly, MAPK signalling and biosynthesis of secondary metabolites pathways were down regulated in *gl1* seeds, in contrast to *erf* counterparts (Figure 3-14B).

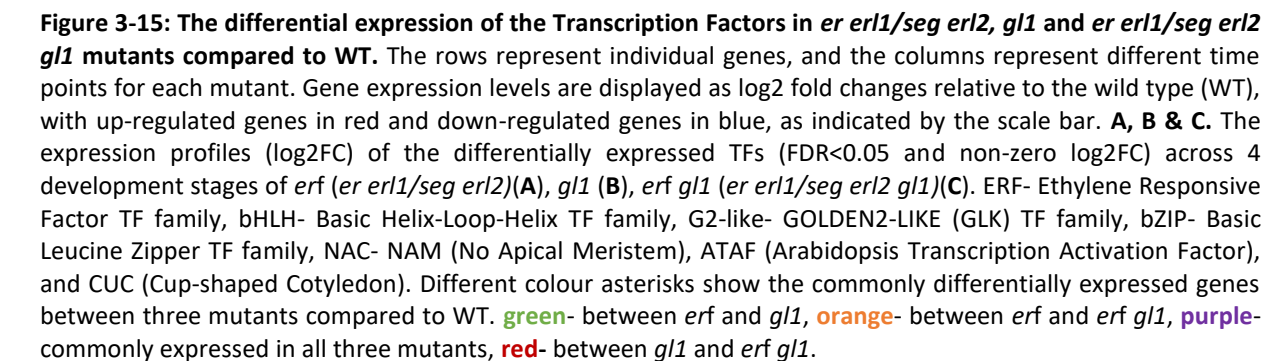
Loss of function of ERf GL1

Quadruple mutant samples, combining loss of *ERf* and *GL1* functions (*er erf1/seg erf2 gl1*) showed very limited differential gene expression compared to *erf* (*er erf1/seg erf2*) at 0 DAFO, similar in that to the single *gl1* mutant (see Figure 3-10). Moreover, all BP terms associated with stress responses related to biotic stress responses, none to abiotic stress; only hormonal auxin signalling pathways BP-terms were enriched and there was no enrichment in terms associated with metabolic processes. But, similar to *erf* seeds, there was an enrichment in genes relating to development and growth (Figure 3-13C; Supp figure 3-5). At 3 DAFO, enrichment analysis yielded similar GO_BP terms as for *gl1*, except for an additional enrichment in terms relating to amino acid biosynthesis, response to water deprivation and response to nutrient level (Supp figure 3-5). At 5 DAFO, there was a more prominent enrichment of terms relating to abiotic and biotic responses compared to *erf* and *gl1* alone, but no more enrichment of growth-related BP terms, and of hormonal signalling terms, except for JA signalling (Supp figure 3-5). At 6DAFO, differential expression preferentially affected abiotic and /biotic stress response genes and metabolic processes, similar to *erf* seeds carrying functional *GL1*, but the deregulation of hormonal signalling was more similar to that seen in *gl1* seeds than *erf* seeds -ABA, Auxin, and brassinosteroids signalling (Figure 3-13F and Supp figure 3-5). A remarkable difference compared DEGs induced by loss of either the *ERf* or *GL1*, was the vastly more prominent enrichment in BP terms relating to cell wall organisation, cell wall loosening and biogenesis, including xylan and xyloglucan biosynthesis. Another was the appearance of an enrichment in GO_BP terms associated with photosynthesis (up-regulation) (Figure 3-13 F).

ADP binding and NAD(P)⁺ nucleosidase activity were significantly downregulated MF terms at 0 and 3 DAFO, and at 5 and 6 DAFO terms associated with ion binding and carbohydrate metabolism were also enriched (Supp figure 3-7). A majority of DEGs are genes reported as expressed in the plasma membrane, chloroplast and extracellular region (GO_CC) (Supp figure 3-6). Consistently, KEGG pathways analysis at 6 DAFO, indicated a majority of up and down regulated genes associated with “unspecified metabolic pathways” (Figure 3-14 C), as well as genes involved in photosynthesis-antenna functions, glycerolipid metabolism, which were up-regulated, as were sulfur metabolism, amino acid biosynthesis, and glucosinolate biosynthesis pathways within the set of the down-regulated genes.

Altogether, these data indicate significant shifts in transcriptional activity associated with *Erf* and *GL1* functions within the first few days of seed development, during the period of expansion of the seed coat to its maximum size, the early differentiation of its specialised mucilage-secreting epidermal cells, and the formation of the endosperm-associated cuticle. Moreover, they suggest overlaps and interactions between the *Erf* and *GL1* transcriptional networks during seed development, in the regulation of genes involved in abiotic stress responses, metabolic processes, hormonal signalling, and also, specifically, of genes involved in photosynthesis, cell wall organisation, and cell wall component biosynthesis. Further analysis such as network reconstruction would be needed to confirm these interactions.

To better understand the *Erf* and *GL1* transcriptional networks and their interactions, I next examined in more detail the identity and functional annotations of the different types of genes involved, first focusing on the analysis of the *Erf*, *GL1* and *Erf-GL1* induced differential expression of transcription factors. Transcription factors are instrumental to the control of tissue patterning, cell proliferation and expansion during early organ development. A number of bHLHs transcription factors are key well-established downstream effectors of *Erf* signalling pathways in aerial organs, first and foremost the cell-fate specifying *SPEECHLESS*, *MUTE* and *FAMA* bHLHs in the sequential phase transitions from protodermal to meristemoid mother cell, meristemoid, guard mother cell, and finally mature stoma, during stomatal development, in interaction with the *SCREAMs* and bHLHs ([MacAlister et al., 2007](#))(Reviewed in ([Pillitteri et al., 2008](#)))([Ohashi-Ito & Bergmann, 2006](#); [Kanaoka et al., 2008](#)). As LRR-RLK, the *Erf* act as direct activators of a MAPK kinase cascade to regulate gene expression in the nucleus. Similarly, in its only known function in trichome initiation in leaves, *GL1* is an indispensable component of a MYB-bHLH-WD40 complex of transcription factors for the initiation of trichomes, which acts to regulate numerous downstream transcription factors, some of which involved in the formation of the seed mucilage too. More broadly, numerous transcription factors, of diverse families, have been reported in studies of transcriptional reprogramming during seed development, for example, *LEAFY COTYLEDON (LEC)* genes ([Lotan et al., 1998](#); [Le et al., 2010](#)); MYB transcription factors (e.g. *TTG1*, *MYB 61*) ([Walker et al., 1999](#)); NAC transcription factors (e.g. *NaRS1* and *NARS2*) ([Kunieda et al., 2008a](#)) and WRKY transcription factors (*WRKY2*, *WRKY 34*) ([Lei et al., 2017](#)).



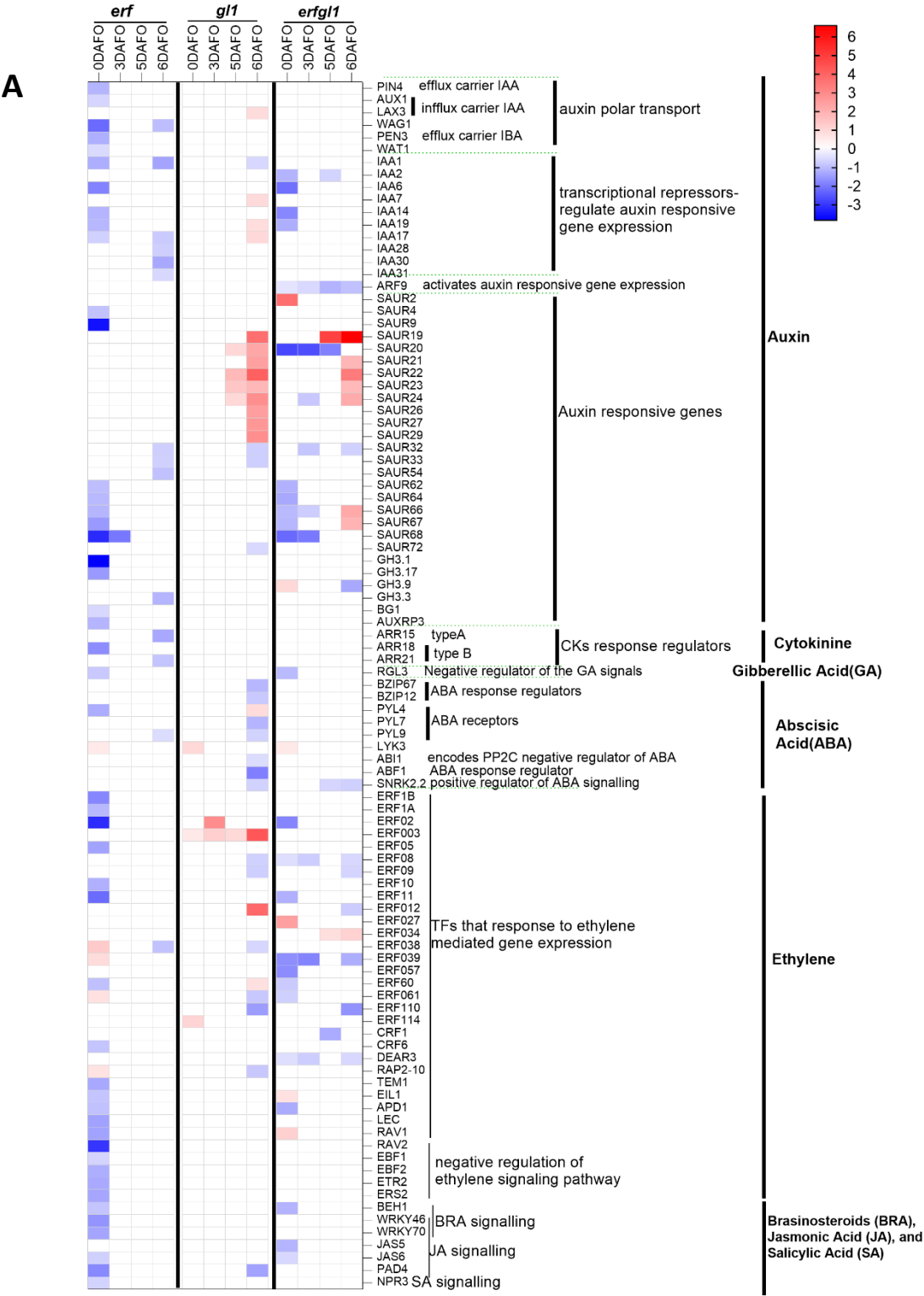
3.3.4 Transcriptional interplay between *ERf* and *GL1*: Key regulators in *Arabidopsis* seed coat development and hormonal signalling

There were 104 differentially expressed transcription factors (FDR<0.05, log2FC>0.5) between *erf* (*erl1/seg erl2*) and WT, representing 19 families among which the Ethylene Response Factor family (*ERF*) (17 genes), basic Helix-Loop-Helix family (*bHLH*) (12 genes), *MYB* family (25 genes), *WRKY* family (9 genes), G2-like family (3 genes) basic leucine zipper (*bZIP*) family (4 genes) and *NAC* family (5 genes) (Figure 3-15A). The vast majority of these TFs showed differential expression at either 0 DAFO or 6 DAFO, with the exception of a few *ERF* and *MYB* transcription factors detected at 3 DAFO or 5 DAFO. Conservation of the types of TF families that are under the transcriptional control of the *ERf* and *GL1*, alone or in combination, but with differences across the three genotypes, in the numbers of genes affected within these families and their relative proportions: most notably *NAC* TFs, much more represented in *gl1*, and even more so in *erf gl1*, than in *erf*- mutant indicating an important role of *GL1* in the expression of *NAC* TFs. Also strikingly: the *ERF*, *bHLH*, members that show differential expression in *erf* are mostly different from family members that are DE in *gl1* or quadruple mutant.

For *NAC*, *bZIP* and *WRKY* families, no overlap between the 3 mutants; different sets of family members are differentially expressed in the 3 mutants. Of note, is the detection of differential expression of *SCRM2* and *PIF4* in *erf gl1*; both TFs are well established key components of the *ERf* pathway in stomatal development, and interact with *SPCH* (reviewed in ([Bergmann & Sack, 2007](#); [Pillitteri & Torii, 2012](#); [Xian Qu et al., 2017](#); [Zoulas et al., 2018](#)));([Chen et al., 2020](#)). Intriguingly they were not detected in *erf* seeds carrying functional *GL1*, but only in the presence of the additional *gl1* mutation.

Overall, these data suggest possible interactions between the *ERf* and *GL1* at the transcriptional level across a wide range of transcription factors. GO enrichment shows a significant enrichment of terms “transcription” and “hormonal signalling regulation” in the three lines (Supp figure 3-8), but also unique features such an enrichment of TFs involved in secondary cell wall biogenesis and lignin biosynthetic processes, particularly involving *MYB* and *NAC* TFs, especially in seeds carrying *gl1* alone or in combination with *erf* (Figure3-13).

I next examined in greater detail the genes involved in the biological processes and KEGG pathways declared as enriched in the above Gene ontology and Gene enrichment analyses, searching key primary genes that are known to play a role in regulating early seed development, or the novel germination and seed coat development and patterning phenotypes uncovered in the experiments reported in the previous Chapter.



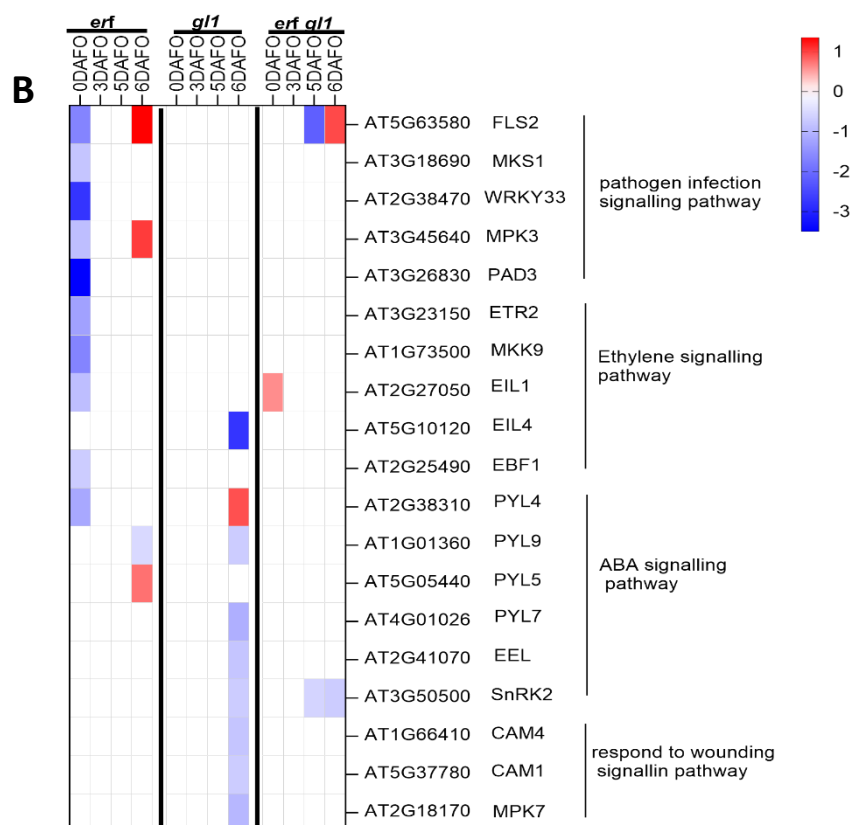


Figure 3-16: Differential gene expression of genes involved in hormonal signalling pathways in three mutants at four developmental stages (0DAFO, 3DAFO, 5DAFO, and 6DAFO). The rows represent individual genes, and the columns represent different time points for each mutant. Gene expression levels are displayed as log2 fold changes relative to the wild type (WT). **A.** Genes involved in different hormonal pathways between *erl1/seg erl2* (*erf*), *gl1*, *erl1/seg erl2 gl1* (*erf gl1*) compared to WT across 4 time points. **B.** Differentially expression genes involved in the MAPK signalling pathway of arabidopsis in *erf*, *gl1* and *erf gl1* compared to WT. The clustering of genes and time points in both heat maps highlights the temporal and genetic regulation of hormonal signalling pathways and MAPK signalling pathways during early seed development in these mutants. Gene IDs and full names of the gene symbols included in the heatmaps were presented in Appendix (3).

3.3.5 Loss of ERf function induces an extensive deregulation of hormonal signalling during early seed development and interacts with GL1 in the regulation of auxin, cytokinin and abscisic acid signalling pathways.

Figure 3-16 gives a description of the extensive deregulation of the *ERf* and *GL1* hormonal signalling transcriptional networks. Cytokinin, Gibberellin (GA), Absciscic acid (ABA), Ethylene, Brassinosteroids (BRA), Jasmonic acid (JA) and Salicylic acid (SA) signalling were all affected, and mostly down-regulated, except for auxin genes which showed significant upregulation at 6DAFO in both *gl1* and *erf gl1* (Figure 3-16 A). Downregulated components of auxin signal transduction pathways included the auxin influx transporter *AUXIN RESISTANT 1* (*AUX1*), three *GRETCHEN HAGEN* (*GH3*), coding for hormones-amino acid conjugating enzymes, 9 *SMALL AUXIN-UPREGULATED RNA* (*SAUR*, early auxin response genes involved in the promotion of cell expansion), five *AUXIN/INDOLEACETIC ACID* (*AUX/IAA*, a large gene family involved in auxin perception and transcription repression), 3 auxin *INDOLE-3-ACETIC ACID* *INDUCIBLE* genes (*IAA14*, *IAA19*, *IAA1*), as well as the auxin-efflux carrier-encoding gene *PIN-FORMED 4* (*PIN4*).

Down-regulated genes in the ethylene pathway included two EIN3/EIL1 ethylene-insensitive transcription factors, which are key transcriptional regulators of ethylene response, and the *ETHYLENE RESPONSE FACTOR 1* (*ERF1*). Notably, the three DEGs associated with abscisic acid signal transduction included: the *PYR1-like 9* and *PYR1-like 4* (*PYL4*), both downregulated at 0DAFO and 6DAFO, respectively, and both members of the *PYR/PYL* family of proteins involved in the formation of the ABA receptor complex, and in the case of *PYL4* is also a documented interactor of the *SPCH* and *ERECTA* kinases in stomatal development ([Gonzalez-Guzman et al., 2012](#); [Yang et al., 2022](#)). The third gene was the *LYSM-CONTAINING RECEPTOR-LIKE KINASE 3* (*LYK3*) which regulates the cross-talk between immunity and abscisic acid responses ([Pietraszewska-Bogiel et al., 2013](#)) and is important for chitin signalling and plant innate immunity in Arabidopsis ([Tanaka et al., 2013](#)).

Remarkably too, the DELLA protein *RGL3*, which acts as a negative regulator of the GA signalling and as a co-activator of *ABI3* to promote seed storage protein at seed maturation, was the only detected component of the gibberellic acid signal transduction pathway in *erf* seeds (down-regulation at 0 DAFO). The 4 DEGs related to the jasmonic acid signalling pathway were downregulated at 0 DAFO; WRKY transcription factors (*WRKY46* and *WRKY70*), *ARABIDOPSIS PHYTOALEXIN DEFICIENT 4* (*PAD4*) that encodes a lipase-like gene, and a gene encoding the *JASMONATE-ZIM-DOMAIN PROTEIN 6* (*JAS6*) (Figure 3-16 A).

As noted earlier, by 3 DAFO, the extensive deregulation of hormonal signalling induced by loss of *ERf* function had disappeared with only one *SAUR*-like auxin-responsive protein (*SAUR 72/AT1G29490*)

showing differential expression, and none at 5 DAFO (Figure 3-16A). At 6 DAFO, a few SAUR- and IAA-encoding genes were detected, but were all different from the SAUR and IAA members detected 6 days earlier, and the *GH3.3* genes, which regulate auxin homeostasis, were also down-regulated. Overall, only 15 genes involved in hormonal pathways were detected, mostly related to auxin metabolism, transport and signalling (10 genes), and secondarily to cytokinin (2 *ARR*), and ABA (*PYL9*) (Figure 3-16A), but remarkably for all these three major hormones, through different variants of the same gene family.

In contrast, loss of *GL1* function interfered with hormonal signalling at a later stage of embryogenesis, only from around 5 DAFO, with the exception of the differential expression of *ERF2* and *ERF3* at 3 DAFO (Figure 3-16A). It mostly affected the auxin and ethylene pathways, as in the *erf* 6 days earlier. However, contrary to observations in *erf* seeds, auxin-associated genes were generally up-regulated and, while also including SAUR and IAA genes (11 and 5 genes, respectively), they mostly consisted of different family members from those that showed differential expression in the *erf* mutant. Interestingly, *LAX3*, which encodes an auxin influx carrier similar to *AUX1*, was specifically upregulated in *gl1*, at 6 DAFO. *LAX 3* is constitutively expressed in various organs, but notably in guard cells, flowers, and globular to torpedo stage embryos ([Basu et al., 2013](#)).

Another major, specific, feature of *GL1* loss of function, was the inhibition of several core upstream components of the ABA signalling pathway, detected for the first time 6 DAFO, and including: a) the differential expression of three members of the PYR/PYL/RCAR family proteins of the ABA receptor complex - *PYL 4* (upregulated), *PYL7* and *PYL9* (both downregulated in *gl1*); b) also the downregulation of *ABI1*, one of the Type A PP2Cs phosphatases which act as ABA co-receptors; c) the down-regulation of one of the downstream *SNF1-RELATED PROTEIN KINASES 2* (SnRK2.2) ; d) the down-regulation of *ABF1*, one of the *ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 1* (*ABF*) transcription factors which, when phosphorylated by SnRK2s, activate ABA response genes; e) the down-regulation of two bZIP transcription factors *BZIP67* and *BZIP12*, a transcription factor homologous to *ABI5* which is well known to promote ABA signalling during seed development ([De Giorgi et al., 2015](#)) and seed germination. These results indicate that *GL1* acts to regulate ABA signalling during early embryogenesis, around or from about 5-6 DAFO.

Similar to its action alone, when combined with *erf*, *gl1* caused the up-regulation of a number of SAUR proteins at 6 DAFO, 4 of which were conserved (SAUR23, 24, 25 and 21), and at that final time-point

also shows the down-regulation of several ERFs, which are typically expressed in response to ethylene-mediated gene expression, although not always. However, most strikingly, the differential expression of the major upstream components of ABA signalling observed in the presence of *gl1* alone at that 6 DAFO time point was abolished, apart from the downregulation of *SnRK2.2*. (Figure 3-16 A). Moreover, in contrast to seeds carrying *gl1* alone, the *erf gl1* quadruple mutant seeds showed significant changes in hormonal networks at 0 DAFO already, similar to the temporal pattern observed in *er erl1/seg ser12 GL1* counterparts. The differential gene expression induced by *erf gl1* also involved IAA family members, as well as *RGL3*, and a few components of the JA and brassinosteroids pathways, as observed at 0 DAFO in *erf* seeds. However, there was no detectable sign of differential expression of cytokinin genes.

Erf and GL1 modulation of MAPK cascade components during Arabidopsis early seed development.

The *ER*, *ERL1* and *ERL2* Leucine Rich Receptor Kinases and LRR Receptor-like Too Many Mouth (TMM) complex functions upstream of a MAPK phosphorylation cascade composed of YDA-MKK4/MKK5-MPK3/MPK6. Upon small peptide ligand binding, the *ERf*-TMM complex heterodimerize with Somatic Embryogenesis Receptor Kinases (SERKs), triggering the phosphorylation and activation of MPK3/MPK6 cascade via phosphorylation of upstream MKK4/MKK5 and MEKKK/YODA to regulate stomatal development, tissue patterning, organ growth and plant morphogenesis, as well as plant defence responses through the activation of a range of substrates – most of which unknown- including transcription factors, protein kinases and other enzymes and structural proteins. I thus next examined the effects of loss of *ERf*- or/and *GL1*- function on the expression of *MPK3/6* and known downstream targets and upstream activators in plant pathogen responses, abiotic stress responses, wounding and hormonal signalling (Supp figure 3-9). *erf* seeds showed downregulation of *MKK9* and *MPK3* expression at 0 DAFO, together with the downregulation of several components of MAPK signalling during pathogen infection and of ethylene signalling and the ABA receptor component *PYL4*, which is also a known interactor of *SPCH* and *ER* in stomatal development. None of these genes showed differential expression any more on 3 DAFO nor 5 DAFO, but within the next 24h, *MPK3* and *FLS2* both now showed a strong upregulation as well as *PYL5*, and *PYL9* was downregulated. All the other DEGs detected at 0 DAFO showed WT expression levels.

Strikingly, in the presence of *gl1*, the above *erf*-induced differential expression was abolished, apart from that of the *FLS2* on 5DAFO and 6DAFO. *FLS2* is a well-studied LRR-RLK involved in innate immunity

and which shares interactors with *ERECTA* such as *BAK1* ([Jorda et al., 2016](#); [Majhi et al., 2021](#); [Chen et al., 2023](#)). Notably also, *SNRK2* (*At3g50500*) was downregulated, in contrast to its unchanged, WT levels, in *erf GL1* seeds (Figure 3-16 B). *gl1* alone had no effect on the transcription of the above genes, but part from downregulating *SNRK2*, *PYL 5*, *7* and *9* as described earlier (Figure 3-16 A), *GL1* alone also downregulated *MPK7*, and two Ca^{2+} binding calmodulin kinases, *CAM1* and *CAM4*, involved in wounding signalling, which were expressed at WT level in *erf gl1* tissues. These results indicate a combination of specificity and strong negative interactions of the *ERf* and *GL1* in the transcriptional regulation of MAPK signalling components during early seed development.

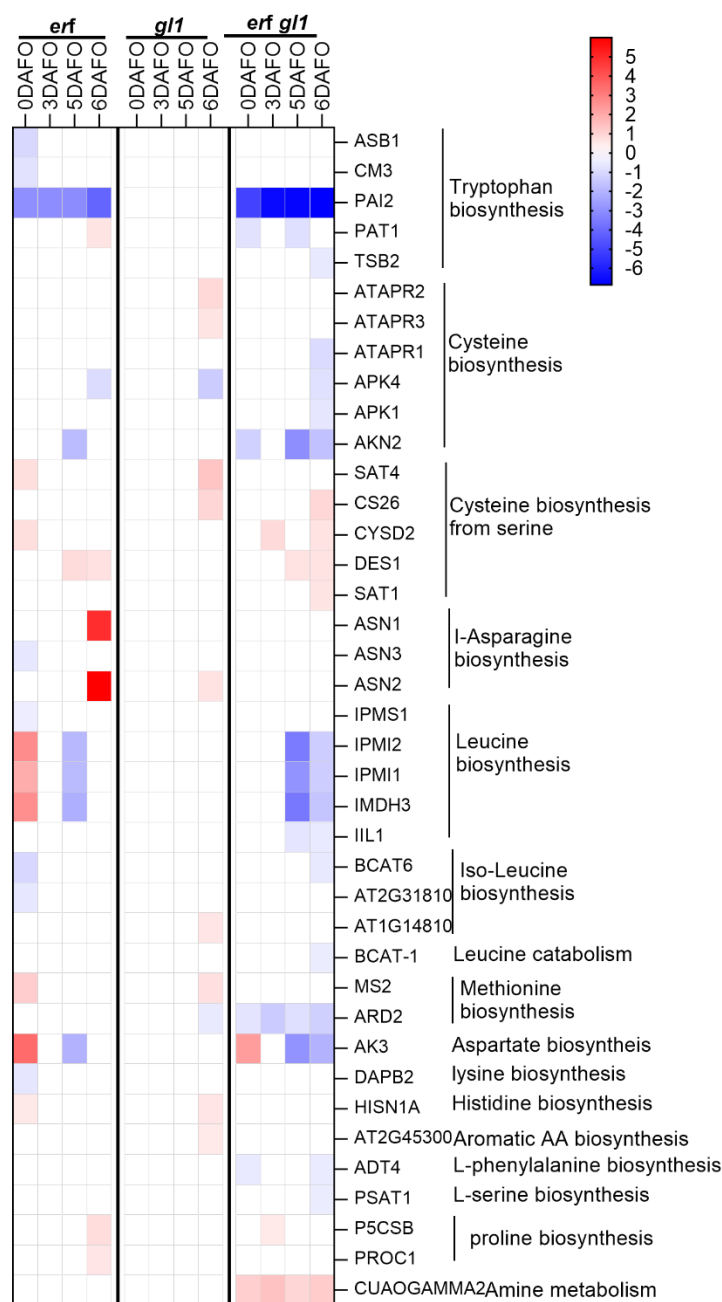


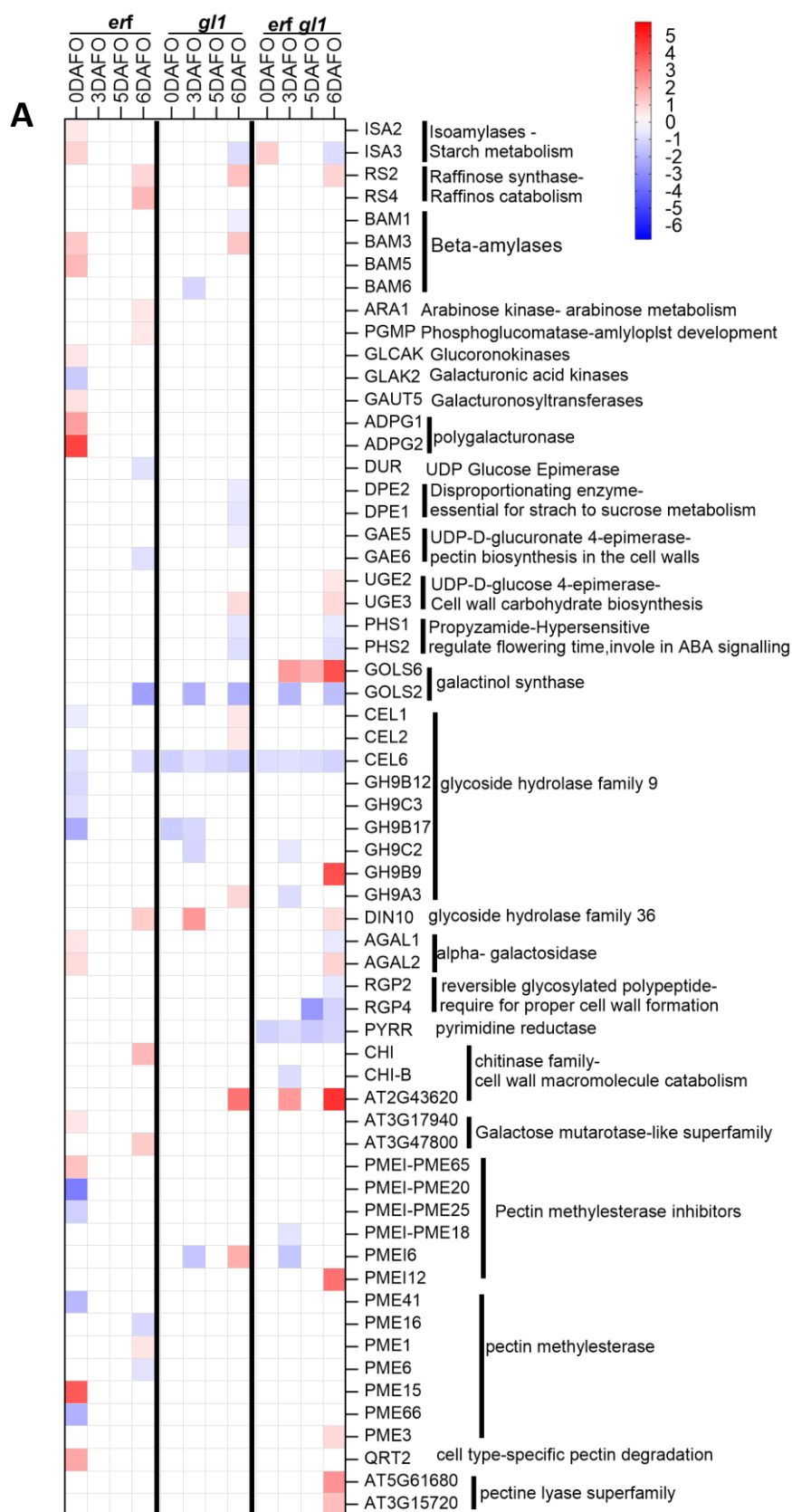
Figure 3-17: Differential gene expression of genes involved in amino acid in three mutants (*erl1/seg erl2* (*erf*), *gl1*, *erl1/seg erl2 gl1* (*erf gl1*)) compared to WT at four developmental stages (0DAFO, 3DAFO, 5DAFO, and 6DAFO). The rows represent individual genes, and the columns represent different time points for each mutant. Gene expression levels are displayed as log2 fold changes relative to the wild type (WT), with up-regulated genes in red and down-regulated genes in blue, as indicated by the scale bar. The clustering of genes and time points in the heat map highlights the temporal and genetic regulation of amino acid metabolism pathways during early seed development in *erf*, *gl1* and *erf gl1* mutants. Gene IDs and full names of the gene symbols included in the heatmap was presented in Appendix (3).

3.3.6 *Three ERf genes regulate amino acid metabolism in interaction with GL1 during early seed development.*

The large sets of differentially expressed genes involved in the regulation of amino acid, lipid, and carbon metabolism detected in the three mutants (Figure 3-17 to Figure 3-19) all showed strong contrast in both the temporal patterns of differential expression and the affected genes. Lipid and carbon metabolism were most affected with respect to multiple regulatory components of the cell wall, lipid polyesters, and wax components.

Differential expression of genes amino acid synthesis in the *erf* samples were detectable as soon as 0 DAFO, and then again at 6 DAFO, except for 7 genes including a trio of genes (*IPMI1*, *IPMI2*, *IMDH3*) involved in Leucine (LEU) biosynthesis and (Methionine) MET chain elongation, implicated in pathogen resistance and thought to have broad roles, as precursors of phenylpropanoids, lignin, hormones (Figure 3-17) ([Lundquist et al., 2014](#); [Tonnessen et al., 2015](#); [Lächler et al., 2020](#)). Notably, IPMI Isopropylmalate isomerases are specifically expressed in epidermal cells, including green siliques (*IPM1*) ([Lächler et al., 2020](#)). These three aromatic amino acid metabolism enzymes were all upregulated at 0 DAFO but downregulated at 5 DAFO and back to WT levels within the next day. Also notable was the *PAI2* phosphoribosyl anthranilate isomerase gene encoding the catalytic enzyme of the third step in the tryptophan biosynthetic pathway ([Zhao, 2012](#)), which showed a consistent downregulation throughout the whole 6 days period covered in this study; and also, specific to *erf* transcriptome, the huge upregulation (FC>10) at 6 DAFO of the asparagine synthetase genes *ASN1* (At3g47340) and *ASN2* (At5g65010) and *ASN3* (At5g10240) (Figure 3-17).

Strikingly, *gl1* seeds showed hardly any differential expression in amino acid metabolism genes compared to WT. However, the transcriptome of the quadruple mutant only partly mirrored that of the *erf* with the downregulation of *PAI2* across all time points, and of the same three leucine biosynthesis genes at 5 & 6 DAFO, but the former was stronger than in *erf GL1* seeds (doubling of log₂FC value) (Figure 3-17), and the downregulation of the leucine biosynthesis genes and the aspartate biosynthesis gene *AK3* extended to 6 DAFO. Moreover, contrary to their upregulation in *erf*, *AS1* and *ASN3* transcripts were at WT levels, as observed in *gl1* samples.



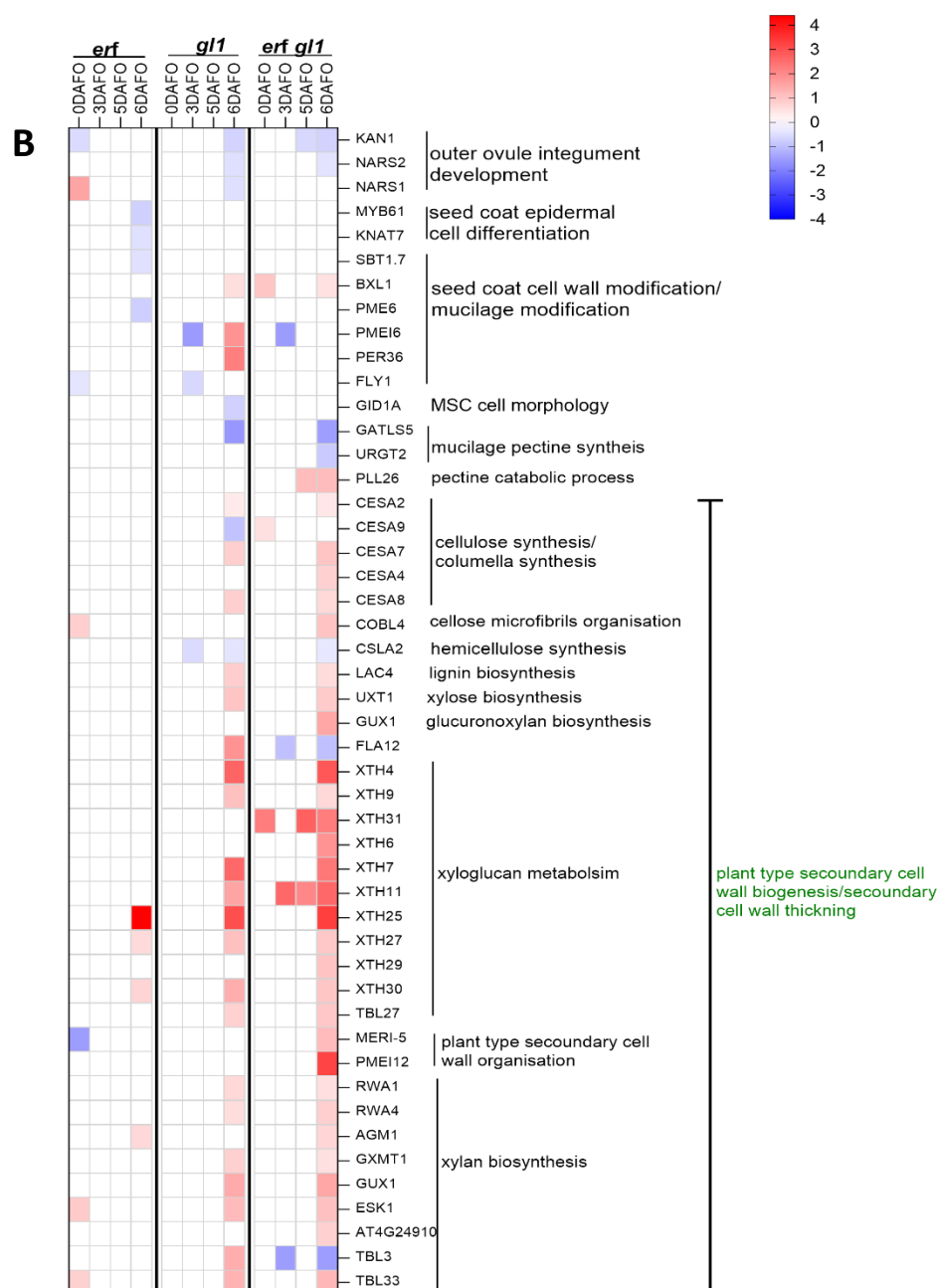


Figure 3-18: Differential gene expression of genes involved in carbohydrate/carbon metabolism (A) and genes involved in Arabidopsis seed coat development, differentiation, and cell wall modification (B) in three mutants (*erl1/seg erl2* (*erf*), *gl1*, *erl1/seg erl2 gl1* (*erf gl1*)) at four developmental stages (0DAFO, 3DAFO, 5DAFO, and 6DAFO). The rows represent individual genes, and the columns represent different time points for each mutant. Gene expression levels are displayed as log2 fold changes relative to the wild type (WT), with up-regulated genes in red and down-regulated genes in blue, as indicated by the scale bar. B. The heatmap illustrates the expression patterns of genes that are directly involved in various aspects of seed coat development and cell wall modification in Arabidopsis. The genes are grouped by their identified functional roles. Gene IDs and full names of the gene symbols included in the heatmaps were presented in Appendix (3) MSC-Mucilage Secretary Cells

3.3.7 The *ERf* and *GL1* are both implicated in the transcriptional control of multiple cell wall genes.

The data presented in Chapter 2 showed that the loss of function of *ERf* regulates the cell proliferation and cell expansion in Arabidopsis developing seed coat (Chapter 2, Figure 2-13) during a critical developmental window of early seed development when the maximum expansion of the seed cavity takes place. In addition to the previously reported modifications of the cell wall and mucilage composition ([Nanda et al., 2019](#)), the microscopy examination of the *erf* mature seed coat epidermis here, also revealed thicker cell walls, and broader columellae (Chapter 2, Figure 2-7), two features significantly enhanced by loss of *GL1*. I hence mined the sets of differentially expressed genes for genes with demonstrated functions in the expansion of the seed coat integuments, the formation and properties of mucilage whose production starts as soon as 4-5DPDAO ([Beeckman et al., 2000](#); [Western et al., 2000](#); [Haughn & Western, 2012](#); [Voiniciuc, Yang, et al., 2015](#)), cell wall biogenesis, remodelling, degradation (Figure 3-18 A and B).

The *erf* seeds showed differential expression in a number of regulators of the cell wall at 0 and 6 DAFO, most of which were not detected in *gl1*. Included were several pectin methylesterase (*PME*) and pectin methylesterase inhibitors (*PMEI-PME*) involved in cell wall biogenesis or degradation, cell wall pectin loosening, as well as loosening of the pectinaceous mucilage that lines the arabidopsis seed epidermis. Among the latter were *At1g23200* (*PEM 6*) and *At2g43050* (*PEM 16*), which have been reported to be highly expressed in the Arabidopsis seed coat 4-7 DAFO ([Levesque-Tremblay et al., 2015](#)) and were down-regulated (FC>1.5) in the *erf* mutant. Notable were also two *ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE* (*ADPG*) family genes, known for their role in cell wall modification during abscission ([González-Carranza et al., 2007](#)), and two *alpha-galactosidase* (*AGAL*) family genes up-regulated at 0DAFO in the *erf* (Figure 3-18 A). Conversely, another group of genes involved in cell wall organisation and remodelling (*PME3*), pectin lysis (pectinases/pectinases- like *At3g15720*, *At5g61680*), or the chitinase *At2g43620*, glycoside-hydrolase *GH9B9* reported to be involved in pollen germination and tube growth, were all upregulated in *erf gl1* tissues but not in *erf GL1*, and not either in *gl1* except for *At2g43620*. Similarly, the UDP arabinose mutase/deaminase *RGP2* and *RGP4* acting in the interconversion of pyranose and furanose, essential for wall formation, and the PyrR gene thought to be involved in the riboflavin biosynthetic pathway and damage repair, were all specifically down-regulated in the quadruple mutant but expressed at WT levels in the two parental mutants.

The transcriptomes of the 3 mutants indicate strong *ERF-GL1* interactions in the transcriptional control of a number of other genes involved in carbohydrate metabolism, cellulose and polysaccharide degradation, which were either down-regulated such as *CELLULASE 1 (CEL1)*, *CELLULASE 6 (CEL6)*, *GLYCOSYL HYDROLASE 9B12 (GH9B12)*, *GLYCOSYL HYDROLASE 9C3 (GH9C3)*, and *GLYCOSYL HYDROLASE 9B17 (GH9B17)*, or highly upregulated such as the pectin lysase-like *At5g61680* and pectin ligase *At3g15720*, or the glycosyl hydrolase *DARK INDUCIBLE 10 (DIN10)*, and some members of the *BAM*((β -amylase) family or starch debranching enzyme Iso-amylase *ISA2*, or the Galactinol Synthase *GOLS6* that catalyses the first, rate-limiting step of raffinose synthesis (Figure 3-18 A). Notably, another *GOLS* gene encoding *GALACTINOL SYNTHASE 2 (GOLS2)* was, on the contrary, downregulated in the three mutants. Galactinol synthase-*GOLS2* is a key enzyme for the synthesis of raffinose family oligosaccharides, which have been implicated in the mitigation of effects of environmental stresses in plants, germination speed and the seedling's tolerance of high salinity and osmotic stress ([Sun et al., 2013](#)),

Figure 3-18 B gives a comparative overview on the differential expression detected on whole suites of additional genes with reported functions in outer ovule integument development, seed coat epidermal cell differentiation, cell wall biogenesis and remodelling, and mucilage production and integrity, across the three mutants and 4 time-points. *KANADI* family member *KAN1* transcription factor was down regulated at ODAFO in *erf*, but thereafter only in *gl1* and *erf gl1*, at 5-6DAFO. NAC family members *NARS1* and *2* were down regulated later, only in *gl1* and *erf gl1* at 5 DAFO, and 5-6DAFO respectively. *KAN1* was reported to regulate outer integument development ([McAbee et al., 2006](#); [Kelley et al., 2009](#)), and *NARS* are involved in outer integument differentiation (*NARS2* Kunieda et al., 2008). Contrary, MYB transcription factor *MYB61*, along with *KNAT7* which encodes a homeodomain transcription factor of the Knotted family and *SBT1.7* were also down-regulated at 6DAFO, but only in the *erf*. *MYB61* is required for proper seed mucilage extrusion upon imbibition ([Penfield et al., 2001](#)), along with *KNAT7*, and the subtilisin-like protease *SBT1.7* is one of the major cell wall proteins necessary for normal mucilage release together with *PMEI6* and class III peroxidase *PRX56* ([Rautengarten et al., 2011](#); [Kunieda et al., 2013](#); [Saez-Aguayo et al., 2013](#); [Ranocha et al., 2014](#)), all expressed in MSC around the torpedo stage.

Apart from the few above genes, there was a large set of genes involved in cell wall formation and modification, detected among DEGs at 6 DAFO only, that were upregulated apart from a few exceptions-furthermore in both *gl1* and *erf gl1*, but not *erf* tissues (Figure 3-18). These included *BETA-*

XYLOSIDASE 1 (BXL1), a gene necessary for normal mucilage release ([Arsovski et al., 2009](#)) up-regulated in both *gl1* at 0 DAFO and *erf gl1* at 0 and 6 DAFO; *PMEI6*, downregulated in both *gl1* and *erf gl1* at 3 DAFO, but upregulated at 6 DAFO in *gl1* only (log2FC 1.86). *PMEI6*, together with the SBT1.7 and transmembrane RING E3 ubiquitin ligase, *FLYING SAUCER 1 (FLY1)* is highly expressed during mucilage deposition in the developing seed coat and whose reduced expression results in a reduction mucilage production ([Rautengarten et al., 2008](#); [Saez-Aguayo et al., 2013](#); [Voiniciuc et al., 2013](#)). These data indicate that *gl1* alone plays a role in mucilage synthesis and modification, independent to the signalling of *ERf*. Interestingly *FLY1* was downregulated at 3 DAFO too in *gl1*, and one of the very few DEGs detected in *erf* at 0 DAFO (down regulated).

The set of genes differentially expressed in both *gl1* and *erf gl1* mutants but not the *erf*, also included *GALACTURONOSYLTRANSFERASE-LIKE 5 (GATLS5/ GATL5)* highly down-regulated in both *gl1* and *erf gl1* which encodes a protein with putative galacturonosyltransferase activity and *UDP-RHA/UDP-GAL TRANSPORTER 2 (URGT2)*, only down- in *erf gl1*: *CSLA2*, a member of the CELLULOSE SYNTHASE-LIKE A protein family, that is involved in the synthesis mucilage hemicellulose component glucomannan ([Yu et al., 2014](#)), down-regulated in both *gl1* and *erf gl1* mutant seeds at 6 DAFO; three secondary cell wall cellulose synthase (CESA) members (*CESA4/IRX5*; *CESA7, IRX3*; *CESA8/IRX1*) involved in the formation of secondary cell walls cellulose and the adherence of the inner mucilage layer to the seed epidermis; *CESA2* upregulated in *gl1* and *erf gl1* at 6 DAFO, and *CESA9* down-regulated in *gl1* at 6 DAFO and up-regulated in *erf gl1* at 0 DAFO, both with a role in the promotion of cell proliferation, of cell wall integrity and secondary cell wall thickness ([Hu et al., 2018](#)); *PER36*, a member of the class III peroxidase family, strongly upregulated in *gl1* but no more in the *erf* background, which is polarly deposited into the outer primary cell wall at the start of mucilage secretion and generates reactive oxygen species that may degrade the major pectins of the seed mucilage, RG I and/or HG ([Kunieda et al., 2013](#)).

Further, enrichment analysis indicated a significant over-representation specific to 6 DAFO and mostly the *gl1* and *erf gl1* transcriptomes of regulatory components of secondary cell wall biogenesis and thickening, especially a large group of genes involved in xyloglucan and xylan biosynthesis, all up-regulated at 6 DAFO (Figure 3-18 B); *UXT1*, involved in xylose transmembrane transport; *LAC4* involved in lignin biosynthesis and co-expresses with secondary cell wall biosynthesis genes; the *COBRA-like 4 (COBL4)* gene, which participates to secondary cell wall assembly and is essential for cellulose deposition in the xylem7 up-regulated members of the *XTH* family in *gl1* and *erf gl1*, 3 additional members, *XTH31*, *XTH6* and *XTH29*, only up-regulated in the quadruple mutant *gl1* indicating an interactive role of *ERf* and *GL1* in the transcriptional regulation of *XTH* genes; the *RWA* genes, including

RWA1 and *RWA4*, *TBL33* and *ESK1*, that are involved in the biosynthesis of xylan acetyl. Altogether these data reveal extensive *GL1*-mediated positive regulation at the transcriptional level of the formation, composition, deposition and modelling of mucilage pectins and of primary and secondary cell wall components, and indicate functional specificity as well as synergy with the *Erf* family in this function.

3.3.8 Cutin and wax synthesis genes were significantly up-regulated by loss of function of *GL1* and *Erf*, alone and/ or in combination.

Lipids play crucial roles in developmental and growth processes, and in the formation of barrier and defence components such as waxes, suberin and cutin, such as present in arabidopsis at the seed surface and boundaries of seed compartments (Figure 2-16, Chapter 2).

Biosynthesis of cutin involves the transfer of acyl-CoA by long-chain acyl-CoA synthetase (LACS) to fatty acid monomers; then, cutin monomer precursors are enzymatically modified by a *CYTOCHROME P450* (*CYP*) family of fatty acyl ω -hydroxylases proteins and of glycerol-3-phosphate O-acyltransferases (*GPAT*) that transfer hydroxyl groups and glycerol groups, respectively ([De Giorgi et al., 2015](#)). Several components of that pathway were differentially expressed in the *erf* mutant, including two genes encoding a *GLYCEROL-3-PHOSPHATE SN-2-ACYLTRANSFERASE* (*GPAT4*, *GPAT1*), up- and down-regulated, respectively; five upregulated *CYTOCHROME P450* genes (*CYP77A9*, *CYP77A6*, *CYP86A4*, *CYP86A7* at 0 DAFO; *CYP86A8* and *CYP86B1* at 6 DAFO), one down-regulated (Figure 3-19); downregulated *LACS 3* and *LACS 1* at 0 and 6 DAFO respectively. In addition, genes involved in wax biosynthesis were also up and down-regulated in *erf* at different time points, including two encoding wax ester synthase (WS) and diacylglycerol acyltransferase (*DGAT*); *WSD1* and *WSD7*, down and up-regulated at 0 and 6 DAFO, respectively; *CYP96A15* encoding midchain alkane hydroxylase during cuticular wax synthesis, downregulated at 0 DAFO. Further, four suberin biosynthesis genes, including two *FATTY ACID REDUCTASE* (*FAR 1* and *FAR7*) and the gene encoding feruloyl-CoA transferase that is required for suberin synthesis (*HHT1*), were differentially expressed in *erf* at 0 and (Figure 3-19).

Remarkably none of those genes, or any other member of their corresponding families showed differential expression in *gl1* at 0 DAFO, or subsequent time-points, until 6 DAFO. At that latest time stage, *GPTA1*, 3, 4, *LACS1*, 3 were all differentially expressed, but all up-regulated.

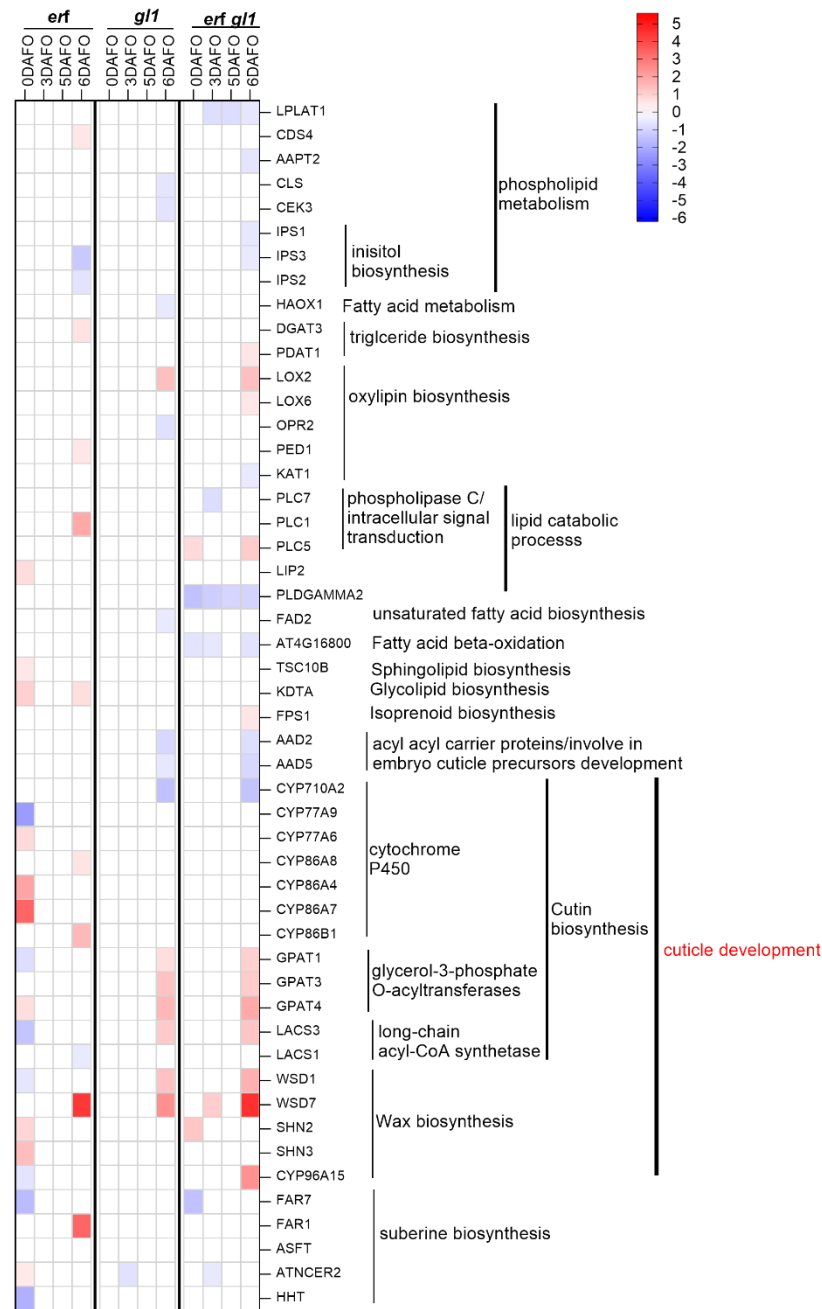


Figure 3-19: Differential gene expression is involved in lipid metabolism, cutin suberin, and wax synthesis across three mutants at four developmental time points. The rows represent individual genes, and the columns represent different time points for *er erl1/seg erl1*, *gl1* and *er erl1/seg erl2 gl1* mutants. Gene expression levels are displayed as log2 fold changes relative to the wild type (WT), with up-regulated genes in red and down-regulated genes in blue, as indicated by the scale bar. Gene IDs and full names of the gene symbols included in the heatmaps were presented in Appendix (3).

Similar to *erf* tissues, *gl1* tissues showed an up-regulation of *GLYCEROL-3-PHOSPHATE SN-2-ACYL TRANSFERASE* family *GPAT4* and *GPAT1* but additionally too of *GPAT3*- as well as of *LACS3*. However, the only CYP among DEGs in *gl1* were *CYP710A2*, a CYTOCHROME P450 involved in the hydroxylation of fatty acids at 6 DAFO, not detected in the *erf*, and again the *WSD1* and 7 genes (Figure 3-19).

Interestingly, *CYP96A15* showing strong upregulation, along with no differential expression of these genes was observed at any other time point except at 6 DAFO, indicating the development stage-specific functional specificity of *GL1*.

The pattern of differential expression in the quadruple *erf gl1* mutant for the above set of genes mirrored that observed in *gl1* (Figure 3-19). The *GPAT4* is specifically expressed in the epidermal cells of the developing embryo and the seed coat. Loss-of-function mutations in *GPAT4* result in reduced cutin deposition in the seed coat, leading to increased permeability and altered water retention properties of the seed (De Giorgi et al., 2015). In addition, *GPAT1* were also highly expressed in seeds and loss of function alter the seed fatty acid composition, and increased the plant height by inducing the GA signalling (Bai et al., 2021). Further the Bai et al (2021) study also reported that reduced *GPAT1* signalling increased the expression of *PMEs*, *PMEI*, *XTHs* and *EXPs*. The upregulation of genes involved in cuticles biosynthesis at 6 DAFO in *gl1* and *erf gl1* suggests that around the beginning of seed maturation and seed coat differentiation, *GL1* plays a role in the transcriptional control of the seed cuticle deposition and composition and also interact with the loss of function of *ERf* in the wax and cuticle biosynthesis gene expression, consistent with the increased cuticle thickness seen in *erf* and its enhancement by combined *erf* and *gl1* mutations. Interestingly, two Acyl carrier proteins, *AAD2* and *AAD5*, involved in the formation of embryo cuticle were also among DEGS in *erf gl1*, a single *gl1*, but not in the *erf*.

Apart from genes involved in the complex suberin, cutin and waxes, the quadruple mutants showed differential expression of genes involved in the synthesis of inositol and phospholipids, oxylipins, and the regulation of PLC expression *PLC1*, 5 and 7. These genes were also detected in the *erf* and *gl1*, and then remarkably, in all cases, at the latest 6 DAFO time point. These results indicate synergistic roles of the *ERf* and *GL1* genes in the transcriptional control of major regulators of cell growth and of signalling lipids (e.g., inositol, *LYSOPHOSPHATIDYLCHOLINE ACYLTRANSFERASE 1* (*LPLAT1s*, or *PHOSPHOLIPASE D GAMMA 2* (*PLDGAMMA2*) and several PLCs). The double *plc5/7* was also reported to be defective in mucilage deposition and *PLC7* expression was detected in trichomes and hydathodes (van Wijk et al., 2018), similar to *GL1* and the *ERf*, respectively. Altogether, these results are consistent with the altered thickness of the endosperm-associated cuticle in *erf* and even more so *erf*

gl1 seeds compared to WT, as well as differential cell proliferation and expansion, and mucilage properties identified in the experiments reported in Chapter 2.

3.4 Discussion

Results in Chapter 2 demonstrated the primary role of the seed coat in mediating the maternally derived functions of the *ERf* and *GL1*, separately and together, in the control of seed germination.

Consistent with this, they revealed multiple effects a promoting role of the *ERf* in the regulation of the rapid expansion of the seed coat that takes place during early embryogenesis (0-6DAFO) and gives rise to the fully expanded seed cavity, through modulatory effects on both cell proliferation and cell expansion in the seed coat integuments. Furthermore, the anisotropy of cell expansion was modified, especially in the inner ii'1 layer, as well as the size and shape of the incipient columellae on the outer-most oi2 epidermal layer of specialised mucilage-secreting cells, and the formation of the endosperm associated cuticle, both involving synergy between the *ERf* and *GL1*. That early phase of seed development from 0 to 6 DAFO hence appeared as a developmental window of choice to understand the early mechanisms of action of the *ERECTA* family and *GL1* transcription factor in their interactive effects on the properties of the mature seed coat epidermal patterning of the mature seed coat; its anatomy and morphology, with most prominently the formation of thicker radial cell walls, and broader columellae in the *er erl1/segerl2 gl1* seeds; the formation of the endosperm-associated cuticle which by day 6 post-pollination is completed.

My aim of the experiments reported in this Chapter was to gain insights on the underlying molecular pathways, and on their interactions in the control of some of the developing and mature seed coat characteristics. Given the scarcity of information on the *ERf* pathway in seed and the previously unsuspected action of *GL1* there, I opted for a genome-wide exploration of transcriptional responses associated to loss of *GL1* function or/and *ERf* signalling in *gl1*, *er erl1/seg erl2 (erf)* and *er erl1/seg erl2 gl1(erf gl1)* seeds, during the above critical developmental window spanning the morphogenetic phase of seed development, from 0 DAFO to late torpedo embryo stage. To that end, I carried out RNAseq on the minute siliques of WT and mutants during a developmental time series from pollination to 6 DAFO.

Given the extremely minute size of seeds, and their attachment to- and communication with the maternal silique through the funiculus during most of that initial developmental phase, I opted for an analysis of the combined transcriptomes of the developing seeds and their enclosing silique, considering they formed an intricately integrated system, essentially maternal at these very early

embryonic stages, and also being beware of minimising disturbance and risks of wounding and confounding contaminations during dissection.

The developing seeds and nourishing siliques consist of several compartments, with specialised tissues, endowed with specific patterns of proliferation, patterning and differentiation. In order to integrate the formation and functions of these tissues, and accurately control cell fate and progression of seed development to completion and maximum seed viability, a precise spatial and temporal coordination of regulatory pathways is needed. The results presented in this Chapter show that the *ERECTA* kinase family and *MYB R2R3 GL1* are key players in the transcriptional control of that coordination. They identify previously unknown wide-spread transcriptional activities of the *ERf* and *GL1* in the regulation of hormonal networks, transcription factors and downstream targets across developmental and biochemical pathways throughout embryogenesis. Furthermore, the functional information available on the differentially expressed genes, and the directions and temporal patterns of the observed differential expression induced by loss of *GL1* and *ERf* function, alone and in combination, are consistent with the differences in seed developmental and germination phenotypes with presented in the previous chapter.

As a whole they indicate complementary and synergistic transcriptional roles of the *ERf* and *GL1*, in their timing and nature, throughout early embryogenesis and the expansion and patterning of the seed cavity, and early differentiation of the specialised mucilage producing epidermal cells. The *ERf* exerts an extensive transcriptional regulation of genes involved in the initial steps of seed development, during the 24h or so following fertilisation (0 DAFO time-point), and then again, but only marginally, around 5-6 DAFO, as the outer oi2 epidermal layer starts to produce carbohydrates, starch and secondary cell wall, mucilage components. In contrast, there no sign of *GL1*-mediated transcriptional regulation until 5-6 DAFO, coinciding with the end of seed coat expansion, and beginning of its differentiation.

Fertilisation is the trigger of massive developmental reprogramming and of active cell proliferation and expansion in the new seed and connected maternal tissues ([Ingouff et al., 2006](#); [Köhler & Makarevich, 2006](#)). The extensive *ERf*-mediated transcriptional up-regulation observed within the 24h following that key event, is consistent with the function of the *ERf* in aerial organs as a master promoter and coordinator of cell growth and proliferation, and a master regulator of cell fate, tissue patterning and organ shape ([Shpak et al., 2004](#); [Pillitteri, 2007](#); [Bemis, 2013](#); [Shpak, 2013](#); [Wu et al., 2022](#)), and its similar roles in the seed coat integuments (Chapter 2: Smaller seed cavity, increased seed coat thickness through integumental layer-specific control of radial cell expansion and reduced cell

proliferation in *erf* vs WT). Also consistent, are the types of genes that were found to be upregulated during that early period, most prominently: auxin related genes, transcription factors involved in auxin and ethylene signalling, a major interacting hormone of auxin in growth regulation, downstream genes of phospholipid signalling and synthesis, biosynthesis and turn-over of sugars, synthesis of primary cell wall components hemicellulose and pectins, necessary to fuel and sustain the active formation and expansion of cellular membranes and cell walls for the *ERf*-mediated promotion of seed coat expansion and thickness (Chapter 2) and seed size (Chapter2; Nanda et al. 2019).

Auxin is indispensable to the trigger of seed-silique development upon ovule fertilisation and its spatio-temporal control ([Vanneste & Friml, 2009](#)). Results here show that the *ERf* acts as a positive regulator of **a) auxin synthesis** (e.g. *PAI2*, phosphoribosylanthranilate isomerase which catalyses the third step in the tryptophan biosynthetic pathway, one of the two pathways of auxin synthesis in plants; the so-called Trp-dependent pathway for IAA synthesis); **b) auxin perception** through up-regulation of a range of IAA transcriptional repressors which act in auxin perception as part of a co-receptor complex with *ARF* (Auxin Response Factor activator proteins). Once bound to *ARFs*, *AUX/IAAs* are targeted for degradation by ubiquitination, thus allowing for the transcription of a large network of auxin-regulated genes; **c) auxin carrier proteins**, among which *PIN4* and *AUX1* which has been reported to be implicated in polar auxin distribution in the seed coat and to be expressed in both *oi1* and *oi2* to regulate seed size ([Redman et al., 2004](#); [Nemhauser et al., 2006](#); [Liu et al., 2023](#)). **d) early auxin response proteins** of the large *SAUR* family of Small Auxin Up-regulated genes ([Kodaira et al., 2011](#)). *SAUR* proteins were only relatively recently discovered and their functions are not well known but converging evidence led to their denomination as “cell elongation” proteins, acting in many organs at different stages ([Stortenbeker & Bemer, 2018](#)) through activation of PM H⁺-ATPase and cell wall acidification. In cotyledons and hypocotyls, *SAURs* were found to be regulated by *PIFs* (Phytochrome Interacting Factors), a significant observation in the context of the present study as *PIF4* is a known interactor of *SPCH* which is a master, direct interactor of *ERECTA* in stomatal development ([Lau et al., 2018](#)). Moreover, some *SAURs* can bind to calmodulins ([Yang & Poovaiah, 2000](#); [Popescu et al., 2007](#)), consistent with the suggestion that some are involved in polar auxin transport ([Vanneste & Friml, 2013](#)) ([Ren & Gray, 2015](#)).

The transience of these very early wide-spread *ERf* transcriptional effects, over the first 24h to 48h of seed development according to the changes in transcriptional networks detected in this study between *erf* and WT tissues (Figure 3-15), is intriguing as it covers only about half of the period of seed coat expansion to its full size, and only the pre-globular to globular stages of embryonic development, about 3-4 days before embryo body plan and patterning are in place. It may indicate that the *ERf* members

are very upstream, primary, regulatory components of the gene network involved in the control of seed coat expansion and patterning, acting to program their unfolding and characteristics during phase change transition from ovular to seed development upon fertilisation? It will be intriguing to test that hypothesis, especially in the context of the importance of the *ERf* as a fate determinant in stomatal development or vascular differentiation in aerial organs.

At first sight, that early *ERf*-mediated transcriptional upon fertilisation does not appear to involve *GL1*. In contrast, *GL1* knock-out did not induce any detectable transcriptional changes at this very early stage (0 DAFO) and even hardly any or very limited changes until massive effects kicked in between 5 and 6 DAFO, most prominently in different sets, or types of genes from those earlier found to be transcriptionally regulated by the *ERf*.

Most notably, the transcriptional changes associated to *GL1* transcriptional activity reveal unsuspected functions, as a positive regulator of very upstream core components of ABA signalling – PYR/PYL-PP2C-SnRK-ABF-; a negative regulator of a set of early auxin response (*SAURs*) genes, notably different from those regulated by the *ERf*, and a few *IAA* genes but otherwise no other type of auxin related genes; a major (mostly positive) regulator of lipid and fatty acid metabolism, biosynthesis of cutin and cuticle formation; synthesis of secondary cell wall material, including lignins; and of several genes reported for their roles in cellulose synthesis for oi2 columellae formation, seed coat cell wall and mucilage modifications; and a regulator of transcription factors reported as involved in outer integument development and epidermal cell differentiation; and, consistently, among transcription factors, a predominance of NAC transcription factors. Strikingly, the transcriptional changes detected in *erf gl1* quadruple mutant showed large overlap with those associated to loss of *GL1* (*gl1 ERf* genotype), rather than to quasi KO *erf* (*er erf1+/- erf2*).

These results provide a set of candidate genes totally consistent with the developmental differences I identified in my microscopy observations of the developing and mature seed coats of the *gl1*, *erf*, and *erf gl1* mutants, in epidermal cell wall thickness, columella size, endosperm associated cuticle thickness, and the roles of both the *ERf* and *GL1* in mucilage properties affecting seed germination (Chapter 2 and Nanda et al.2019). These candidates need to be confirmed, starting with independently confirming their *ERf*- and/or *GL1*-dependent transcriptional regulation.

However, they already allow several important novel insights:

- As inferred from my physiological data in Chapter 2, *GL1* is not an integral part of the *ERF* signalling pathway during seed development, but operates in a separate, intersecting pathway, downstream of the *ERF*.
- *GL1* signalling in seeds indicates much broader roles than its only previously established role in aerial organs, not only in mucilage synthesis, but much more broadly in the formation and biophysical properties of epidermal cell wall, waxes and cuticles, i.e., core structural and protective components of seeds. It will be very interesting in future studies to examine whether those are specific to the seed or also exist in leaves and stems, where *GL1* acts to initiate trichome development.

Finally, as mentioned earlier the present study provides an analysis of the combined transcriptomes of seeds during the determination of seed size and embryo patterning, and of the enclosing silique walls. Its results and the developmental and physiological data reported in the previous Chapter, together with the recent technological developments in laser microscopy dissection, single-cell omics, and big data analysis, give the means of spatially and temporally targeted investigations, in particular of the oi2 testa layer, the ii1' pigmented and endosperm associated cuticle that is derived from the inner ii1 layer, and separately of the seed chalazal and funiculus connective tissues to the maternal silique wall.

3.5 Supplementary Figures

3.5.1 Supplementary Figure 1

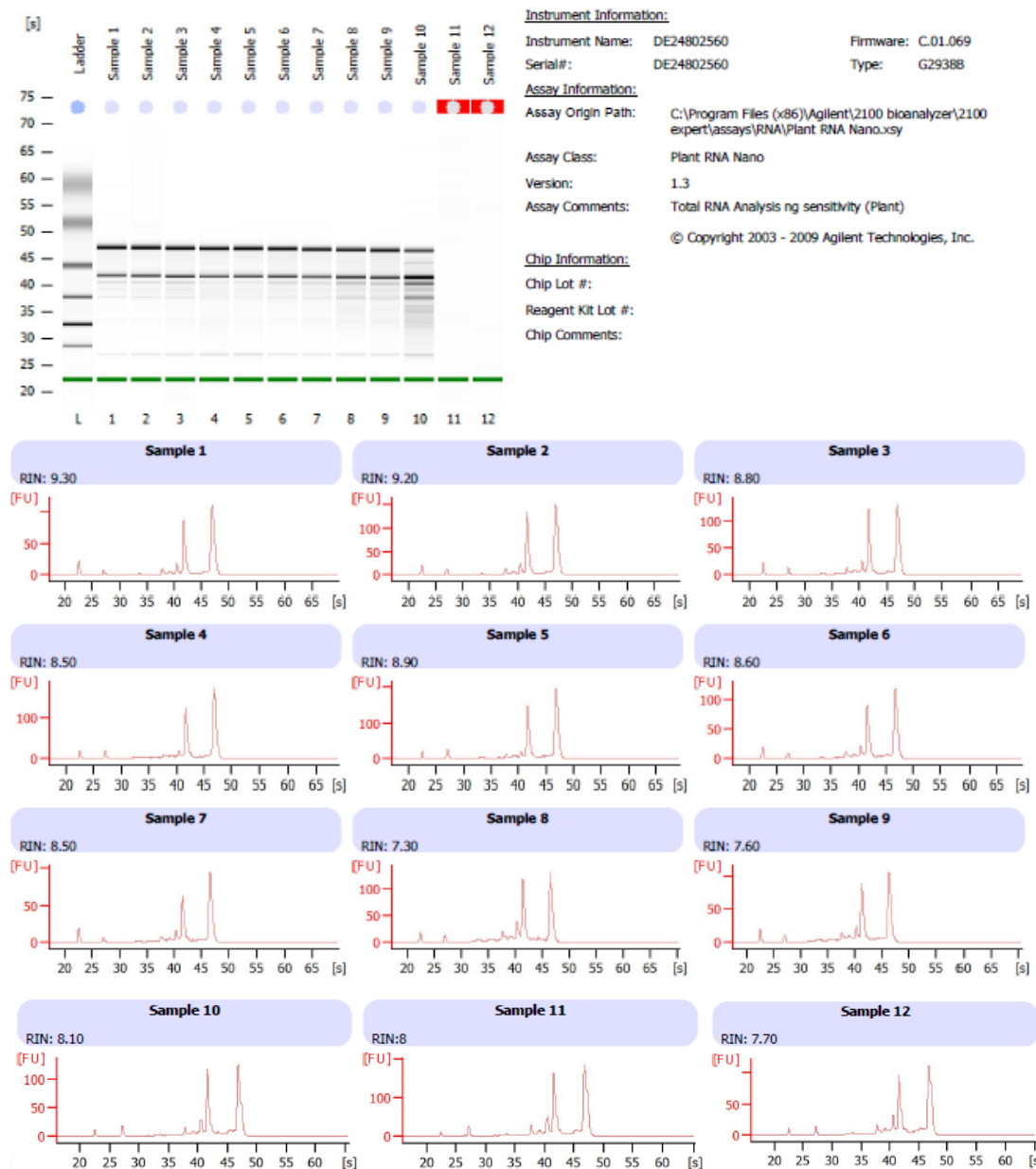
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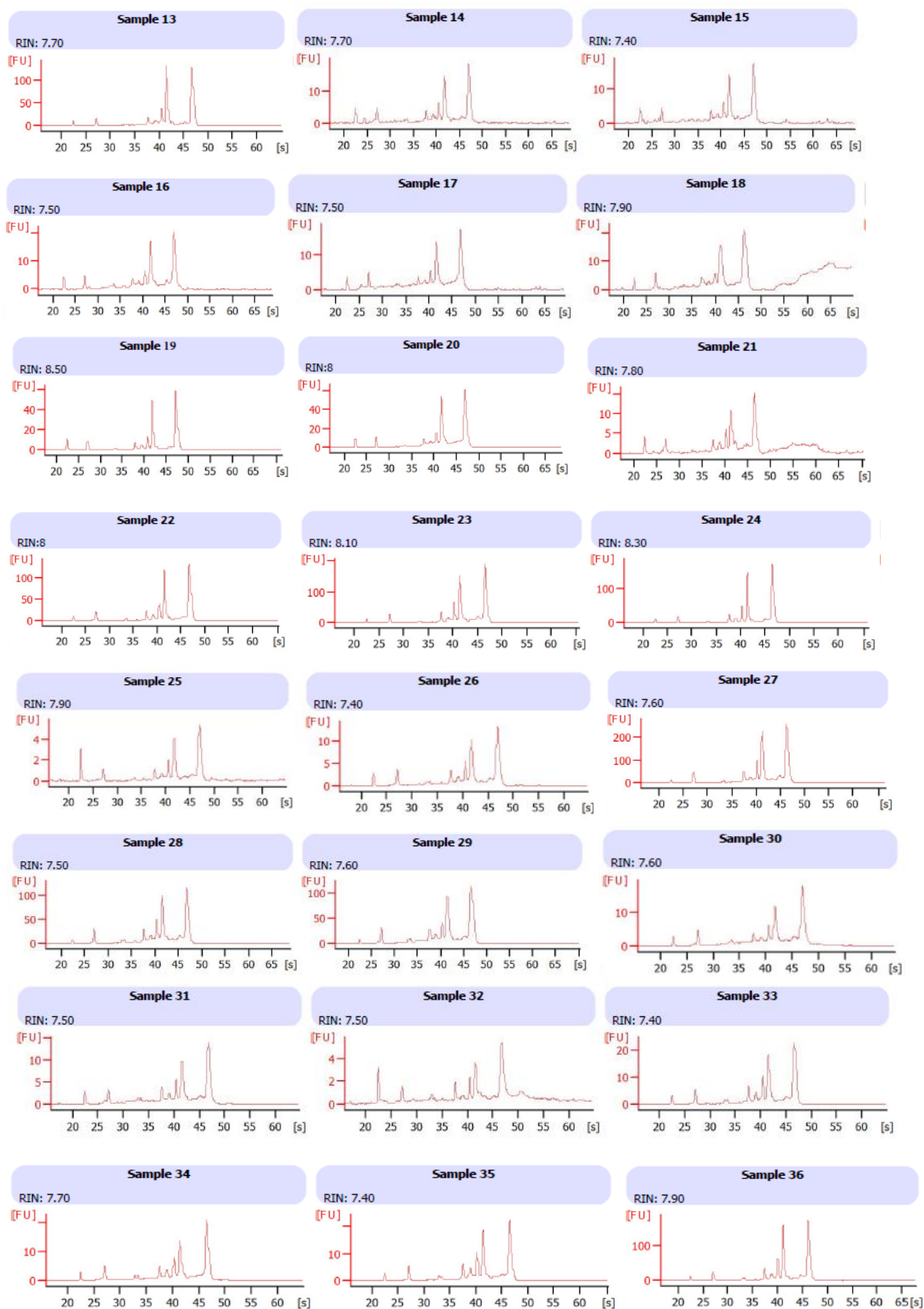
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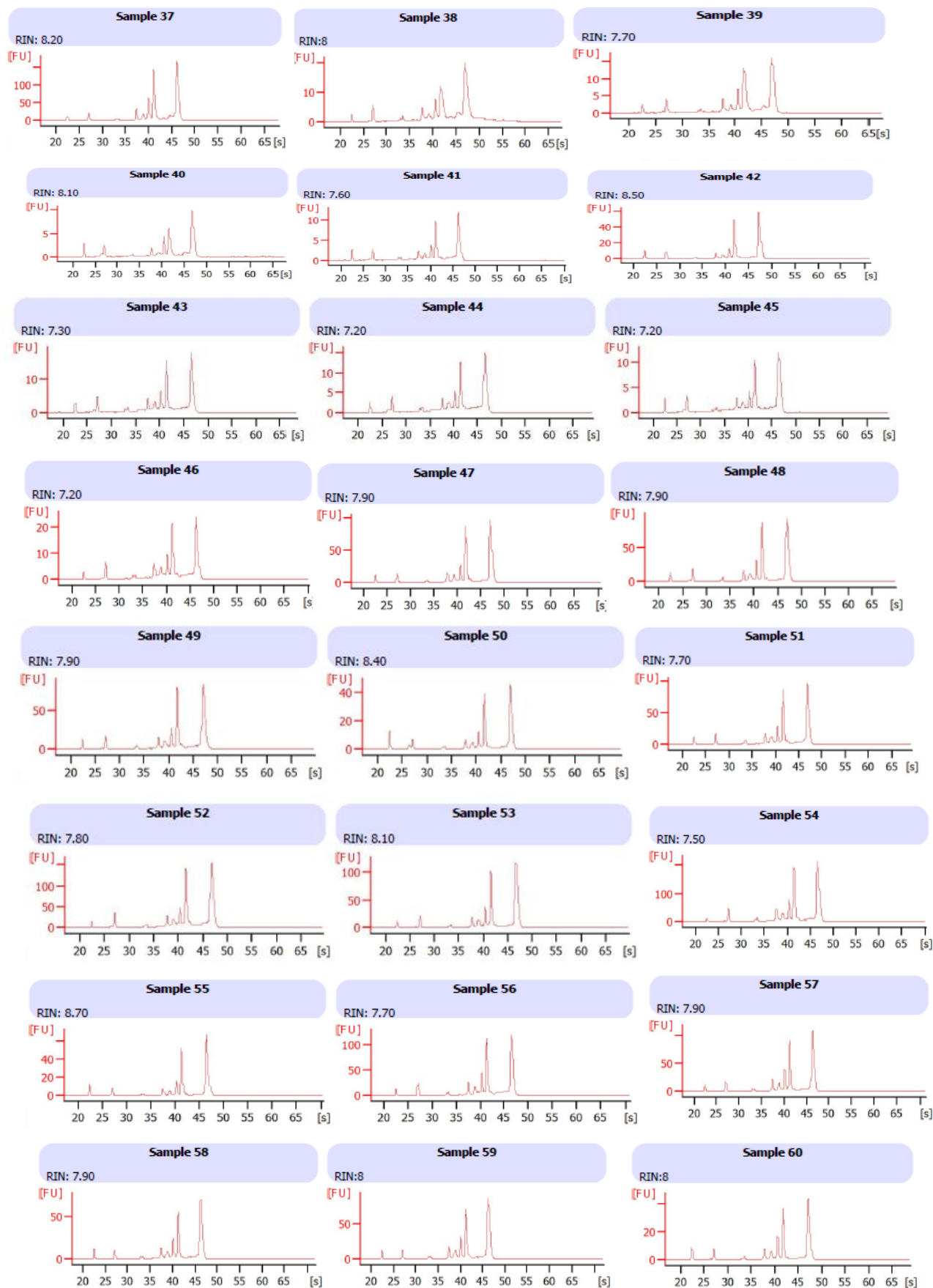
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Created: 2/02/2022 11:12:42 AM
 Modified: 2/02/2022 11:36:34 AM

Electrophoresis File Run Summary

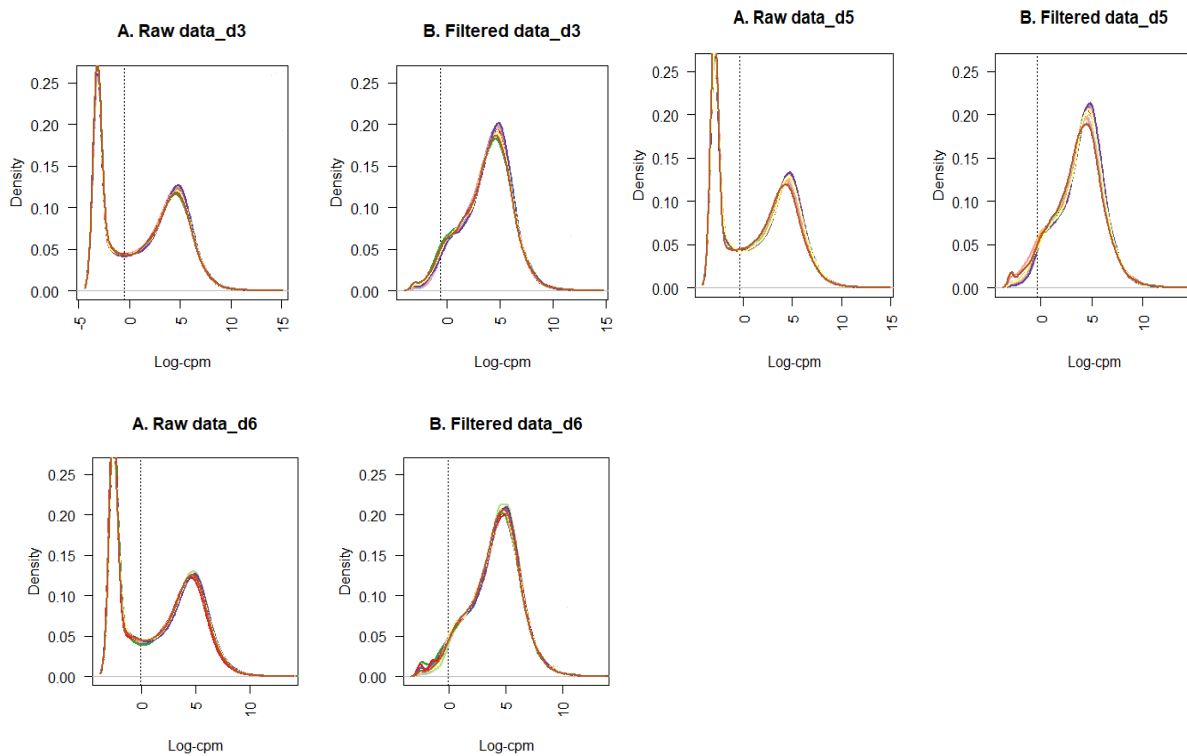






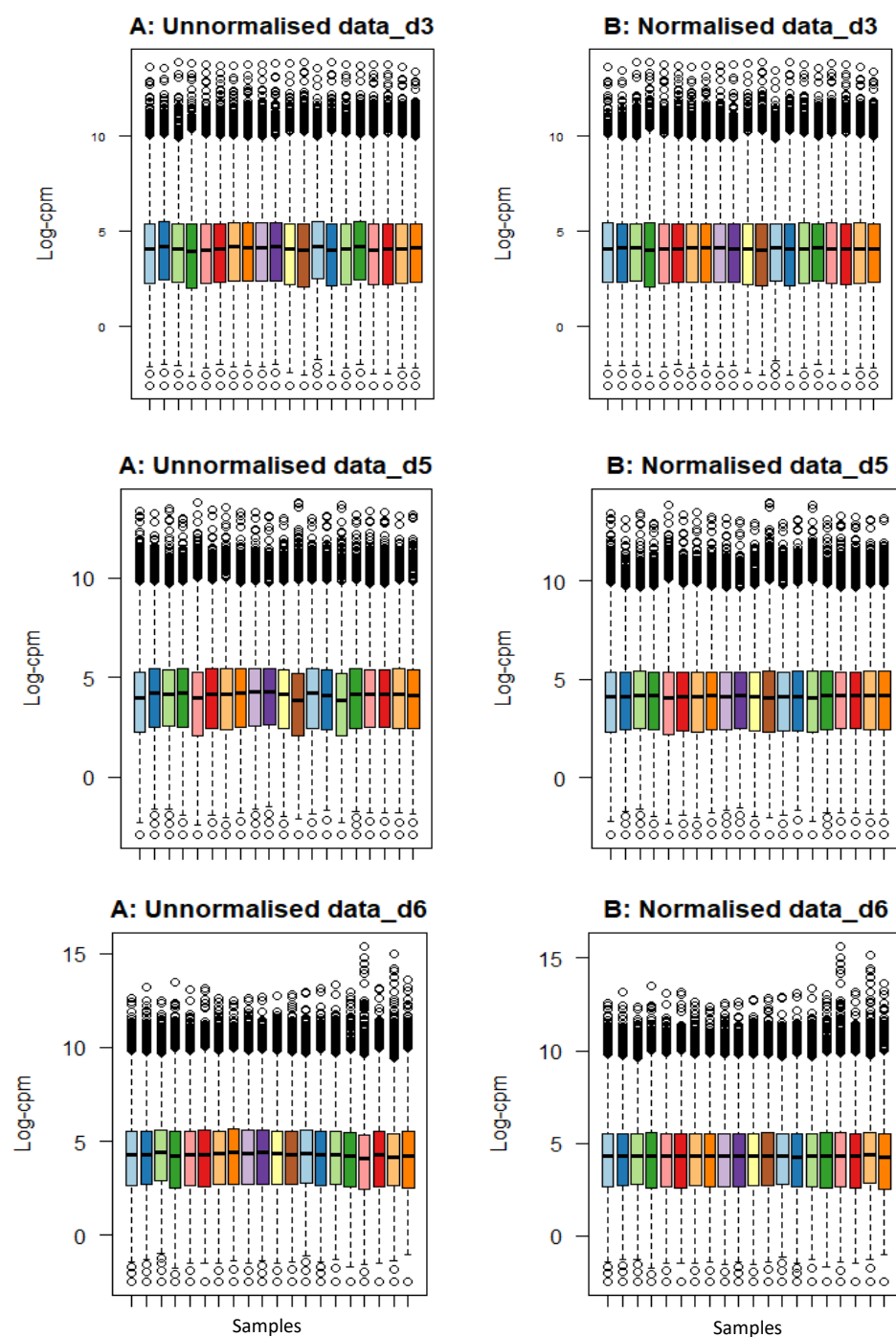
Supp figure 3-1. Qulaity control (QC) results from bio analyser for all the samples used in RNA seq analysis. distinct sharp 18S (small) and 25S (large) rRNA peaks preceded by a flat base indicate the low noise level and tight integrity of RNA used in this study. Sharp corresponding bands are visible on the gel image (shown for only few samples) .

3.5.2 Supplementary Figure 2



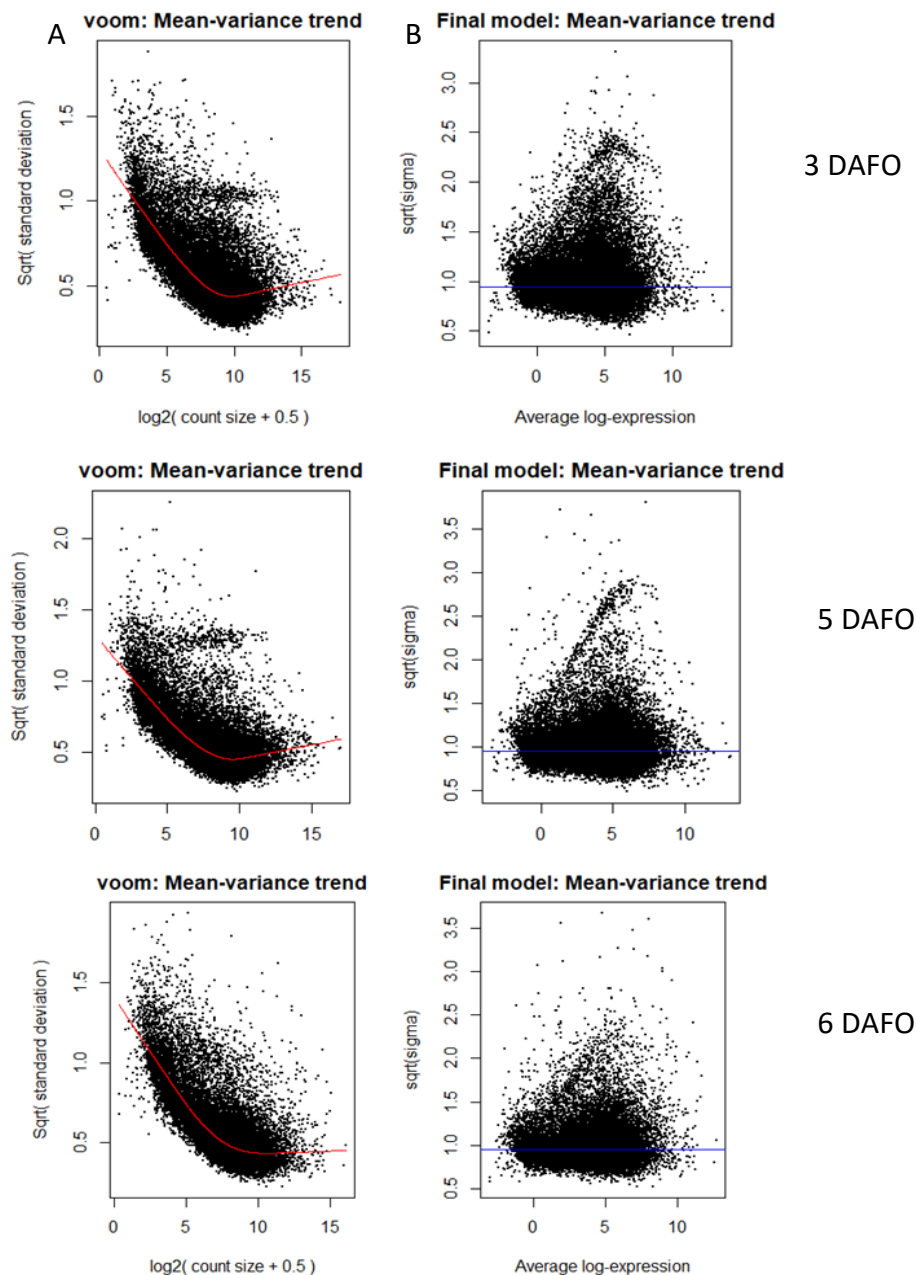
Supp figure 3-2. log-CPM density plot of before (A) and after (B) filtering out unexpressed or lowly expressed genes OF 3-5DAFO samples. Default function used the filtering of keeping at least the 10 gene count in 5 samples (sample number is defined according to the biological replicates used in the experiment), actual filtering used the CPM values -hence, the cut off equivalent to a CPM value of about 0.5 ($\log\text{CPM} = -1$) i.e., $10/20(\text{library size}) = 0.5$. Dotted vertical lines mark the log-CPM threshold used in the filtering step

3.5.3 Supplementary Figure 3



Supp figure 3-3. Boxplots (3-5DAFO) of log-CPM values showing expression distributions for unnormalized data (A) and normalised data after applying TMM normalisation (B). X axis of the box plot; samples and y axis; the log CPM value distribution. mild normalisation across all the samples as the scaling factor close to 1

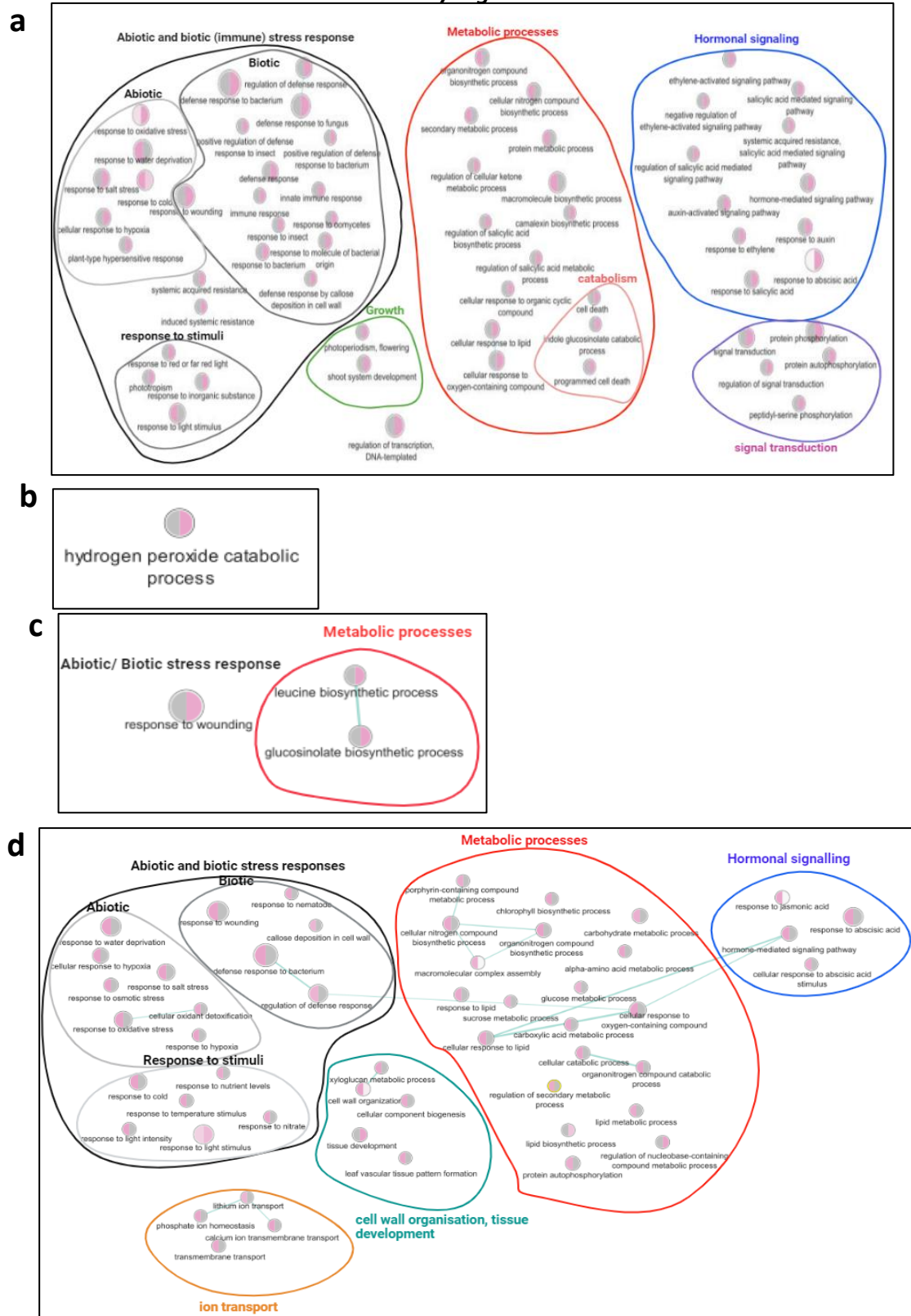
3.5.4 Supplementary Figure 4

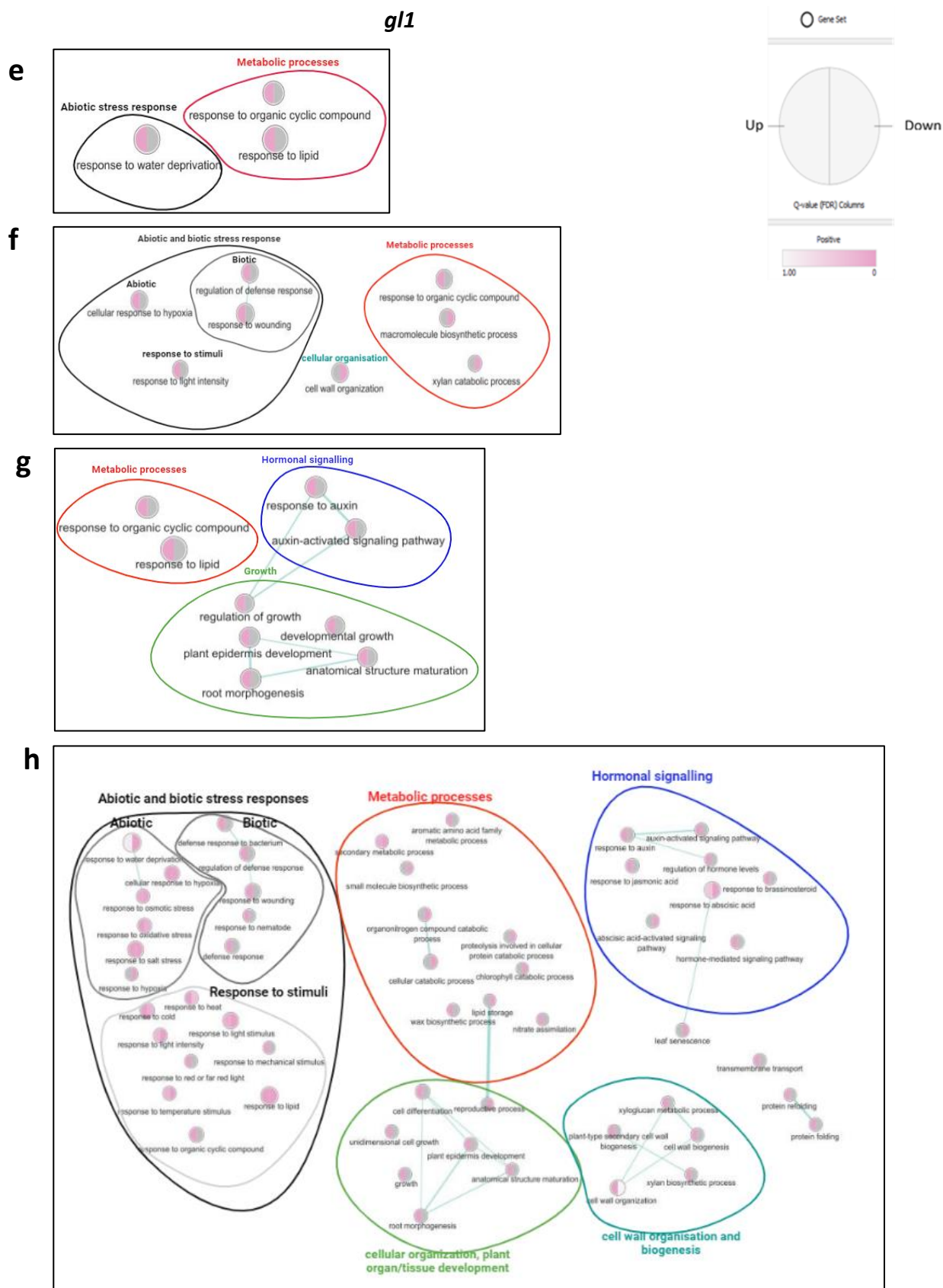


Supp figure 3-4. Removing the heteroscedasticity of the data using the vroom function.

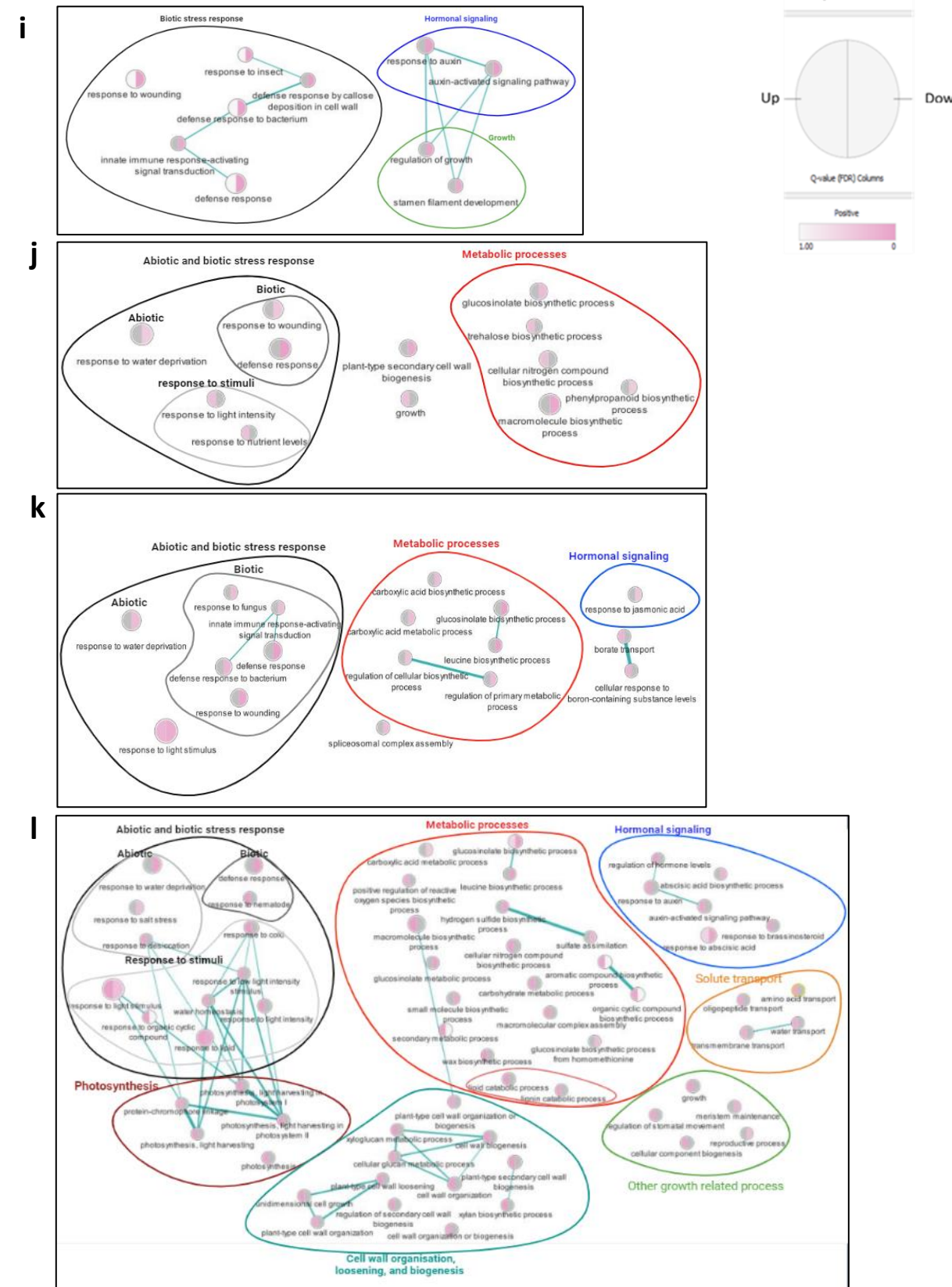
The mean (x-axis) -variance (y-axis) plot of each gene in -time points (3,5, and 6 DAFO) were plotted before (A) and after (B) applying the vroom precision weights. The vroom function estimates the mean-variance relationship for the log-CPM values, and then the estimated variance is used to calculate the precision weights for each gene. A. created within the vroom function and displays the mean expression level of each gene (log2-transformed mean-counts) with an offset of 0.5 to handle zero counts (x-axis) against the square root of the standard deviation (y-axis). B. the average log-transformed expression levels of genes (x-axis) against the log 2 square root of the residual standard deviation from the linear model fit (y-axis). For both plots, each dot represents a gene, and a fitted red curve (a) shows the overall trend of the mean-variance relationship and blue line (B) average log 2 residual standard deviation.

3.5.5 Supplementary Figure 5

er erl1/seg erl2

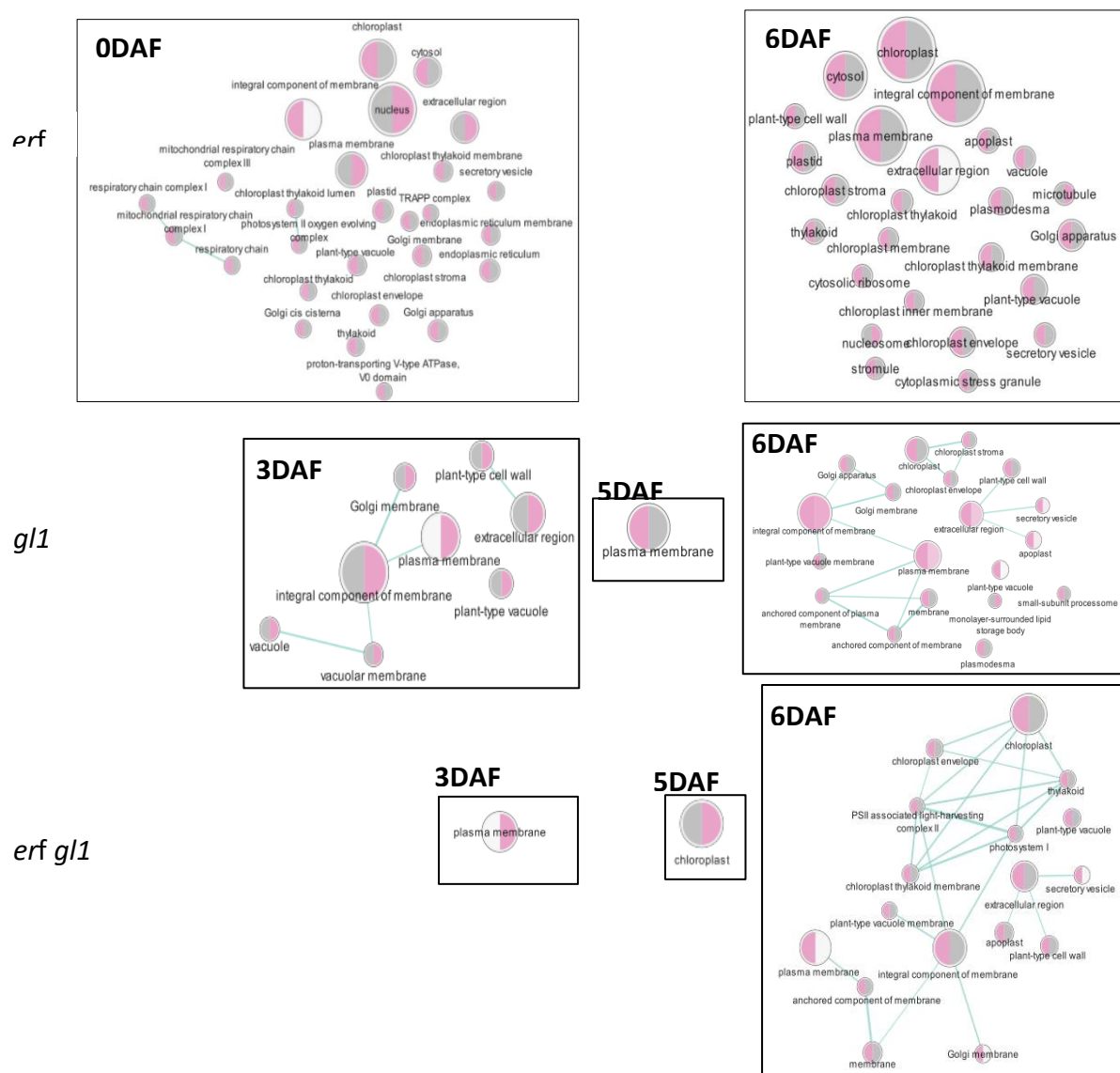


er erl1/seg erl2 gl1 (erf gl1)



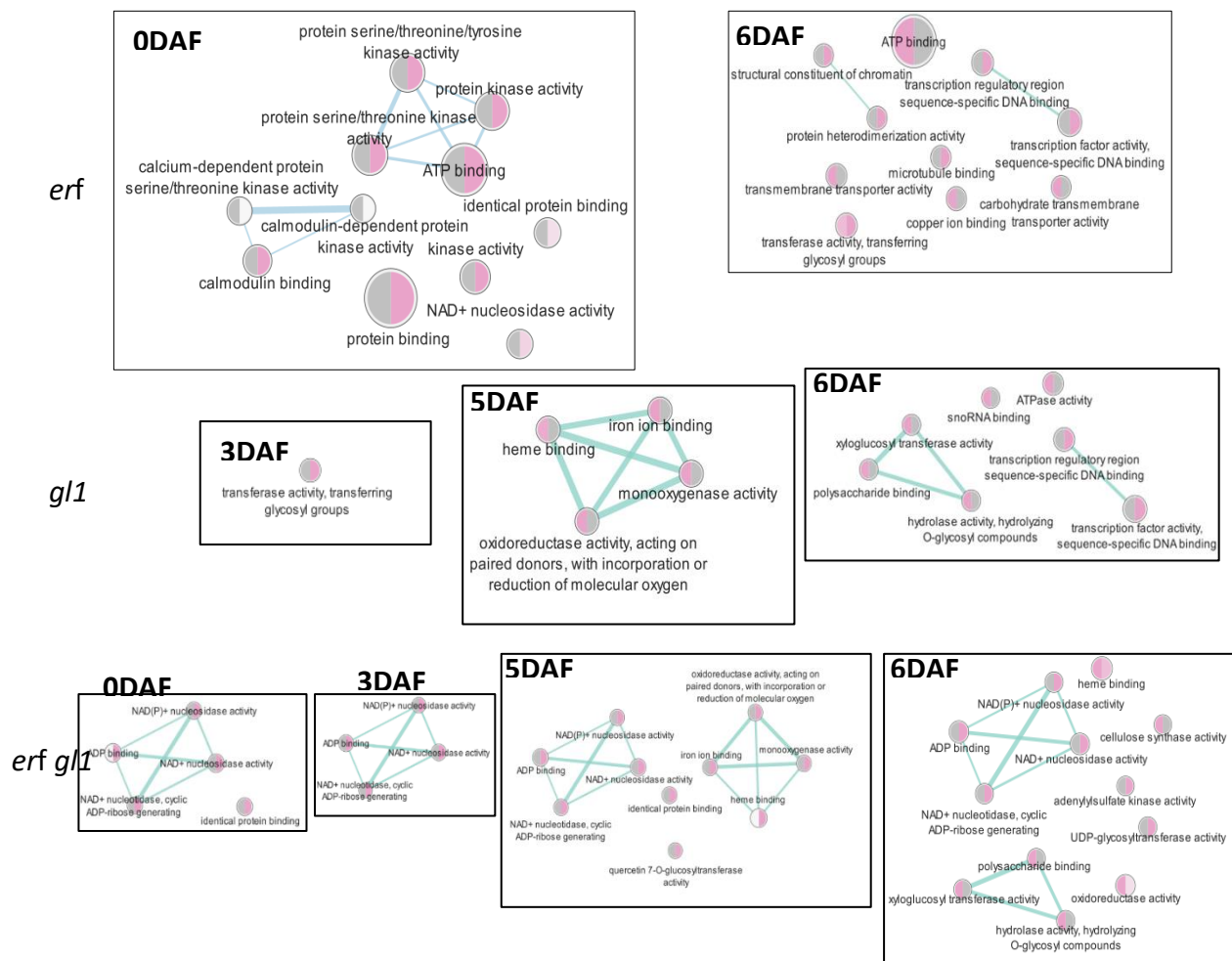
Supp figure 3-5. Gene ontology terms (Biological Process (GO_BP)) enrichment analysis of significant DEGs (FDR<0.05 and non-zero log₂FC) in *er erl1/seg erl2*(a, b, c, d), *gl1*(e, f, g, h), and *er erl1/seg erl2 gl1*(i, j, k, l) compared to Col (WT) across 0, 3, 5, and 6 DAFO respectively. The functional annotation from DAVID was uploaded to Cytoscape, and the enrichment map of significant ($p < 0.01$ and $FDR < 0.05$) GO_BP terms was created using the -EnrichmentMAP plugin. Nodes (pink/grey circles) in the map represent the up (left) and down (right) regulated genes set, and the size of the circle is proportional to the number of genes associated with the term. The shading of Grey/white to pink represents the FDR (white/grey to pink- highest to lowest FDR). Genetic links between nodes were represented by the green line. The related functional terms were then manually grouped according to their broader functional group (e.g., functional terms related to abiotic and biotic stress responses were grouped under Biotic and abiotic stress responses) for easy comparison between the mutants.

3.5.6 Supplementary Figure 6



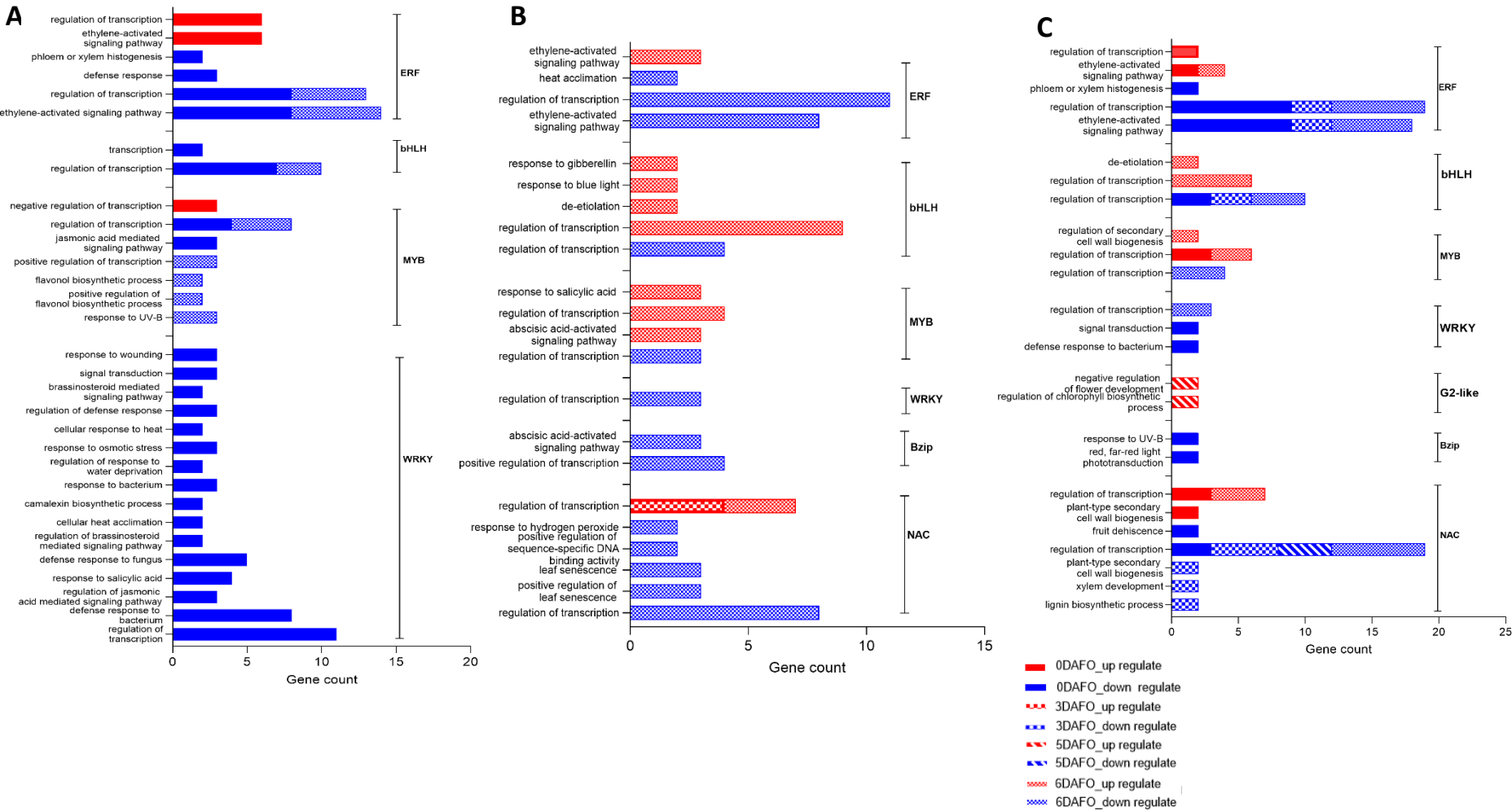
Supp figure 3-6. Gene ontology terms (Cellular Component (GO_CC) enrichment analysis of significant DEGs (FDR<0.05 and non-zero log2FC) in *er erl1/seg erl2(erf)*, *gl1* and *er erl1/seg erl2 gl1 (erf gl1)* compared to Col (WT) across 0, 3, 5, and 6 DAF respectively. The functional annotation from DAVID was uploaded to Cytoscape, and the enrichment map of significant (p < 0.01 and FDR<0.05) GO_CC terms was created using the -EnrichmentMAP plugin. Nodes (pink/grey circles) in the map represent the up (left) and down (right) regulated genes set, and the size of the circle is proportional to the number of genes associated with the term. The shading of Grey/white to pink represents the FDR (white/grey to pink- highest to lowest FDR). Genetic links between nodes were represented by the green line.

3.5.7 Supplementary Figure 7

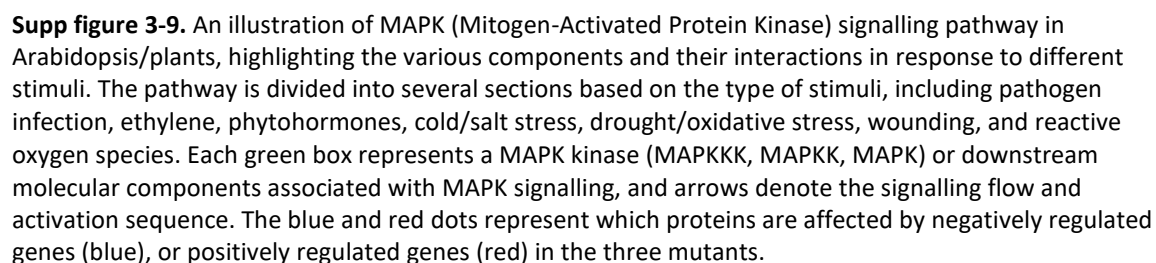


Supp figure 3-7. Gene ontology terms (Molecular Function GO_MF) enrichment analysis of significant DEGs (FDR<0.05 and non-zero log2FC) in *erl1/seg erl2*, *gl1* and *erl1/seg erl2 gl1* compared to Col (WT) across 0, 3, 5, and 6 DAFO respectively. The functional annotation from DAVID was uploaded to Cytoscape, and the enrichment map of significant ($p < 0.01$ and FDR<0.05) GO_MF terms was created using the -EnrichmentMAP plugin. Nodes (pink/grey circles) in the map represent the up (left) and down (right) regulated genes set, and the size of the circle is proportional to the number of genes associated with the term. The shading of Grey/white to pink represents the FDR (white/grey to pink- highest to lowest FDR). Genetic links between nodes were represented by the green line.

3.5.8 Supplementary Figure 8



Supp figure 3-8. Functional enrichment of the differential expression of the Transcription Factors (TFs) in *er erl1/seg erl2*, *gl1* and *er erl1/seg erl2 gl1* mutants compared to WT. A.B&C. significantly enriched biological processes (GO_BP) associated with differentially expressed TFs of *er erl1/seg erl2* (A), *gl1* (B), *er erl1/seg erl2 gl1*(C) . ERF- Ethylene Responsive Factor TF family, bHLH- Basic Helix-Loop-Helix TF family, G2-like- GOLDEN2-LIKE (GLK) TF family, bZIP- Basic Leucine Zipper TF family, NAC- NAM (No Apical Meristem), ATAF (Arabidopsis Transcription Activation Factor), and CUC (Cup-shaped Cotyledon).



3.6 Supplementary Tables

3.6.1 Supplementary Table 1

Supp table 3-1. QC data for total RNA (qubit and bioanalyzer results). Samples with absorbance260/280 ratio ≥ 2.0 were analysed in the bio analyser. RNA Integrity Number (RIN) were obtained for all the samples using Agilent Bioanalyzer (2100).

<i>Sample ID</i>	<i>Sample name</i>	<i>A260/230</i>	<i>A260/280</i>	<i>Qubit con ng/ul</i>	<i>RIN value</i>
1	1. Col_1_d0	2.207	2.112	124	9.3
2	2. Col_2_d0	2.056	2.126	146	9.2
3	3. Col_3_d0	2.136	2.098	144	8.8
4	4. Col_4_d0	2.27	2.172	182	8.5
5	5. Col_5_d0	2.109	2.172	168	8.9
6	6. gl1_1_d0	1.883	2.113	136	8.6
7	7. gl1_2_d0	2.423	2.134	126	8.5
8	8. gl1_3_d0	2.1	2.134	154	7.3
9	9. gl1_4_d0	1.83	2.132	138	7.6
10	10. gl1_5_d0	2.22	2.157	252	8.1
11	11. er erl1/seg erl2_1_d0	2.213	2.151	268	8
12	12. er erl1/seg erl2_2_d0	2.245	2.166	220	7.7
13	13. er erl1/seg erl2_3_d0	1.788	2.178	216	7.7
14	14. er erl1/seg erl2_4_d0	2.136	2.167	184	7.7
15	15. er erl1/seg erl2_5_d0	2.168	2.198	124	7.4
16	16. er erl1/seg erl2 gl1_1_d0	1.852	2.191	132	7.5
17	17. er erl1/seg erl2 gl1_2_d0	2.123	2.18	118	7.5
18	18. er erl1/seg erl2 gl1_3_d0	2.165	2.164	142	7.9
19	19. er erl1/seg erl2 gl1_4_d0	2.245	2.176	110	8.5
20	20. er erl1/seg erl2 gl1_5_d0	1.896	2.169	102	8
21	21. Col_1_d3	1.89	2.124	158	7.8
22	22. Col_2_d3	1.923	2.103	235	8
23	23. Col_3_d3	1.896	2.138	256	8.1
24	24. Col_4_d3	1.957	2.033	168	8.3
25	25. Col_5_d3	1.768	2.092	174	7.9
26	26. gl1_1_d3	1.809	2.098	194	7.4
27	27. gl1_2_d3	1.871	2.127	356	7.6
28	28. gl1_3_d3	2.123	2.139	122.8	7.5
29	29. gl1_4_d3	1.904	2.114	292	7.6
30	30. gl1_5_d3	2.115	2.142	296	7.6
31	31. er erl1/seg erl2_1_d3	2.005	2.127	198	7.5
32	32. er erl1/seg erl2_2_d3	1.891	2.133	186	7.5
33	33. er erl1/seg erl2_3_d3	1.921	2.104	181.2	7.4

34	34. <i>erl1/seg erl2_4_d3</i>	1.864	2.06	212	7.7
35	35. <i>erl1/seg erl2_5_d3</i>	1.835	2.068	184.4	7.4
36	36. <i>erl1/seg erl2 gl1_1_d3</i>	1.956	2.145	256	7.9
37	37. <i>erl1/seg erl2 gl1_2_d3</i>	2.204	2.122	220	8.2
38	38. <i>erl1/seg erl2 gl1_3_d3</i>	1.988	2.138	400	8
39	39. <i>erl1/seg erl2 gl1_4_d3</i>	2.009	2.144	332	7.7
40	40. <i>erl1/seg erl2 gl1_5_d3</i>	1.895	2.013	144	8.1
41	41. <i>Col_1_d6</i>	1.966	2.06	112	7.6
42	42. <i>Col_2_d6</i>	1.985	2	88.6	8.5
43	43. <i>Col_3_d6</i>	1.932	2.011	136	7.3
44	44. <i>Col_4_d6</i>	1.899	2.043	96.2	8
45	45. <i>Col_5_d6</i>	1.954	2.053	126	9.3
46	46. <i>gl1_1_d6</i>	1.896	2.033	132	8
47	47. <i>gl1_2_d6</i>	1.884	2.106	118	7.9
48	48. <i>gl1_3_d6</i>	1.944	2.048	110	7.9
49	49. <i>gl1_4_d6</i>	1.987	2.01	130	7.9
50	50. <i>gl1_5_d6</i>	1.968	2.04	91.8	8.4
51	51. <i>erl1/seg erl2_1_d6</i>	1.869	2.067	136	7.7
52	52. <i>erl1/seg erl2_2_d6</i>	1.899	2.09	152	7.8
53	53. <i>erl1/seg erl2_3_d6</i>	1.896	2.065	90	8.1
54	54. <i>erl1/seg erl2_4_d6</i>	1.888	2.071	146	7.5
55	55. <i>erl1/seg erl2_5_d6</i>	1.963	2.087	96.8	8.7
56	56. <i>erl1/seg erl2 gl1_1_d6</i>	1.872	2.147	172	7.7
57	57. <i>erl1/seg erl2 gl1_2_d6</i>	1.986	2.137	140	7.9
58	58. <i>erl1/seg erl2 gl1_3_d6</i>	1.966	2.1	122	7.9
59	59. <i>erl1/seg erl2 gl1_4_d6</i>	1.873	2.17	136	8
60	60. <i>erl1/seg erl2 gl1_5_d6</i>	1.864	2.111	88.8	8

3.6.2 Supplementary table 2

Supp table 3-2. Sequencing quality metrics for paired-end RNA sequencing raw data. The sequencing reads have a length of 150 base pairs (bp)/paired end. A total of ~20-25 M read pairs were generated for each sample used in the study. The total number of bases sequenced, bases with quality score (Q20) of 20 or higher, indicating a 1% error probability, and bases with quality score (Q30) of 30 or higher, corresponding to a 0.1% error probability, The GC content of the reads and occurrence of unknown bases (N) in parts per million (ppm) were shown in the table.

Sample	Length (paired- end, bp)	Total Reads	Total Bases	Q20 (%)	Q30 (%)	GC (%)	N (ppm)
1-Col-1-do	150	44071714	6610757100	98.04	94.17	45.1	4.68
2-Col-2-do	150	51268348	7690252200	98.01	93.98	46.42	5.04
3-Col-3-do	150	50144554	7521683100	98.34	94.92	46.19	5.39
4-Col-4-do	150	44842588	6726388200	98.17	94.47	44.93	4.85
5-Col-5-do	150	48380930	7257139500	98.39	94.98	44.96	5.09
6-gl1-1-do	150	45824774	6873716100	98.25	94.69	44.81	4.91
7-gl1-2-do	150	45364140	6804621000	98.24	94.61	44.62	5.42
8- gl1-3-do	150	45278348	6791752200	98.35	94.94	45.17	5.39
9- gl1-4-do	150	56475090	8471263500	98.2	94.53	44.91	5.41
10-Colgl-5-do	150	44732826	6709923900	98.08	94.23	45.52	4.9
11-er erl1/seg erl2-1-do	150	48008116	7201217400	98.22	94.55	45.37	5.36
12- er erl1/seg erl2-2-do	150	46303136	6945470400	98.27	94.7	44.87	14.93
13- er erl1/seg erl2-3-do	150	43644196	6546629400	98.16	94.49	46.77	5.39
14- er erl1/seg erl2-4-do	150	43230902	6484635300	98.49	95.3	45.35	5.33
15- er erl1/seg erl2-5-do	150	45491686	6823752900	98.25	94.65	45.26	5.46
16- er erl1/seg erl2 gl1-1-do	150	50299518	7544927700	98.19	94.48	44.99	5.36
17- er erl1/seg erl2 gl1-2-do	150	54677120	8201568000	98.2	94.52	44.9	5.44
18- er erl1/seg erl2 gl1-3-do	150	53966972	8095045800	98.21	94.55	44.98	5.39
19- er erl1/seg erl2 gl1-4-do	150	54017126	8102568900	98.25	94.65	44.91	5.42
20- er erl1/seg erl2 gl1-5-do	150	45891038	6883655700	98.39	95.06	44.87	4.92
21-Col-1-d3	150	41037260	6155589000	97.92	94.36	46.06	5.42
22-Col-2-d3	150	43190036	6478505400	98.36	94.97	47.21	5.37
23-Col-3-d3	150	43880750	6582112500	97.42	93.55	46.5	23.86
24-Col-4-d3	150	55818572	8372785800	98.27	94.7	47.06	5.37
25-Col-5-d3	150	56656602	8498490300	98.07	94.23	48.15	5.39
26-gl1-1-d3	150	44063532	6609529800	97.45	93.85	47.49	5.39
27- gl1-2-d3	150	50887644	7633146600	97.62	93.71	46.84	22.84
28- gl1-3-d3	150	44373464	6656019600	98.25	94.68	46.99	4.67
29- gl1-4-d3	150	45774174	6866126100	97.78	94.04	46.57	15.3
30- gl1-5-d3	150	44543240	6681486000	98.23	94.69	47.55	4.71
31- er erl1/seg erl2-1-d3	150	51127730	7669159500	98.28	94.76	48.31	5.43
32- er erl1/seg erl2-2-d3	150	45674258	6851138700	97.57	93.66	47.03	15.03
33- er erl1/seg erl2-3-d3	150	44415618	6662342700	98.24	94.7	47.44	4.91
34- er erl1/seg erl2-4-d3	150	47682590	7152388500	97.79	94.19	46.16	14.76
35- er erl1/seg erl2-5-d3	150	51407636	7711145400	98.2	94.56	47.41	5.35
36- er erl1/seg erl2 gl1-1-d3	150	41006120	6150918000	98.41	95.08	47.15	5.4
37- er erl1/seg erl2 gl1-2-d3	150	49578362	7436754300	98.2	94.54	46.62	5.44
38- er erl1/seg erl2 gl1-3-d3	150	49245030	7386754500	98.42	95.15	47.16	5.39
39- er erl1/seg erl2 gl1-4-d3	150	46365888	6954883200	98.33	94.88	46.44	5.39
40- er erl1/seg erl2 gl1-5-d3	150	46925430	7038814500	97.58	93.79	46.25	23.07

41-Col-1-d5	150	45655404	6848310600	97.76	94.22	48.72	4.93
41-Col-1-d6	150	45598094	6839714100	98.37	94.95	47.54	4.06
42-Col-2-d5	150	45011570	6751735500	97.78	94.09	47.25	5.26
42-Col-2-d6	150	45483800	6822570000	98.21	94.7	48.7	4.19
43-Col-3-d5	150	45016064	6752409600	97.92	94.52	47.44	4.7
43-Col-3-d6	150	45204424	6780663600	98.42	95.16	49.25	4.01
44-Col-4-d5	150	45559880	6833982000	97.55	93.65	47.52	5.24
44-Col-4-d6	150	45621176	6843176400	98.57	95.49	46.93	3.99
45-Col-5-d5	150	44662152	6699322800	97.51	93.88	46.64	5.32
45-Col-5-d6	150	45200464	6780069600	98.4	95.07	49.47	3.97
46-gl1-1-d5	150	45920918	6888137700	97.79	94.07	47.49	5.06
46- gl1-1-d6	150	45775172	6866275800	98.26	94.92	52.8	4.26
47- gl1-2-d5	150	45942502	6891375300	97.54	93.56	46.64	4.89
47- gl1-2-d6	150	44279980	6641997000	98.13	94.51	49.3	4.68
48- gl1-3-d5	150	45829034	6874355100	97.49	93.93	48.51	5.05
48- gl1-3-d6	150	44102654	6615398100	98.32	95.05	50.5	4.35
49- gl1-4-d5	150	45937290	6890593500	97.76	94.19	46.97	4.62
49- gl1-4-d6	150	45069888	6760483200	98.28	94.75	49.42	4.02
50- gl1-5-d5	150	45507812	6826171800	97.51	93.93	48.04	5.29
51- er erl1/seg erl2-1-d5	150	45717912	6857686800	97.68	93.83	46.32	5.1
52- er erl1/seg erl2-2-d5	150	44116374	6617456100	97.55	93.79	48.15	4.58
53- er erl1/seg erl2-3-d5	150	44435564	6665334600	97.77	94.32	48.18	4.71
54- er erl1/seg erl2-4-d5	150	45143458	6771518700	97.82	94.15	48.27	4.77
55- er erl1/seg erl2-5-d5	150	44837826	6725673900	97.41	93.46	48.12	5.18
56- er erl1/seg erl2 gl1-1-d5	150	45559338	6833900700	97.48	93.88	46.56	5.29
57- er erl1/seg erl2 gl1-2-d5	150	45161540	6774231000	97.53	93.86	50.08	4.99
58- er erl1/seg erl2 gl1-3-d5	150	45667908	6850186200	97.72	94.09	47.67	4.61
59- er erl1/seg erl2 gl1-3-d5	150	44729502	6709425300	97.86	94.34	49.39	5.18
60- er erl1/seg erl2 gl1-5-d5	150	45374968	6806245200	97.58	94.13	47.76	4.94
50- gl1-5-d6	150	44021152	6603172800	98.56	95.51	47.9	3.96
51- er erl1/seg erl2-1-d6	150	44534756	6680213400	98.36	95.18	51.15	4.47
52- er erl1/seg erl2-2-d6	150	45017248	6752587200	98.28	94.87	49.62	4.22
53- er erl1/seg erl2-3-d6	150	45003046	6750456900	98.24	94.94	52.76	4.26
54- er erl1/seg erl2-4-d6	150	44287766	6643164900	98.34	95.06	50.71	4.72
55- er erl1/seg erl2-5-d6	150	44069304	6610395600	98.07	94.51	53.48	4.49
56- er erl1/seg erl2 gl1-1-d6	150	44370540	6655581000	98.38	95.13	49.14	4.52
57- er erl1/seg erl2 gl1-2-d6	150	45061300	6759195000	98.24	94.78	50.11	4.29
58- er erl1/seg erl2 gl1-3-d6	150	45279746	6791961900	98.19	94.62	49.9	4.18
59- er erl1/seg erl2 gl1-4-d6	150	44379994	6656999100	98.34	95	47.25	4.44
60- er erl1/seg erl2 gl1-5-d6	150	45912994	6886949100	98.13	94.56	51.03	4.51

3.6.3 Supplementary Table 3

Supp table 3-3. Mapping statistics. total reads- total number of sequencing reads generated, Total mapped: the total number of sequencings reads that have been successfully aligned to references genome, Multiple mapped: The number of reads that have been aligned to multiple locations in the reference genome, Uniquely mapped : The number of reads that have been aligned to a unique location in the reference genome, Read1 and Read2: number of reads in paired-end sequencing that sequenced from both ends, resulting in two reads per fragment: Read1 & Read 2 (the forward and reverse read respectively, Reads map to '+' and '-' : This indicates the directionality of the alignment of the reads. Reads that map to the '+' strand align in the same direction as the reference genome, while reads that map to the '-' strand align in the opposite direction. Non-splice reads: Reads that align to the reference genome without spanning any splice junctions. These reads align entirely within exonic regions. Splice reads: Reads that span splice junctions, indicating the presence of introns in the transcribed RNA molecules. Reads mapped in proper pairs: Paired-end reads where both Read1 and Read2 align to the reference genome in the expected orientation and at an appropriate distance from each other.

Samples	Total reads	Total mapped	Multiple mapped	Uniquely mapped	Read1	Read2	Reads map to '+'	Reads map to '-'	Non_splice reads	Splice reads	Reads mapped in proper pairs
1-Col-1-do	43982534	42865116 (97.4594%)	2434945 (5.53616%)	40430171 (91.9232%)	20319411	20110760	20216709	20213462	23979767	16450404	39265230
2-Col-2-do	51179730	49894386 (97.4886%)	6994418 (13.6664%)	42899968 (83.8222%)	21555643	21344325	21458524	21441444	26686881	16213087	41555374
3-Col-3-do	50058876	48953534 (97.7919%)	6162815 (12.3111%)	42790719 (85.4808%)	21446369	21344350	21402415	21388304	26760638	16030081	41777906
4-Col-4-do	44757166	43648898 (97.5238%)	2906736 (6.49446%)	40742162 (91.0294%)	20438546	20303616	20368544	20373618	25380187	15361975	39577856
5-Col-5-do	48298406	47256276 (97.8423%)	3134337 (6.48952%)	44121939 (91.3528%)	22093269	22028670	22061066	22060873	26882957	17238982	43282924
6-gl1-1-do	45727230	44633206 (97.6075%)	2388133 (5.22256%)	42245073 (92.3849%)	21190293	21054780	21117711	21127362	26136565	16108508	41348596
7-gl1-2-do	45284342	44228183 (97.6677%)	2212788 (4.88643%)	42015395 (92.7813%)	21069651	20945744	21003121	21012274	26088740	15926655	40929142
8- gl1-3-do	45200084	44190570 (97.7666%)	3654874 (8.08599%)	40535696 (89.6806%)	20308272	20227424	20266071	20269625	25862966	14672730	39648852
9- gl1-4-do	56371784	55020607 (97.6031%)	3759108 (6.66842%)	51261499 (90.9347%)	25716368	25545131	25628037	25633462	32111487	19150012	49928764
10-Colgl-5-do	44642156	43542103 (97.5358%)	3196834 (7.16102%)	40345269 (90.3748%)	20265645	20079624	20175680	20169589	23707146	16638123	39244572

11-er erl1/seg erl2-1-do	47905672	46796394 (97.6845%)	3338585 (6.96908%)	43457809 (90.7154%)	21797616	21660193	21728084	21729725	25974483	17483326	42489716
12- er erl1/seg erl2- 2-do	46224236	45078260 (97.5208%)	3387432 (7.32826%)	41690828 (90.1926%)	20882302	20808526	20840863	20849965	26384821	15306007	40701864
13- er erl1/seg erl2- 3-do	43563750	42389859 (97.3053%)	8050447 (18.4797%)	34339412 (78.8257%)	17216881	17122531	17172751	17166661	23313868	11025544	33399700
14- er erl1/seg erl2- 4-do	43158252	42252261 (97.9008%)	2654978 (6.15173%)	39597283 (91.749%)	19808582	19788701	19796946	19800337	23574302	16022981	38928714
15- er erl1/seg erl2- 5-do	45403178	44298684 (97.5674%)	2499735 (5.50564%)	41798949 (92.0617%)	20959444	20839505	20894628	20904321	24718709	17080240	40795402
16- er erl1/seg erl2 gl1-1-do	50206374	48471719 (96.545%)	2512612 (5.00457%)	45959107 (91.5404%)	23054665	22904442	22985513	22973594	27599557	18359550	44465654
17- er erl1/seg erl2 gl1-2-do	54584106	52755191 (96.6494%)	2603952 (4.77053%)	50151239 (91.8788%)	25164165	24987074	25083913	25067326	29889530	20261709	48637298
18- er erl1/seg erl2 gl1-3-do	53877460	51995164 (96.5063%)	3188578 (5.9182%)	48806586 (90.5881%)	24478838	24327748	24410314	24396272	29790138	19016448	47306824
19- er erl1/seg erl2 gl1-4-do	53913802	52082664 (96.6036%)	2574386 (4.775%)	49508278 (91.8286%)	24826838	24681440	24760323	24747955	29397133	20111145	47930644
20- er erl1/seg erl2 gl1-5-do	45804898	44256903 (96.6205%)	2171088 (4.73986%)	42085815 (91.8806%)	21087136	20998679	21049366	21036449	25150667	16935148	40887496
21-Col-1-d3	40731020	39415000 (96.769%)	3959867 (9.72199%)	35455133 (87.047%)	17852331	17602802	17731764	17723369	22860242	12594891	34504576
22-Col-2-d3	43111010	42161101 (97.7966%)	7582563 (17.5885%)	34578538 (80.2081%)	17316253	17262285	17297913	17280625	22953386	11625152	33804090
23-Col-3-d3	43015728	40830305 (94.9195%)	6177311 (14.3606%)	34652994 (80.5589%)	17454002	17198992	17330885	17322109	24005503	10647491	33814830
24-Col-4-d3	55713300	54540929 (97.8957%)	7588149 (13.62%)	46952780 (84.2757%)	23525263	23427517	23485732	23467048	29179278	17773502	45954022

25-Col-5-d3	56552956	55381770 (97.929%)	11131421 (19.6832%)	44250349 (78.2459%)	22214290	22036059	22138984	22111365	28671991	15578358	43306292
26-gl1-1-d3	43414400	41957807 (96.6449%)	8970108 (20.6616%)	32987699 (75.9833%)	16668895	16318804	16504183	16483516	22793768	10193931	32193020
27- gl1l-2-d3	50101232	48575787 (96.9553%)	8795632 (17.5557%)	39780155 (79.3996%)	20040398	19739757	19899667	19880488	27524311	12255844	38903618
28- gl1-3-d3	44279870	43331293 (97.8578%)	7543713 (17.0364%)	35787580 (80.8213%)	17938024	17849556	17901337	17886243	23910456	11877124	35165374
29- gl1-4-d3	45106190	43777804 (97.055%)	6283496 (13.9305%)	37494308 (83.1245%)	18869080	18625228	18754223	18740085	25110044	12384264	36618742
30- gl1-5-d3	44446970	43322232 (97.4695%)	8594066 (19.3355%)	34728166 (78.1339%)	17415595	17312571	17375610	17352556	23055396	11672770	33902384
31- er erl1/seg erl2- 1-d3	51040288	49840685 (97.6497%)	12876033 (25.2272%)	36964652 (72.4225%)	18519148	18445504	18493433	18471219	25785170	11179482	36127764
32- er erl1/seg erl2- 2-d3	45201872	43381414 (95.9726%)	7026858 (15.5455%)	36354556 (80.4271%)	18352870	18001686	18186160	18168396	24005541	12349015	35125212
33- er erl1/seg erl2- 3-d3	44324684	43293114 (97.6727%)	7062294 (15.9331%)	36230820 (81.7396%)	18168444	18062376	18124554	18106266	22994320	13236500	35320174
34- er erl1/seg erl2- 4-d3	46850216	45139396 (96.3483%)	4279793 (9.13506%)	40859603 (87.2133%)	20547521	20312082	20432586	20427017	26631013	14228590	39654610
35- er erl1/seg erl2- 5-d3	51312870	50184512 (97.801%)	8301167 (16.1776%)	41883345 (81.6235%)	21000692	20882653	20951453	20931892	26607820	15275525	40941766
36- er erl1/seg erl2 gl1-1-d3	40926114	39760983 (97.1531%)	6313194 (15.4258%)	33447789 (81.7273%)	16737184	16710605	16736577	16711212	21293011	12154778	32585410
37- er erl1/seg erl2 gl1-2-d3	49465508	47954538 (96.9454%)	5297833 (10.7102%)	42656705 (86.2353%)	21400551	21256154	21342530	21314175	26216370	16440335	41463482
38- er erl1/seg erl2 gl1-3-d3	49157096	47640038 (96.9139%)	8559372 (17.4123%)	39080666 (79.5016%)	19567366	19513300	19553854	19526812	25655901	13424765	38031768

39- er erl1/seg erl2 gl1-4-d3	46266378	44758081 (96.74%)	4846236 (10.4746%)	39911845 (86.2653%)	19993577	19918268	19970154	19941691	24529106	15382739	38950458
40- er erl1/seg erl2 gl1-5-d3	46103364	43978815 (95.3918%)	4071092 (8.83036%)	39907723 (86.5614%)	20081983	19825740	19960852	19946871	25838745	14068978	38634710
41-Col-1-d5	45274964	43906795 (96.9781%)	11734112 (25.9174%)	32172683 (71.0606%)	16157365	16015318	16110606	16062077	22552760	9619923	31541322
42-Col-2-d5	44433574	43200559 (97.225%)	8309218 (18.7003%)	34891341 (78.5247%)	17558382	17332959	17459257	17432084	23074598	11816743	34125528
43-Col-3-d5	44180878	42775636 (96.8193%)	8610372 (19.4889%)	34165264 (77.3304%)	17166382	16998882	17096944	17068320	23310795	10854469	33333304
44-Col-4-d5	44666980	43153077 (96.6107%)	9318750 (20.8627%)	33834327 (75.748%)	17043785	16790542	16932639	16901688	23142497	10691830	32973986
45-Col-5-d5	43769326	42333246 (96.719%)	5490775 (12.5448%)	36842471 (84.1742%)	18540801	18301670	18425428	18417043	24356206	12486265	36145746
46-gl1-1-d5	45190804	43474938 (96.2031%)	9321130 (20.6262%)	34153808 (75.5769%)	17168389	16985419	17093069	17060739	23750837	10402971	33294678
47- gl1-2-d5	45510690	43987490 (96.6531%)	6365884 (13.9877%)	37621606 (82.6654%)	18994516	18627090	18819716	18801890	24239600	13382006	36678964
48- gl1-3-d5	44751584	43477318 (97.1526%)	12553503 (28.0515%)	30923815 (69.1011%)	15548967	15374848	15479793	15444022	22382618	8541197	30316130
49- gl1-4-d5	45379524	44126290 (97.2383%)	9224104 (20.3266%)	34902186 (76.9117%)	17559747	17342439	17464558	17437628	24113832	10788354	34146222
50- gl1-5-d5	44442466	43252461 (97.3224%)	11200738 (25.2028%)	32051723 (72.1196%)	16110062	15941661	16041712	16010011	22844695	9207028	31376762
51- er erl1/seg erl2- 1-d5	45130052	43808980 (97.0727%)	5379517 (11.92%)	38429463 (85.1527%)	19356669	19072794	19217477	19211986	24671621	13757842	37551602
52- er erl1/seg erl2- 2-d5	43696884	42067953 (96.2722%)	11725079 (26.8328%)	30342874 (69.4394%)	15291572	15051302	15190624	15152250	21811921	8530953	29623636
53- er erl1/seg erl2- 3-d5	43539394	42256867 (97.0543%)	12410870 (28.5049%)	29845997 (68.5494%)	14993803	14852194	14939300	14906697	21674377	8171620	29253872

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54- er erl1/seg erl2- 4-d5	44725096	42820877 (95.7424%)	11186669 (25.0121%)	31634208 (70.7303%)	15901501	15732707	15839827	15794381	21666978	9967230	30328826
55- er erl1/seg erl2- 5-d5	43821902	42371697 (96.6907%)	10855233 (24.7713%)	31516464 (71.9194%)	15879103	15637361	15772012	15744452	21859884	9656580	30805318
56- er erl1/seg erl2 gl1-1-d5	44850300	43039196 (95.9619%)	7272203 (16.2144%)	35766993 (79.7475%)	17992134	17774859	17895137	17871856	24562234	11204759	34937960
57- er erl1/seg erl2 gl1-2-d5	44442246	42529928 (95.6971%)	16290754 (36.656%)	26239174 (59.0411%)	13204781	13034393	13141879	13097295	19320476	6918698	25616314
58- er erl1/seg erl2 gl1-3-d5	45166246	43418434 (96.1303%)	10314813 (22.8374%)	33103621 (73.2928%)	16656274	16447347	16574384	16529237	23023942	10079679	32201760
59- er erl1/seg erl2 gl1-3-d5	44286056	42094234 (95.0508%)	14564331 (32.8869%)	27529903 (62.1638%)	13815128	13714775	13798044	13731859	19389579	8140324	26328894
60- er erl1/seg erl2 gl1-5-d5	44641698	42873222 (96.0385%)	9447120 (21.1621%)	33426102 (74.8764%)	16806828	16619274	16725432	16700670	23308151	10117951	32670326
41-Col-1-d6	45518888	44586211 (97.951%)	7979342 (17.5297%)	36606869 (80.4213%)	18338630	18268239	18312750	18294119	24064642	12542227	36093892
42-Col-2-d6	45386404	44394940 (97.8155%)	11082438 (24.418%)	33312502 (73.3975%)	16705881	16606621	16671746	16640756	22991488	10321014	32670388
43-Col-3-d6	45138748	44326186 (98.1999%)	13153216 (29.1395%)	31172970 (69.0603%)	15589919	15583051	15615645	15557325	21910860	9262110	30733950
44-Col-4-d6	45543782	44706432 (98.1614%)	6528357 (14.3342%)	38178075 (83.8272%)	19097904	19080171	19098628	19079447	24744781	13433294	37569250
45-Col-5-d6	45134724	44239533 (98.0166%)	12933834 (28.6561%)	31305699 (69.3606%)	15663469	15642230	15676123	15629576	22341379	8964320	30858310
46- gl1-1-d6	45696136	44799715 (98.0383%)	21111724 (46.2002%)	23687991 (51.8381%)	11850448	11837543	11883662	11804329	19196146	4491845	23328380
47- gl1-2-d6	44189548	43205919 (97.7741%)	12871667 (29.1283%)	30334252 (68.6458%)	15204244	15130008	15192861	15141391	21413376	8920876	29807316
48- gl1-3-d6	44008642	43159982 (98.0716%)	14650828 (33.2908%)	28509154 (64.7808%)	14268907	14240247	14283808	14225346	20512976	7996178	28061208

49- gl1-4-d6	45014912	44077443 (97.9174%)	12727834 (28.2747%)	31349609 (69.6427%)	15693666	15655943	15699183	15650426	21969263	9380346	30599210
50- gl1-5-d6	43935424	43185122 (98.2923%)	9396028 (21.386%)	33789094 (76.9063%)	16900683	16888411	16911722	16877372	22309379	11479715	33394330
51- er erl1/seg erl2- 1-d6	44462100	43525657 (97.8938%)	17572420 (39.5222%)	25953237 (58.3716%)	12977219	12976018	13005236	12948001	20007613	5945624	25448832
52- er erl1/seg erl2- 2-d6	44940536	43977996 (97.8582%)	11987096 (26.6732%)	31990900 (71.185%)	16018180	15972720	16014789	15976111	22872713	9118187	31310088
53- er erl1/seg erl2- 3-d6	44900542	43604465 (97.1134%)	21074396 (46.9357%)	22530069 (50.1777%)	11268081	11261988	11304387	11225682	18114856	4415213	22178408
54- er erl1/seg erl2- 4-d6	44207796	43092838 (97.4779%)	15479163 (35.0146%)	27613675 (62.4634%)	13821505	13792170	13832882	13780793	20504697	7108978	26944226
55- er erl1/seg erl2- 5-d6	43982386	42780987 (97.2685%)	24023133 (54.6199%)	18757854 (42.6486%)	9381098	9376756	9420578	9337276	16192168	2565686	18443130
56- er erl1/seg erl2 gl1-1-d6	44295738	43151437 (97.4167%)	12155511 (27.4417%)	30995926 (69.975%)	15510037	15485889	15524905	15471021	21375529	9620397	30250518
57- er erl1/seg erl2 gl1-2-d6	44963012	43715246 (97.2249%)	15213644 (33.8359%)	28501602 (63.389%)	14275651	14225951	14277993	14223609	20590035	7911567	27990436
58- er erl1/seg erl2 gl1-3-d6	45206486	43945640 (97.2109%)	14634779 (32.3732%)	29310861 (64.8377%)	14696557	14614304	14681268	14629593	20935824	8375037	28688958
59- er erl1/seg erl2 gl1-4-d6	44294722	42975468 (97.0216%)	7183701 (16.218%)	35791767 (80.8037%)	17922888	17868879	17915739	17876028	22861241	12930526	35069740
60- er erl1/seg erl2 gl1-5-d6	45813116	44294865 (96.686%)	17894070 (39.0588%)	26400795 (57.6272%)	13234393	13166402	13231385	13169410	19487855	6912940	25904160

3.7 References

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Chapter 4

***ERECTA* as a key model for genetic improvement of BARLEY (*Hordeum vulgare*) seed germination under changing salinity**

4.1 Introduction

Yield loss due to germination failure or early seedling death for lack of a hospitable environment is an important issue for major crop species worldwide, especially in areas prone to drought or salinity. Seed fitness is also a significant component of biodiversity in natural communities.

A recent study by ([Nanda et al., 2019](#)) established that the Arabidopsis *ERECTA* gene family controls the timing and the speed of seed germination under salinity and osmotic stress while playing little role under optimal conditions. Germination of seeds with reduced *ERECTA* family signalling was slowed down, delayed or even halted by salinity and osmotic stress and then generally resumed when conditions became more favourable again, without significant loss of viability of the germinating seed and/or seedling death. The experiments presented here confirmed these findings and established that the R2-R3 MYB *AtGL1* (*GLABRA1*) trichome initiation transcription factor was a key player in that function, and synergistically interacted with the *ERf* in promoting germination under salinity and regulation of the development of the seed coat (Chapters 2 & 3). These interactions involve the three *ERf* members, in a partly specific manner.

The *ERf* family is widely conserved across the plant kingdom, as a small family of generally three, but sometimes two members, and indications of some functional conservation but also divergence among species, particularly eudicots and monocots ([Shen et al., 2015](#); [Zakhrabekova et al., 2015](#); [Y. Zhang et al., 2018](#)). The Arabidopsis seed presents important structural, biochemical and regulatory differences compared to seeds of other species, including major crop species, even eudicots such as legumes, Solanaceae such as tomato, or even other closely related Brassicaceae such as canola (*Brassica napus*), and most strikingly compared to grain crops. Functional information obtained on Arabidopsis seed germination and underlying mechanisms cannot be assumed to necessarily apply to other species. It is therefore, critical not to consider Arabidopsis as a universal plant model system to assess the adaptive and agronomic relevance of findings in that species and to unravel them. Such concern is, in fact, also emerging from recent studies of stomatal development itself, in which the *ERf* is a key, intensively studied player ([Doll et al., 2021](#); [Doll et al., 2023](#)).

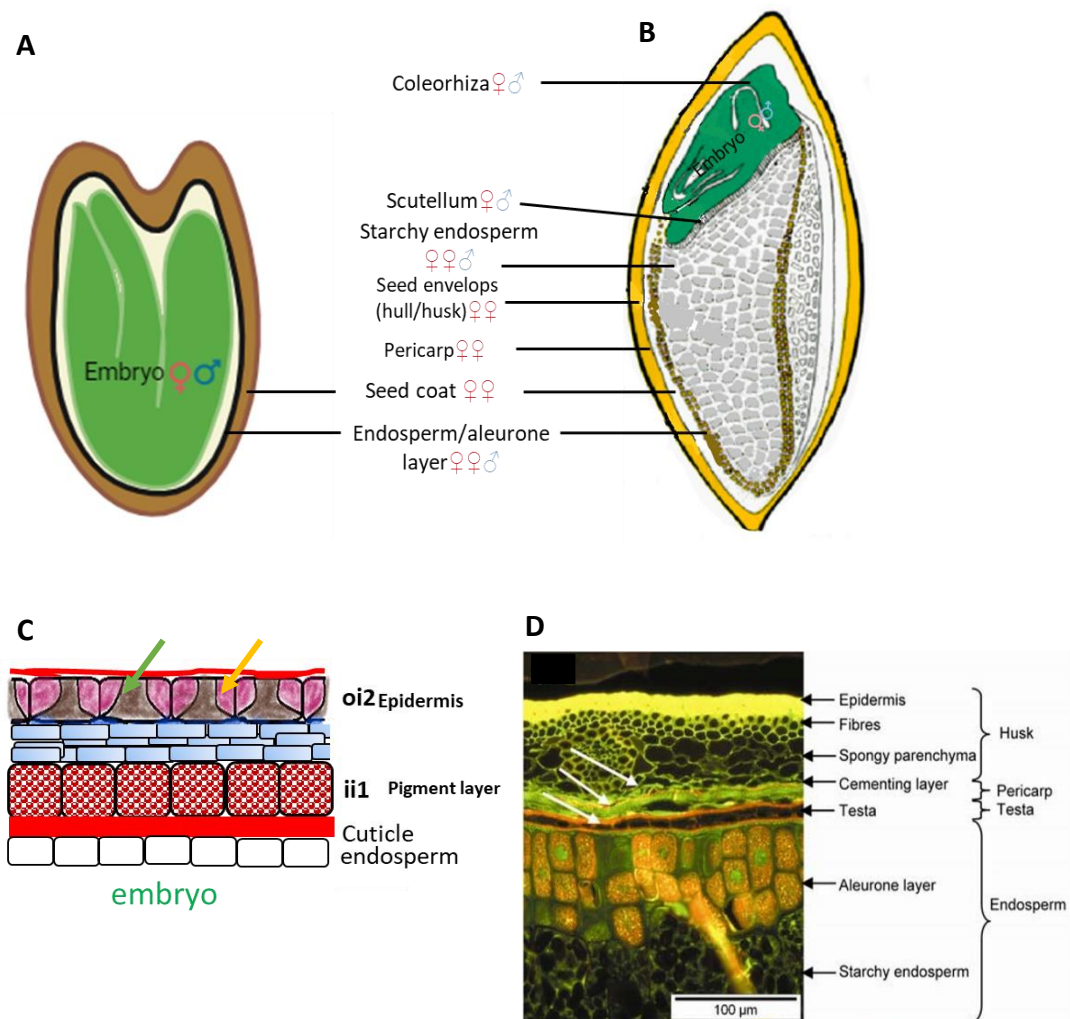


Figure 4-1. Anatomical differences of seed as a whole and embryo covering tissues of Arabidopsis (A and C) and barley (B and D). **A & B.** Longitudinal section of mature Arabidopsis (A) and barley seed (B) illustrating diploid embryo (♀♂) and maternally derived embryo covering tissues. **C.** Cross-sectional view across the middle of the seed of Arabidopsis. The outer epidermal cells consist of columellae (green arrow, secondary cell wall depositions) to attach the mucilage pockets (yellow arrow). All three integument layers below the oi2 were crushed during the seed coat differentiation. Maternally origin thick cuticle lined in between the endosperm and seed coat, associated with the endosperm at maturity. **D.** Cross sectional view across the middle of the seed of barley caryopsis. White arrows pointed to the three cuticle layers between the husk and pericarp (cementing layer), between the pericarp and testa and between the nucellus and endosperm (Figure D adapted from (Oikku et al., 2005)).

The barley caryopsis, as the wheat or rice, and more broadly the seeds of grain cereal is a caryopsis, i.e., a propagation unit consisting of a seed surrounded by a pericarp which adheres tightly to the external seed envelope. It is quite different from the Arabidopsis seed with respect to anatomy and structure, nature of maternal tissues, embryo size and seed reserves, and regulation of germination (Figure 4-1).

The mature *Arabidopsis* seed is characterized by an embryo which fills most of the space available within the seed envelope, having absorbed most of the endosperm for its expansion and the accumulation of reserves necessary to fuel germination and sustain the newly emerged seedling until it becomes a complete autotroph. These reserves are mostly lipidic, with also include a fraction of soluble proteins. The embryo is only separated from the seed envelope/coat/testa by a one cell-thin surrounding layer of residual live endosperm. The overlaying seed coat inner integuments are crushed and no more distinguishable and impregnated with tannic compounds -mostly proanthocyanidins (PAs)- while the outer integument is highly suberized with an outer epidermal layer of mucilage-bearing cells ([Beeckman et al., 2000](#); [Western et al., 2000](#); [Voiniciuc, Yang, et al., 2015](#); [L. Demonsais et al., 2020](#)) (Figure 4-1). By contrast, cereal caryopses as their propagation units (Figure 4-1 B), i.e., fruits with one seed. Barley (*Hordeum vulgare*) was chosen as a species of choice to probe the conservation of *Arabidopsis* *ERECTA*-family function to major crop species. It carries three putative *ERECTA* family members -similar to *Arabidopsis* and many other species, including wheat and rice- that have not been characterized. In addition, barley has been the most studied among major cereals for dormancy and constitutive germination mechanisms and molecular control, possibly due to its for malting which demands a very tight control of germination. However, hardly anything is known about the mechanisms involved in the germination of non-dormant seeds, how they sense the environment and adjust germination accordingly.

As opposed to *Arabidopsis*, the barley mature caryopsis has a large compartment of dead starchy endosperm, separated from a small embryo by a specialized transfer tissue, the scutellum, and surrounded by a 3-cell layer thick living aleurone layer. Upon seed imbibition, the starchy endosperm undergoes degradation under enzymatic action, providing growth substrates to the expanding embryo through the scutellum. The diploid embryo and the two triploid endosperms -starchy and aleurone endosperm- are covered by two externally fused envelopes, lemma and palea, collectively known as “husk”, which encloses an inner 4-5 cell layer-thick pericarp covered by a two-cell layer-thick testa. At maturity, the husk is tightly adherent to the caryopsis, and the pericarp and testa are highly compressed/ crushed. The barley testa exhibits a pigmented tannic proanthocyanidin layer, appressed to the aleurone layer ([Aastrup et al., 1984](#)), similar to the tannic “pigment layer” in the *Arabidopsis* (Figure 4-1 D).

The lemma and palea of the mature barley seed both exhibit four cellular layers, namely from outside to inside, outer epidermis, sclerenchyma, parenchyma and inner epidermis. The mechanical strength of the lemma and palea is mostly determined by the outer epidermal and sclerenchyma cells which have highly suberized cell walls. The vascular bundles are embedded into the thin wall parenchyma

layer. The husk adheres strongly to the underlying four-cell layer thick pericarp ([Cochrane & Duffus, 1979](#); [Freeman & Palmer, 1984](#)). Strong adhesion between the seed envelope and the pericarp is agronomically and industrially important for malting as it avoids grain skinning. It is brought about by a cementing layer made from lipid-rich material secreted from the outer epidermal cells of the pericarp ([Gaines et al., 1985](#)) and sandwiched between the pericarp cuticle and the husk cuticle ([Harlan, 1920](#); [Gaines et al., 1985](#); [Olkku et al., 2005](#); [Hoad et al., 2016](#); [Brennan et al., 2017](#); [Brennan, Hedley, et al., 2019](#)) (Figure 4-1 D). The formation of the cementing layer starts two days after flowering (DAF), just after the deposition of secondary cell wall materials in the pericarp outer epidermal cells ([Gaines et al., 1985](#)). The husk protects the growing embryo throughout the grain-filling period and also contributes to photosynthesis together with the pericarp, providing carbohydrates to the inner compartments. It also protects from premature germination on the mother plant, preserves seed vigour, and prevents caryopsis dehydration during seed germination. Seeds carrying a loosely attached or damaged husk are improper for the malting process.

Barley germination initiates with water absorption from the surrounding environment (imbibition) causing the seed tissues to swell, soften, and the metabolic processes to resume (Bewley and Black, 1994). Hydration activates enzymes that break down stored endospermic reserves, providing energy and building blocks to the growing embryo (Bewley, 1997). The coleorhiza, a protective sheath covering the radicle (embryonic root), emerges first through the husk, breaking the palea-lemma (husk) at the micropylar end, typically after about 16 h of imbibition in the water. In the second step, within the next 6-8 h, the radicle ruptures the coleorhiza and comes into direct contact with the seed surroundings. This defined the time and considered germination as completed. In parallel to the radicle elongating downwards, the shoot breaks its covering coleoptile and elongates upwards.

The experiments reported in this chapter were designed to test the conservation in barley of the *ERF* +/-*GL1* functions in the regulation of seed germination.

4.2 Material and Methods

4.2.1 Plant material and growth conditions

Wild-type barley plants (*Hordeum vulgare* cv. “Golden Promise”-GP) were germinated on Whatman 1 filter paper, and the germinated seedlings were transferred to soil and grown in a controlled environment in growth chambers under controlled conditions: 13°C day/night, 12-hour photoperiod at 150 $\mu\text{E m}^{-2} \text{s}^{-1}$ and 70% relative humidity for 10 weeks and then changes to 18/15°C day/night, and same photoperiod and humidity until maturity.

4.2.2 Characterization of putative barley orthologs of the Arabidopsis *ERf* and *GL1*.

The barley orthologous were identified through a homology search of the barley draft genome (Barley MorexV1, IBSCv2 genome assembly (2017)). In addition, the gene sequences of selected dicots and monocot species were obtained through the NCBI gene bank. The protein sequence of Arabidopsis *ER* and *ERL1* and *ERL2* and *GL1* were used to search for *ERf* and *GL1* in all the selected species through NCBI tblstn. (Altschul et al., 1990). The protein sequences of all identified *ERf*s were aligned by MUSCLE (Edgar, 2004) with default parameters. A phylogenetic tree was constructed using the Neighbour-Joining method through MEGA 6 (Tamura et al., 2013). Each identified gene was classified as *ER* or *ER-like* (*ERL*) based on the similarity to Arabidopsis *ER* /*ERL* genes and further confirmed by its position in the phylogenetic tree and by synteny analysis. Two putative *ER* orthologs, later referred to as *HvER* and *HvER2*, with to *AtER*, with a similar gene structure, typical of LRR-RLKs, as well as the number of exons and introns and 1 putative ortholog to *AtERL1* and *AtERL2* referred as *HvERL* were identified as indicated in Table 4-1. *HORVU6Hr1G058620* in chr 6H (*MYB30-like*) were selected as the gene targets for the *HvGL1* in this study.

Table 4-1. Identification of putative Arabidopsis *ERf* and *GL1* orthologues in barley

Arapidopsis query gene ID	Barley orthologs _ensemble gene ID -Morex.V3(2021)	Gene symbol (NCBI)	gene description	Given gene name/symbol
<i>ERL1</i> (AT5G62230.1)	HORVU.MOREX.r3.7HG06445	LOC123413545	LRR receptor-like serine/threonine-protein kinase SIK1	<i>HvERL</i>
<i>ER</i> (AT2G26330.1)	Ert-m- HORVU.MOREX.r3.7HG0662 630	LOC123413216	LRR receptor-like serine/threonine-protein kinase ER1	<i>Hv ER</i>
<i>ER</i> (AT2G26330.1)	HORVU.MOREX.r3.6HG06201	LOC123401964	LRR receptor-like serine/threonine-protein kinase ER2	<i>HvER2</i>
<i>GL1</i> (AT3G27920.1)	HORVU.MOREX.r3.6HG05963	LOC123406059	transcription factor MYB30-like	<i>HvGL1</i>

4.2.3 Engineering CRISPR/cas9 construct for *HvERf* and *HvGL1* editing and function knock-out, individually or in combination.

The CRISPR vector was constructed according to a method described by Ma et al. 2015. Firstly, the fragments carrying the target sequence and U3 or U6 promoter were synthesized (IDT, USA). The integrated sgRNA(s) expression cassette was then amplified by PCR using the primers (Appendix 1). Subsequently, the amplicons containing each sgRNA with different BsaI-cutting sites were cloned by the Golden Gate method into the CRISPR/Cas9 Multi-targeting vector pYLCRISPR/Cas9Pubi-H at a BsaI site (Figure 4-2 A). All final binary constructs were confirmed by the Sanger sequence and then transferred into *Agrobacterium tumefaciens* strain AGL-1 ([Lazo et al., 1991](#)) by electroporation for barley transformation ([Harwood, 2014](#)).

4.2.4 Embryo Isolation and Agrobacterium-mediated transformation in barley

Agrobacterium culture preparation for transformation

200µl of glycerol inoculum (*A. tumefaciens* strain AGL-1 carrying vector pYLCRISPR/Cas9Pubi-H) were added to the 5ml MG/L (without antibiotics) under sterilized conditions. The *Agrobacterium* culture was then incubated at 28°C, shaking (250rpm), overnight (17-20h).

Immature embryo isolation

Approximately 10 barley heads of 2mm diameters of the embryo were harvested 12-16 days from pollination. The seeds were surface sterilized by rinsing them with 100% ethanol (less than one minute) for 20 minutes in 20% commercial bleach with gentle shaking. Seeds were thoroughly rinsed with sterile distilled water (about 5-8 rinses) for a few minutes until all the bleach was rinsed off under the lamina hood. A few drops of water were kept in the falcon tubes with the sterilized seeds to maintain the moisture, and the tubes were placed at 4°C overnight. The embryos were excised using a sharp scalpel under the binocular. Holding the excised embryo by forceps, the axis of the embryo was removed by placing the scalpel blade in the groove between the scutellum and the axis.

*Inoculation of the explant (embryo without axis) with *A. tumefaciens*.*

Approximately 50µl of *Agrobacterium* culture was poured on the freshly excised embryo, ensuring all embryos were contacted with the solution. The explants were incubated for 1-3 minutes at room temperature. Transfer the explants to freshly prepared BCI-DM plates with the cut side of the embryo facing up at evenly spaced intervals (25-30 per plate). The petri plates were sealed with parafilm,

covered with aluminium foil to provide the dark conditions, and placed in a closed box. Boxes were placed in the growth room at 24°C for 2-3 days.

Callus induction, regeneration and selection.

All the growing calli were transferred to freshly prepared BCI-DM containing hygromycin (for the selection of successful transformant) and Timentin (to prevent further growth of *Agrobacterium*). Calli were left in the medium for 6 weeks with a transfer to fresh medium every two weeks. The calli were transferred to FHG medium after 6 weeks for the shoot generation (Figure 4-2 B). Then, the shoots were transferred to pots containing BCI medium for root generation. After sufficient roots and shoot formation, plants were transferred to sterilized soil containing osmocots and nitrogen-rich fertilizer and grown to maturity under controlled environment conditions.

4.2.5 Screening of Successful Transformants

Barley gDNA extraction

Barley genomic DNA was extracted from young barley leaves regenerated on the selection medium with hygromycin and grown on soil (T_0). Leaf tissues were ground using Tissue Lyser II at 221/s for 1.35 minutes, and Genomic DNA was extracted from the leaf samples using the cetyltrimethyl ammonium bromide (CTAB) method ([Murray & Thompson, 1980](#)). The extracted DNA was quantified and quality-checked using a Nanodrop spectrophotometer (Thermo Scientific Nanodrop 2000c).

Screening for Hpt-positive plant

The PCR reaction mixture (20ul total volume containing 1ul of 100ng of DNA template, 0.25ul (10uM) of each forward and reverse primers, 4ul (5x) green buffer (Promega), 2ul (2mM) dNTPs, 0.1ul of GoTaq (Promega) DNA polymerase) with *Hpt* primers, Initial denaturing at 94°C for 1 minute, followed by 35 cycles of 94°C denaturation for 30 seconds, annealing at 58°C for 30 seconds, 72°C extension for 1 minute, and then a final extension of 10 minutes at 72°C. *Hpt*-positive plants were selected using agarose gel electrophoresis: 20ul PCR products; gel runs at 130 volts for 40 minutes.

4.2.6 Screening for homozygous progeny of T₀ and T₁ plants

Sequencing for the target gene edition in Hpt-positive plants

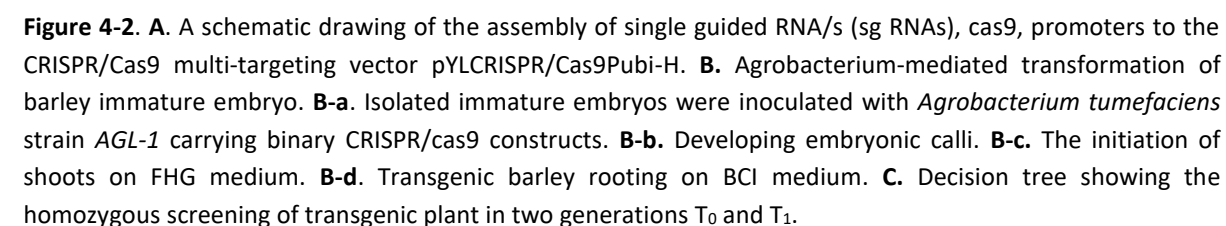
The fragment of the target region was extracted amplified and PCR restriction enzyme digestion and T7E1 cleavage assay were carried out by gene-specific primers from the *Hpt*-positive plants. Gene-specific primers were designed using primer-BLAST (Appendix 1). Then, the target region was amplified and purified (Promega-Pcr-Cleanup-Kit) from the selected positive plants from the T7E1 cleavage assay and were sequenced using Sanger sequencing at the Biomolecular Resource Facility (BRF), The John Curtin School of Medical Research (JCSMR), Australian National University (ANU). Applied Biosystems 96-capillary 3730 DNA Analyzer was used for Sanger sequencing, and sequences at each target site were analysed manually using Geneious (version 8.19). Plants that were successfully edited for the barley Arabidopsis orthologues *ERECTA* and *GL1* genes were kept to harvest the seeds (T₀ plants->T₁ seeds). The absence of the target gene/s of the successfully edited plants was further confirmed by RT-PCR using gene-specific primers.

Genotyping of T₁ barley progenies

15 seeds from each edited (homozygous and heterozygous for the targeted gene/s) T₀ plants were shown in Petri plates with Whatman 1 filter paper, and the successfully germinated seedlings (T₁ plants) were transferred to soil pots with similar conditions as mentioned above. Gene edition and homozygosity were screened on cas9 absence plants using the Sanger sequencing. Homozygous and interested heterozygous edited progenies were kept until maturity to harvest the T₂ seeds (Figure 4-2 C).

4.2.7 Phenotypic characterization of homozygous Barley *ERf +/GL1* lines

Successfully edited plants for three *ERf* genes and/ *GL1* plants grown alongside the Golden Promise (GP) and transgenic WT plants as controls were phenotypically characterized for their vegetative and reproductive development. Agronomically important traits such as tiller number, plant height, number of heads per plant, and number of seeds per head were obtained from T₀ and T₁ plants under optimal growth conditions. Plant height was measured from the top of the soil to the youngest ligule, for three tillers per plant. Leaf blade length was measured from ligule to blade tip, and the blade width was measured at the widest point.



4.2.8 Germination assay of T₂ seeds

Mature seeds were collected and stored together for the same length of time after harvest, at least one month, to release dormancy. The average weight of the seeds used for each replicate was recorded for all the *Hverf +/-GL1* mutant lines, WT and Null, and the seeds with approximately similar weight range were chosen for the assay. Seeds were surface sterilized in 3% sodium hypochlorite for 15 minutes and thoroughly washed three times with sterile distilled water. Seeds were sown on petri plates containing Whatman 1 filter paper and space according to the same grid. The plates were imbibed with 6mL of respective solution (0 and 150mM NaCl). Plates were then placed in room temperature (21°C), dark. Germination was scored daily, individually for testa and endosperm rupture under a binocular microscope, at 3h intervals until all the seeds completed germination on control medium (0mM NaCl) and 3 times to once daily at 150mM, until no change in germination score were observed. Data plotted as percentages of seeds having Coleorhiza Emergence (CE) and Coleorhiza Break (CB) as a function of incubation time post-sowing, or as T₅₀ values, corresponding to the times (h post-sowing) when seeds reached 50% of CE or CB. For the dehusk assay the husk were carefully removed around the embryo using blunt end forceps under the binocular following sterilized conditions and germination scoring were done in every three hours from the 12 h post-sowing for both control and 150mM medium. CE- visible elongation of Coleorhiza through all the inner tissues, germ aleurone, testa and pericarp and CB- primary and seminal roots elongation through breaking the coleorhiza were recorded separately.

4.2.9 Water uptake kinetics

The initial seed weight of each seed was recorded, and similar weight ranges across the genotypes were selected for the assay. The assays were carried out as described in ([Cozzolino et al., 2013](#)). Seeds were imbibed in 0mM NaCl and 150mM NaCl, and seed weight was recorded every hour for 7 hours, then every second hour for 24 hours, every third hour for 36 hours and then accordingly until all the lines completed the CB. Seed water content was recorded as a function of initial dry weight ((seed weight- seed initial weight)/ seed initial weight)X 100.

4.2.10 Microscopy

Barley seeds were cleared in 12% commercial bleach for 2h in a bench-top rotator and washed three times in miliq water to remove all the bleach. Basic Fuchsin 0.2% was prepared in miliq water, and cleared seeds were stained for 30min at room temperature. Then, the seeds were stained in 0.1% Calcofluor white overnight at room temperature. After completing the staining procedure, seed

maternal tissues-husk, seed coat, and pericarp were removed carefully using blunt end forceps, and tissues were placed immediately on the glass slide with a drop of miliq water. Tissues were imaged using a Leica TCS-SP8 confocal microscope, with the following settings: gain `600, smart offset 0.1%, format 1024X1024 pixel, excitation at 405,458 and 514nm and emission acquisition window 400-540nm and 570-750nm. Images were processed using the Fiji Image J software. (n=3, 5 microscopic fields for given location).

4.3 Results

4.3.1 *ERECTA* family genes are conserved in different species, including barley, in terms of gene and protein structure

Conservation of *ER* family genes across several species, including rice, was previously reported by Zhang et al. (2018). Also, Zakhrebekova et al. (2015) study identified and characterized a gene (accession no AK375830/MLOC_34350) with high similarity to arabidopsis *ERECTA* (named *Ert-m*), on chromosome 7H. In this study, two putative orthologous to Arabidopsis *ER* (*HvER* and *HvER2*) mapping to chromosomes 7H and 6H, respectively, have 27 exons encoding a 977 and 863-amino acid (aa) protein, respectively, were identified as having similar gene structure as *AtER* (27 exons, encoding a 976 aa protein) (Figure 4-6 & Figure 4-7). *HvER* corresponds to the *Ert-m* gene identified by Zakhrebekova et al. (2015). The query of the newest draft of the barley genome (Barley Morex IBSC 2021_V3) with the *AtER* protein sequence returns 72-73% and 96-97% sequence identity between the Arabidopsis protein and *HvER*, and *HvER2* translated proteins, respectively. Homology search returned a third locus thereafter named *HvERL*, with the highest homology to *AtERL1* /*AtERL2* (69% and 70% sequence identity and query cover), and a similar structure. Hence, its 26 exons encode a 982aa protein, compared to 26 exons of *AtERL1* and 918 aa *AtERL1* encoded protein (Figure 4-8). The most closely related sequence to the *AtGL1*/*AtMYB0* transcription factor, was barley transcription factor MYB30-like, encoding a 255 aa protein with 74% sequence identity to the *AtGL1*. That gene was chosen as a putative candidate functional orthologue of the Arabidopsis gene, and was targeted for function knock-out editions, along with the 3 *HVER/ERL* genes, using the CRISP/Cas9 gene editing technology (Table 4-1; Figure 4-4 B and Figure 4-9).

Ancestry relationships and sequence conservation of the identified *ERECTA* family genes in other cereals were analyzed using Synteny analysis (Figure 4-3). Wheat (*Triticum aestivum*) *TaERL*, *TaER1* and Rice (*Oryza sativa*) *OsERL*, *OsER2* were declared syntenic with our selected *HvERL* and *HvER* candidate sequences (Figure 4-3 A), and so too *TaER2* and *OsER1* with *HvER2* (Figure 4-3 B). That implies the evolutionary relationships and conservation of the *ERECTA* family genes across the crops. These results are consistent with earlier phylogenetic analyses of *ERECTA* genes (Y. Zhang et al., 2018), and the ER/ERL protein phylogenetic tree shown in (Figure 4-4 A), obtained through Neighbour-Joining analysis. Eudicot and monocot sequences group together, in different clades, for both ER and ER-Like sequences. ER and ERL copy numbers vary little across species, whether eudicot or monocot, between generally 2 – 3 per diploid genomes, as a whole depending on the occurrence of whole genome duplication (WGD) in each species and combination of several genomes, such as in tetraploid canola or hexaploidy *Triticum aestivum* (Figure 4-4 A).

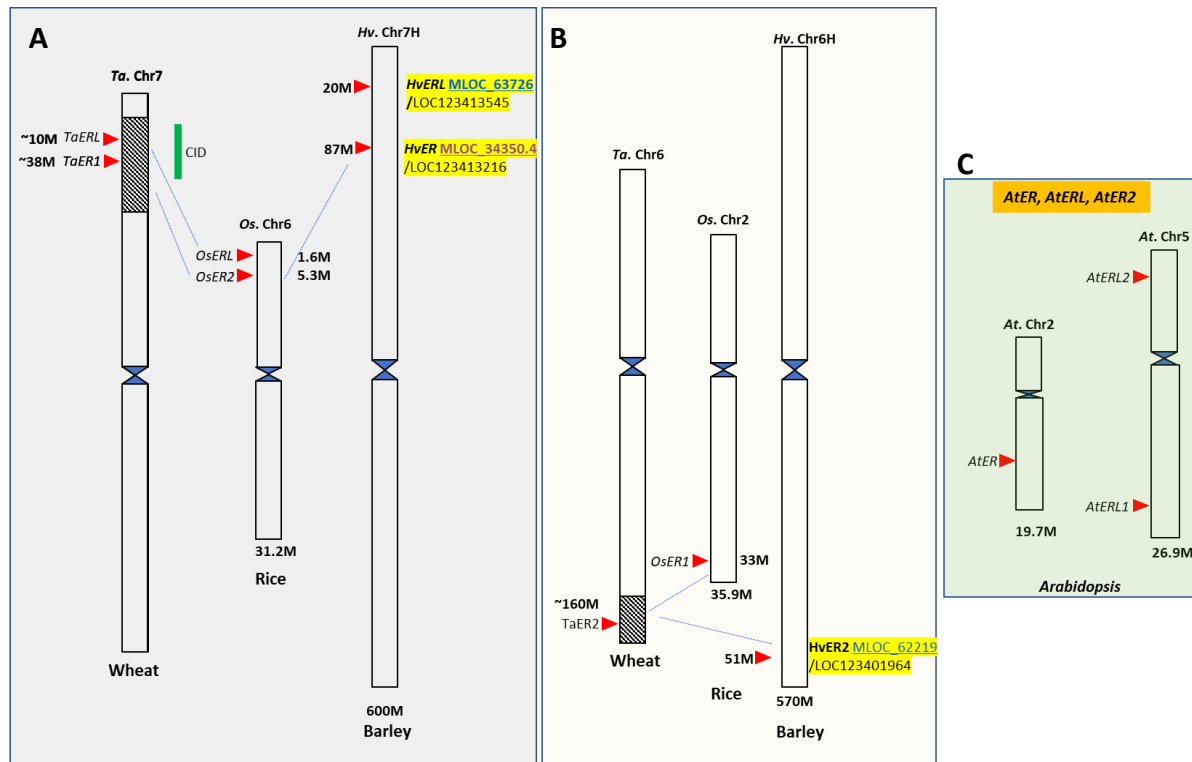


Figure 4-3. Synteny analysis of wheat, barley vs rice to illustrate the conserved regions of *HvER*, *HvER2* and *HvERL*. Syntenic relationship between wheat and barley chromosome 7 and rice chromosome 6 showing the conserved blocks of *HvERL* and *HvER* (A), chromosome 6 for barley and wheat and chromosome 2 for rice showing conserved blocks of *HvER2* (B), and the *AtERf* genes positions in chromosome 2 and 5 (C).

A phylogenetic analysis of *AtGL1*-related protein sequences across the barley (*Hordeum vulgare*) and bread wheat (*Triticum aestivum*) genomes (Morex.v3 and IWGSC v1.0,2018) was also generated (Figure 4-4). The *HvGL1* (MYB30-like) sequence selected for gene editing at the beginning of this thesis as the best candidate orthologue to *AtGL1* remains to this date the most closely related gene to *AtGL1*. The expression of the four genes *HvER*, *HvER2*, *HvERL*, and *HvGL1* in cultivar *Golden Promise* were tested to understand that these previously uncharacterized genes are actually expressed, and hence likely functional, and that their expression is detected in organs and at stages that are consistent with where and when the *ERf/GL1* genes characterized in other species have been found to be expressed. The gene expression data shows approximately similar expression in the young (2-month-old) leaves and elevated expression of the *HvER2* in the spikes compared to the other three genes. (Figure 4-5 C).

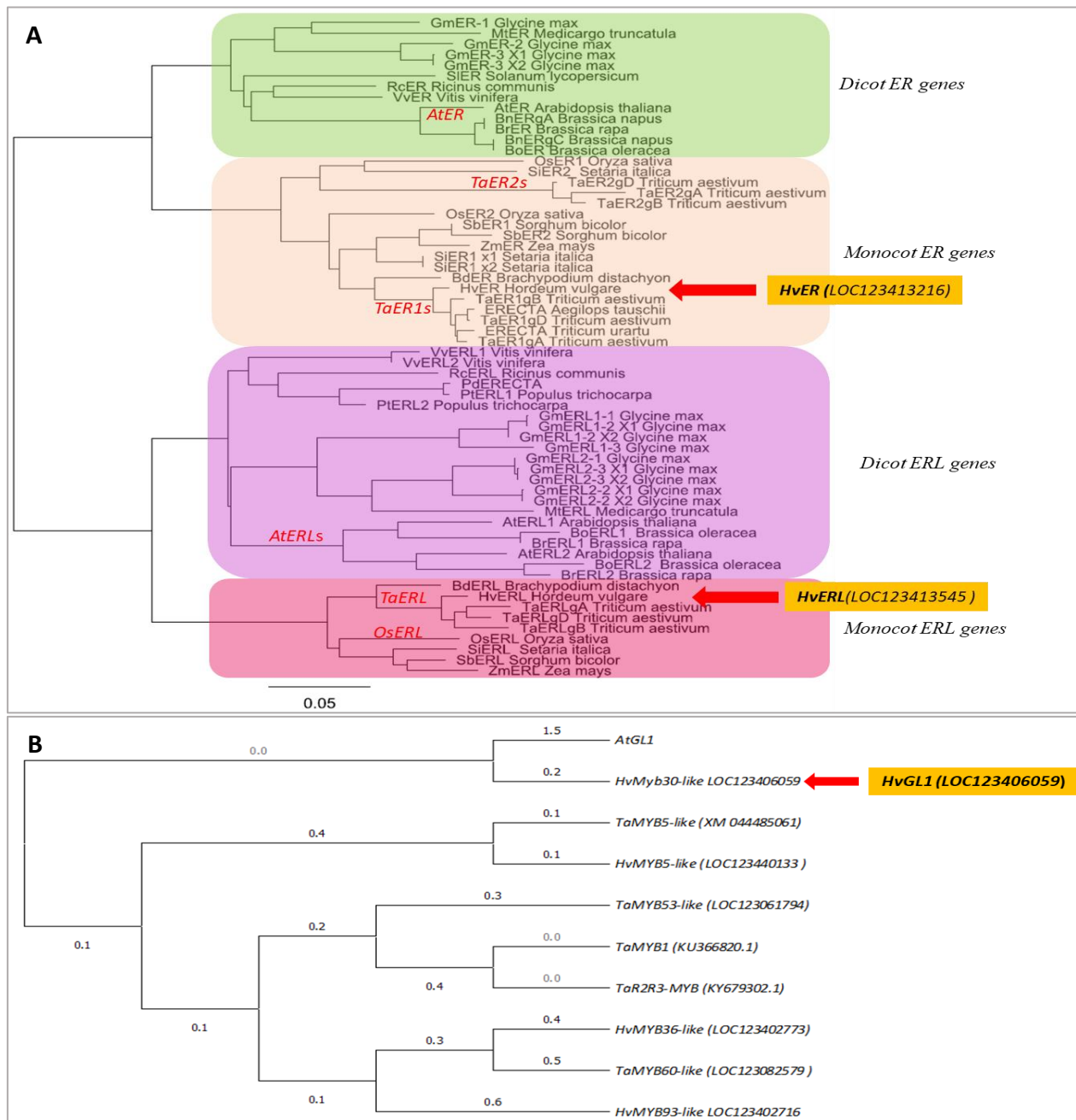


Figure 4-4. Phylogenetic analysis of whole proteins for ERECTA and closest GL1 sequence orthologous in different species. **A.** A phylogenetic tree illustrating the evolutionary relationships between ERECTA proteins across various monocot and dicot species. Bar = 0.05 (5 substitutions per 100 nucleotide positions). **B.** A phylogenetic tree illustrating the closet protein sequence paralogous of AtGL1 in barley (Morex.v3) and wheat (IWGSC v1.0,2018). The position of the selected sequence for the HvERf and HvGL1 (Myb 30-like) in this study was pointed out by the red arrow. Phylogenetic trees were constructed based on neighbour-joining analysis using the built-in ClustW of the Genious 7.1.3 software program.

4.3.2 Generation of barley *erf* and *gl1* mutants by CRISPR/Cas9 gene editing

Successful primary transformant edited for HvERf genes and HvGL1 in Barley

The CRISPR/cas9 construct was designed to edit one, two and three genes at a time among *HvER*, *ER2*, *ERL* and *HvGL1*, guided by a single guide RNA/s (sgRNAs) specific of the gDNA fragment targeted for editing. For the *HvERf* gene, the first exon targeting the leucine-rich repeat (LRR) domain was selected for the edition. The LRR domain in *ERECTA* is vital for ligand recognition and binding, which are central to its role in signal transduction. By targeting this domain, we aimed to impair the protein's interaction with ligands and co-receptors. Similarly, for *HvGL1*, the DNA binding domain was targeted for editing. The position of the target fragment in the gene sequence, the gene-specific primers for genotyping, sgRNAs and protospacer adjacent motif (PAM) used in this study are shown in Figure 4-6 to Figure 4-9 a& b for *HvER*, *HvER2*, *HvERL* and *HvGL1*, respectively. Immature barley embryos (cultivar Golden Promise) were independently transformed with the confirmed binary constructs carrying the sgRNA from Golden Promise (GP). Plants from the T₀ generation were screened for hygromycin resistance; positive plants were further screened by PCR-based amplification of the antibiotic-resistant *Hpt* gene (e.g., Figure 4-5 B). The presence and integrity of the *Hpt* gene in the individual genomic DNA extracts were confirmed by agarose gel electrophoresis (e.g., Figure 4-5 B). gDNA from each *Hpt*-positive T₀ plant was then used to amplify the target fragment (Figure 4-5 C) and sequence it to determine whether and where it had effectively been edited. From here onwards, each mutant lines were denoted by an identifier, for an example *H2#1.4*, H2 is the construct ID as in tabel2, #1 denoted the independent transformant number in the T₀ generation and .4 denoted for the individual plant number in the T₁ generation (Table 4-2). A total of 12 successfully edited T₀ lines across the four target *HvER*, *ER2*, *ERL* and *HvGL1* genes, and the nature and position of the mutation(s) each carried, were identified (Table 4-2); **a)** One heterozygous *Hver*^{+/−} mutant (*H8#1*); **b)** Two mutants differently edited in *HvER2* (*H3#4* and *H3#5*, both with a homozygous insertion at 810 and a heterozygous 9bp deletion at 916; and QH 3#9 with a homozygous substitution); **c)** Three lines edited in *HvERL* (*H2#2* and *H14#6* both carrying a heterozygous but different *erl*^{+/−} mutation; and the homozygous *erl* *H14#1* mutant) (Table 4-2). **d)** Two mutants, each presenting a homozygous mutation in *HvGL1*, were identified (*H10#1*, *H10#2*); and bi-allelic mutants with a heterozygous edition *gl1*^{+/−} mutation, different between the two alleles, (*QH2#1*, and , *QH3#2*) (Table 4-2). **e)** Additionally, two T₀ transformants, successfully edited in two target genes *HvER2* and *HvGL1* were rescued, with furthermore 2 mutated sites in *HvER2*, one homozygous and one bi-allelic mutation (double mutants *er2 er2*^{+/−} *gl1*^{+/−} *QH 3#1* and *QH3#3*) (Table 4-2).

The target sites, the actual editions in the gDNA sequence detected in the above T_0 primary transformants, and their downstream effects on the translated protein (frameshift, premature stop codon, and/or missense mutation) were manually analysed using the Geneious visualization software (Geneious 7.1.3) or comparison of the edited and WT sequences (Figure 4-6 to Figure 4-9; Table 2).

The protein products of the edited genes were used as queries in NCBI blastp, and the sequence identity and coverage of the WT protein sequence were analysed to confirm the successful knockout of the gene function (Table 4-2). Deletions and insertions occurred at higher frequencies than substitutions for all the target genes. The largest deletion (34 bp) was observed in *HvGL1*. (Table 4-2 and Figure 4-6 to Figure 4-9 c). Successfully edited homozygous lines for *HvER*, *HVER2*, *HvERL* and *HvGL1* were further analysed to confirm the absence of a functional target gene with qPCR using gene-specific primers (see a sample of the results obtained in Figure 4-5 C- complete results are provided in Appendix 2). All T_0 lines that were homozygous or heterozygous for the desired editions were grown to maturity to recover the T_1 seed progeny.

Isolation of HvERf homozygous T_1 progeny from confirmed T_0 mutant lines

Since the CRISPR/Cas9 system functions in the somatic cells of the T_0 transgenic plants, mutations would also occur in gametes and embryonic cells, implying that, as long as the Cas9 protein remained present and active, it could continue to further edit the target genes in subsequent generations ([Feng et al., 2013](#)), reviewed in ([Zhang et al., 2019](#)). Moreover, the heterozygous mutations identified in the T_0 generation would segregate in T_1 . A detailed molecular characterization of the T_1 progeny of each positive T_0 line was hence necessary to establish the exact editions they carried in the target genes and identify additional homozygous mutant lines (see decision tree Figure 4-2 C).

Fifteen seeds from the progeny of each T_0 plant were germinated (T_1 seeds), and the seedlings were tested for the presence of Cas9 gene. The plants where it could not be detected were then analysed to test for the presence of a CRISPR/cas9-induced mutation at the target site(s). The PCR restriction enzyme digestion and the T7E1 cleavage assay of the PCR products amplified from the target region were conducted. The T7E1 enzyme recognizes and cleaves mismatched base pairs in DNA, which form when edited and unedited DNA strands hybridize during denaturation and reannealing, creating small fragments, thereby confirming successful genome editing. Sanger sequencing of PCR amplicons for all the T_1 plants was then performed. We identified positive T_1 plants and successfully edited for the three barley *ERECTA/ERECTA-like* genes and *HvGL1*, individually or in combination, for at least two independent transformant events, except for *HvER*. The editions detected in the T_1 progeny of positive

T₀ plants are listed in Table 4-2, together with their consequence on the translated protein (frameshift; truncation; non-synonymous nucleotide substitutions that may allow the protein to retain partial functionality. (Table 4-2; Figure 4-6 to Figure 4-9 b). Moreover, the translated proteins of all detected edited sequences were blasted against the barley protein database in order to confirm the complete knockout of target gene(s) or potentially partial maintenance functionality. That BLAST search yielded either “no-hit “(query sequence did not find any significant matches or alignments in the database) or homology with only very short stretches of DNA (% query cover ≤ 26 across edited genes (Table 4-2).

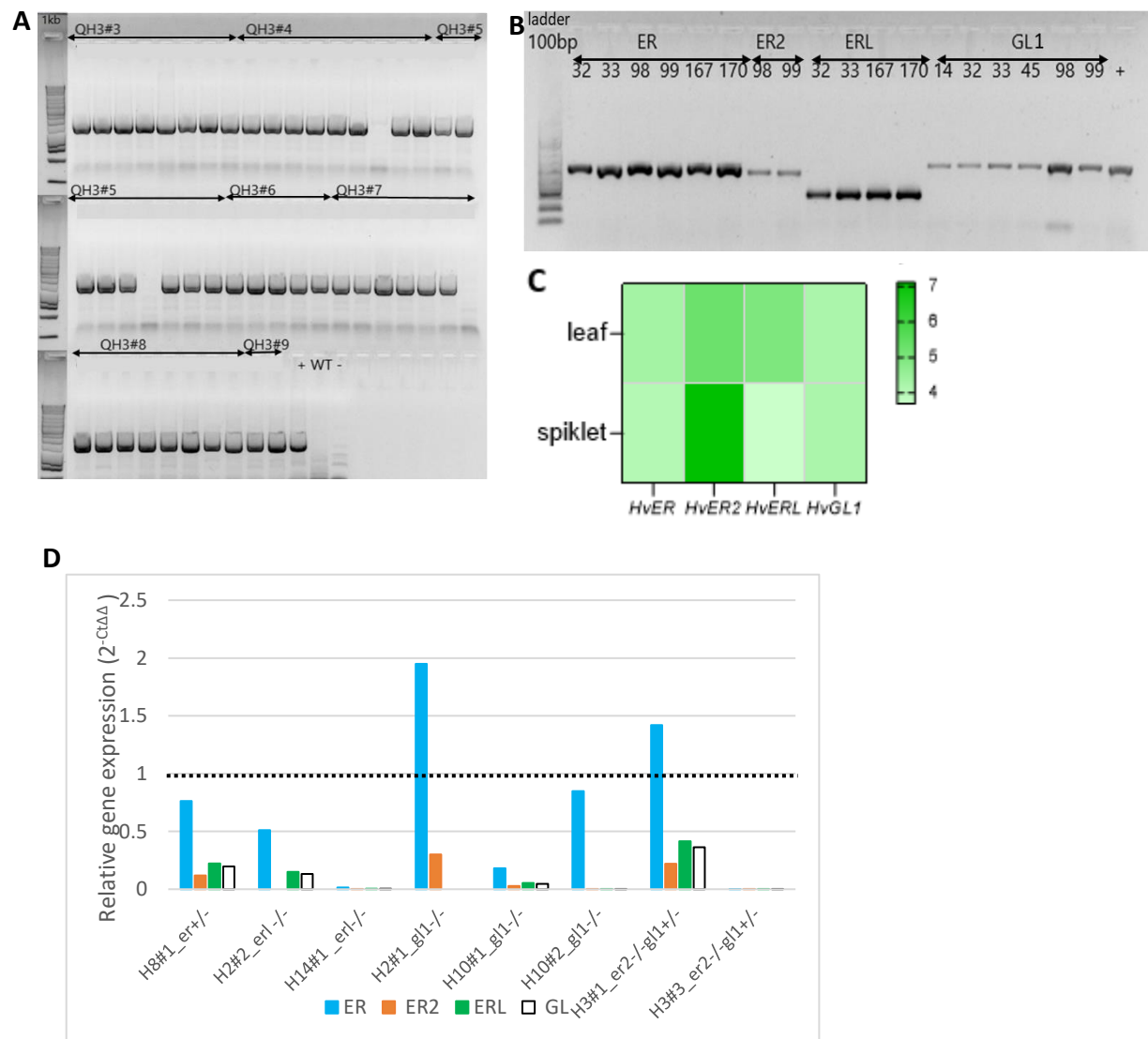


Figure 4-5. Screening for successful transformant edited for *HvERf* genes and *HvGL1* in barley. **A.** An example agarose gel electrophoresis image showing the amplified products of the *Hpt* gene (998 bp) in T₁ plants. WT (Golden Promise) was used as the negative control. Sample groups were labelled according to their plant ID (table 2). **B.** An example of *HvER*, *HvER2*, *HvERL*, and *HvGL1* fragment amplification with gene-specific primers for genotyping of T₁ *Hpt* positive plants. The T₁ plant numbers (as per Table 4-2) are shown in the figure. **C.** Relative gene expression (2^{-ΔCt} - normalised to reference genes) of *HvER*, *HvER2*, *HvERL* and *HvGL1* in WT-Golden promise young leaves and spikelets. **D.** An example of relative *HvER*, *HvER2*, *HvERL*, and *HvGL1* expression (2^{-ΔΔCt} normalised to reference genes and WT) of successfully edited T₀ plants. The reference line shows the expression level of WT. Anything above or below indicates a higher or lower expression than that of WT.

Table 4-2. -List of T₀ and T₁ transgenic CRISPR-Cas9 barley mutant lines successfully edited in *HvERf* / and *HvGL1*.

Construct ID	Targeted gene(s)	Generation	Plant ID	Genotype	Editions in target gene(s)	Mutation(s) position(as) in protein sequence (aa no)	Downstream effect on protein		Affected protein domain	Translated protein BLAST-hit(s) in protein database	
							Frameshift	Truncation (aa no)		Identity (%)	Query Cover (%)
Hv#2	ER+ERL+GL1	T ₀	H2#1	<i>gl1</i> bi-allelic	<i>gl1</i> bi-allelic:34bp DEL at 130; 3bp SUB at 172	aa58; aa44-55	—	133	DNA binding domain	no hit	no hit
		T ₁	H2#1.4	<i>erl gl1</i>	<i>erl</i> 13bp DEL at 217 <i>gl1</i> 2bp INS at 38 & 144	aa73-77 aa13-48	—	91 144	LRR-1 DNA binding domain	87 no hit	18 no hit
		T ₀	H2#2	<i>erl erl+/-</i>	<i>erl</i> 1bp DEL at 107; 13bp DEL at 216 <i>erl+/-</i> 1bp SUB (T to G) at 205	—	at aa 36	119	LRR-1	87	18
		T ₁	H2#2.11 H2#2.12 H2#2.13 H2#2.14	<i>erl gl1</i>	<i>erl</i> 1bp DEL at 107; 13bp DEL at 217 <i>gl1</i> 5bp DEL at 93	aa35; aa73-77 aa33- 35	— —	154 133	LRR-3 DNA binding domain	75 no hit	18 no hit
		T ₀	H2#4	NULL	<i>ERf GL1</i>	—	—	—	—	—	—
		T ₁	H2#4.32 H2#4.33 H2#4.34	<i>erl</i>	<i>erl</i> 13bp DEL at 217	aa73-77	—	91	LRR-1	82	17
Hv#3	ER+GL+ER2	T ₀	H3#1	<i>er2 er2+/- gl1+/-</i>	<i>er2</i> 1bp INS at 810 <i>er2 +/-</i> 2bp INS at 919 & 936 <i>gl1 +/-</i> 1bp INS at 155	aa270 (Glu-Asp) —	at aa307 at aa52	350 171	LRR-8 DNA binding domain	90 no hit	20 no hit
		T ₁	H3#1.38 H3#1.39	<i>er2</i>	<i>er2</i> 2bp INS at 918&936	aa306 & aa312	—	329	LRR-9	91	26
		T ₀	H3#2	<i>gl1</i> bi-allelic	<i>gl1</i> bi-allelic 1bp INS (C&T) at 144	—	at aa42	171	DNA binding domain	no hit	no hit
		T ₁	H3#2.46 H3#2.47	<i>er2 gl1</i>	<i>er2</i> 1bp SUB at 810 <i>gl1</i> 1bp INS at 144	aa270(Glu-Asp) —	— at aa48	— 171	LRR-7 DNA binding domain	91 no hit	26 no hit
		T ₀	H3#3 H3#4 H3#5	<i>er2 er2+/- gl1+/-</i> <i>er2 er2+/-</i>	<i>er2</i> 1bp INS at 810 <i>er2 +/-</i> 9bp DEL at 916 <i>gl1 +/-</i> 1bp INS at 147 <i>er2</i> 1bp INS at 810 <i>er2 +/-</i> 9bp DEL at 916	aa270(Glu-Asp) — aa270(Glu-Asp)	at aa305 at aa49 at aa305	345 171 345	LRR-8 DNA binding domain LRR-8	91 no hit 91	26 no hit 26
		T ₀	H3#9								
		T ₁	H3#9.95 H3#9.100	<i>er2</i>	<i>er2</i> 1bp SUB at 810	aa270(Glu-Asp)	—	—	LRR-7	91	26
		T ₁	H3#9.99	<i>er2 er2+/- gl1+/-</i>	<i>er2</i> 1bp SUB at 810 <i>er2 +/-</i> 1bs INS at 921 <i>gl1</i> 1bp INS at 144	aa270(Glu-Asp) —	at aa307 at aa48	328 171	LRR-8 DNA binding domain	35 no hit	28 no hit

Construct ID	Targeted gene(s)	Generation	Plant ID	Genotype	Editions in target gene(s)	Mutation(s) position(as) in protein sequence (aa no)	Downstream effect on protein		Affected protein domain	Translated protein BLAST-hit(s) in protein database	
							Frameshift	Truncation (aa no)		Identity (%)	Query Cover (%)
Hv#8	ER	T ₀	H8#1	er+/-	er+/- 1bp INS at 969	—	at aa323	346	LRR-11	no hit	no hit
		T ₁	H8#1.108	er	er 1bp INS at 969	—	at aa323	346	LRR-11	no hit	no hit
			H8#1.111								
			H8#1.112								
		T ₀	H8#7	NULL	ERf GL1	—	—	—	—	—	—
		T ₁	H8#7.121								
			H8#7.122								
			H8#7.124								
Hv#10	ER+GL1	T ₀	H10#1	g/l1	g/l1 18bp DEL at 90	aa30-36	—	133	DNA binding domain	no hit	no hit
		T ₁	H10#1.135 H10#1.136								
		T ₀	H10#2	g/l1	g/l1 3bp DEL at 89	aa29-30	—	133	DNA binding domain	no hit	no hit
		T ₁	H10#2.147 H10#2.148								
Hv#14	ER+ERL	T ₀	H14#1	erl	erl 1bp INS at 107; 1bp SUB at 146:1bp DEL at 150	—	at aa48	123	LRR-2	77	18
		T ₁	H14#1.153								
			H14#1.154								
		T ₁	H14#1.155	erl	erl 13bp DEL at 217 erl+/- 2bp INS at 121,126	—	at aa40	92	LRR-1	87	18
			H14#6								
			H14#6.166								
		T ₁	H14#6.169	erl	erl 13bp DEL at 217; 1BP INS at 158	aa53;aa73-77	—	149	LRR-3	75	18
			H14#6.173								

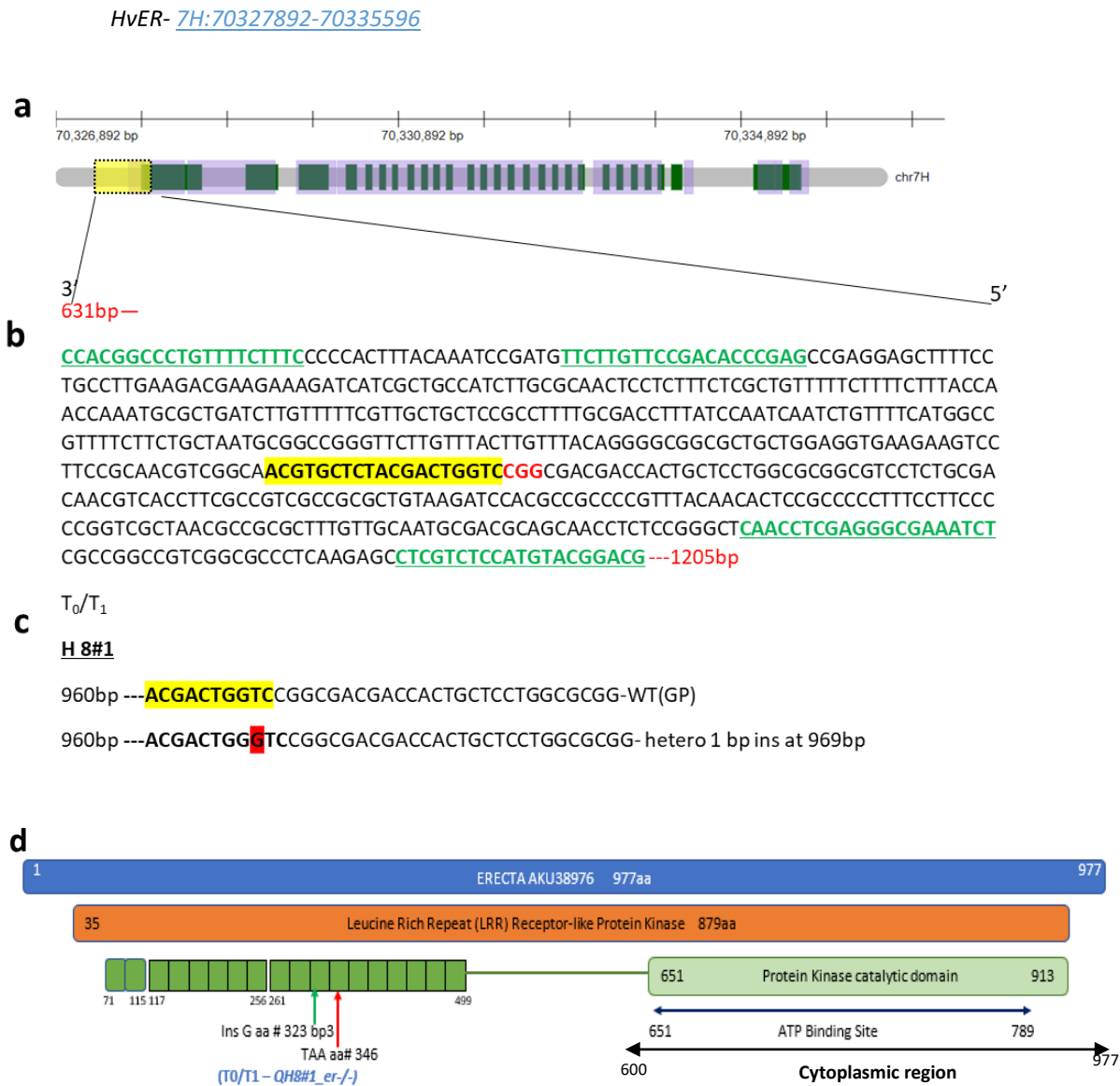


Figure 4-6. Gene structure (a); target region for CRISPR/Cas9 gene editing (b); gene edits and their positions in gDNA (c); and corresponding protein modifications in CRISPR/Cas9-edited *HvER*. **a.** Schematic diagram of *HvER* (*LOC123413216*) gene structure and the location of the target region (highlighted in yellow) for CRISPR/cas9 gene editing. **b.** Target region showing single guided RNA (sgRNA) target site of *HvER* (highlighted in yellow) with the protospacer adjacent motif-PAM (highlighted in red) and gene-specific primers (highlighted in green) for genotyping. **c.** 1bp insertion location in gDNA (highlighted in red) **d.** The protein product of *HvER* and the location of the edition in the protein.

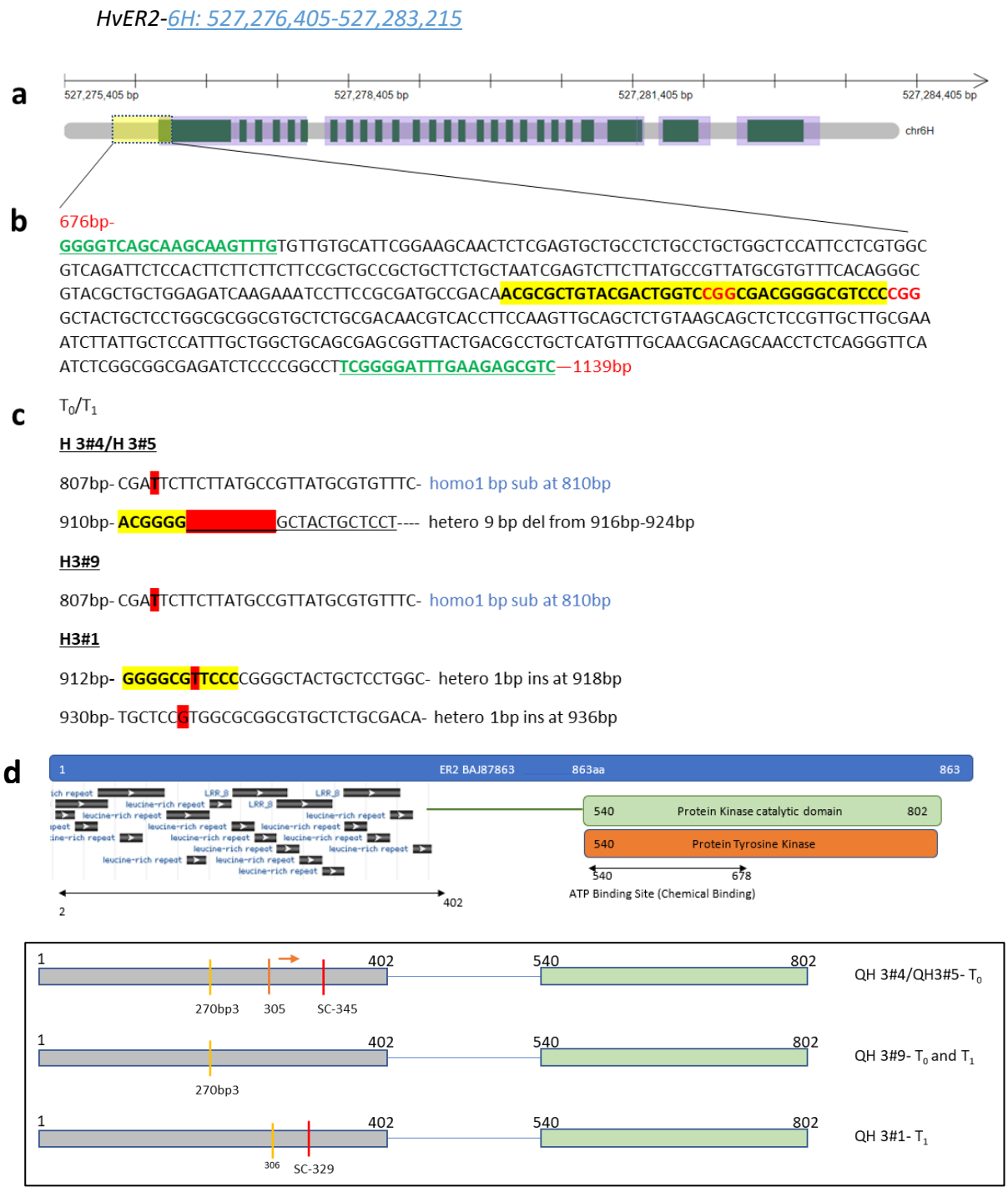


Figure 4-7. Gene structure (a); target region for CRISPR/Cas9 gene editing (b); gene edits and their positions in gDNA (c); and corresponding protein modifications in CRISPR/Cas9-edited *HvER2*. a. Schematic diagram of *HvER2* (*LOC123401964*) gene structure and the location of the target region (highlighted in yellow) for CRISPR/cas9 editing. **b.** Target region showing single guided RNA (sgRNA) target site of *HvER2* (highlighted in yellow) with the protospacer adjacent motif-PAM (highlighted in red) and gene-specific primers (highlighted in green) for genotyping. **c.** Insertions and deletions in the target region of *HvER2* (highlighted in red) according to the gene edits mentioned in (table2). **d.** The protein product of *HvER2* and the location of the edition in the protein for each edition. yellow line- point mutation, orange line-frame shift, red line -premature stop codon.

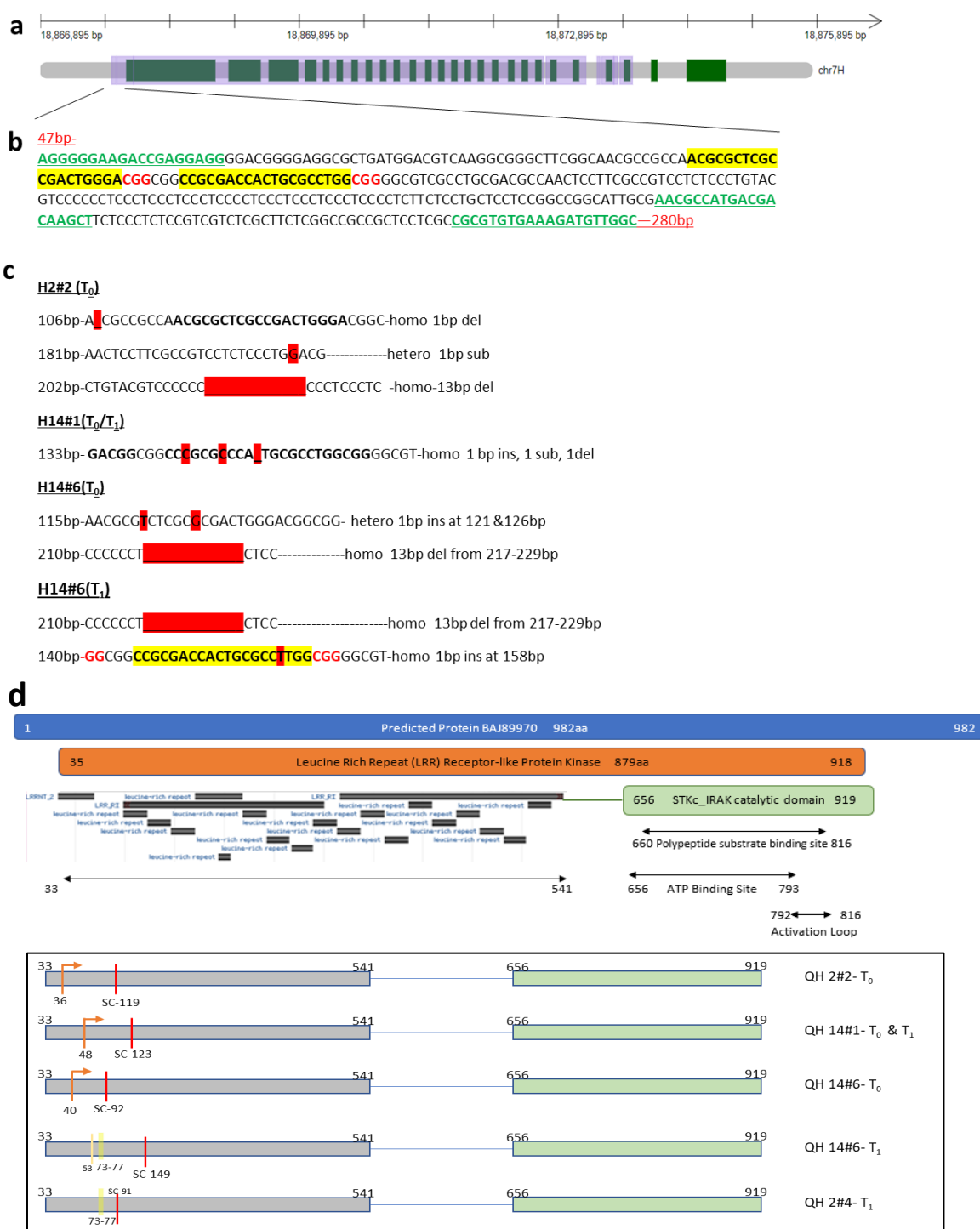
HvERL- 7H: 18,867,895-18,874,842

Figure 4-8. Gene structure (a); target region for CRISPR/Cas9 gene editing (b); gene edits and their positions in gDNA (c); and corresponding protein modifications in CRISPR/Cas9-edited *HvERL*. **a.** Schematic diagram of *HvERL* (*LOC123413545*) gene structure and the location of the target region (highlighted in yellow) for CRISPR/cas9 editing. **b.** Target region showing single guided RNA (sgRNA) target site of *HvERL* (highlighted in yellow) with the protospacer adjacent motif-PAM (highlighted in red) and gene-specific primers (highlighted in green) for genotyping. **c.** Insertions and deletions in the target region of *HvERL* (highlighted in red) according to the gene edits mentioned in (table2). **d.** the protein product of *HvERL* and the location of the edition in the protein for each edition.

HvGL1 -6H: 376,234,142-376,235,039

Figure 4-9. Gene structure (a); target region for CRISPR/Cas9 gene editing (b); gene edits and their positions in gDNA (c); and corresponding protein modifications in CRISPR/Cas9-edited *HvGL1*. **a.** Schematic diagram of *HvGL1* (*LOC123406059*) gene structure and the location of the target region (highlighted in yellow) for CRISPR/cas9 editing. **b.** Target region showing single guided RNA (sgRNA) target site of *HvERL* (highlighted in yellow) with the protospacer adjacent motif-PAM (highlighted in red) and gene-specific primers (highlighted in green) for genotyping. **c.** Insertions and deletions in the target region of *HvGL1* (highlighted in red) according to the gene edits mentioned in (table2). **d.** the protein product of *HvGL1* and the location of the edition in the protein.

4.3.3 The barley *ERECTA* gene family plays a role in vegetative and reproductive development

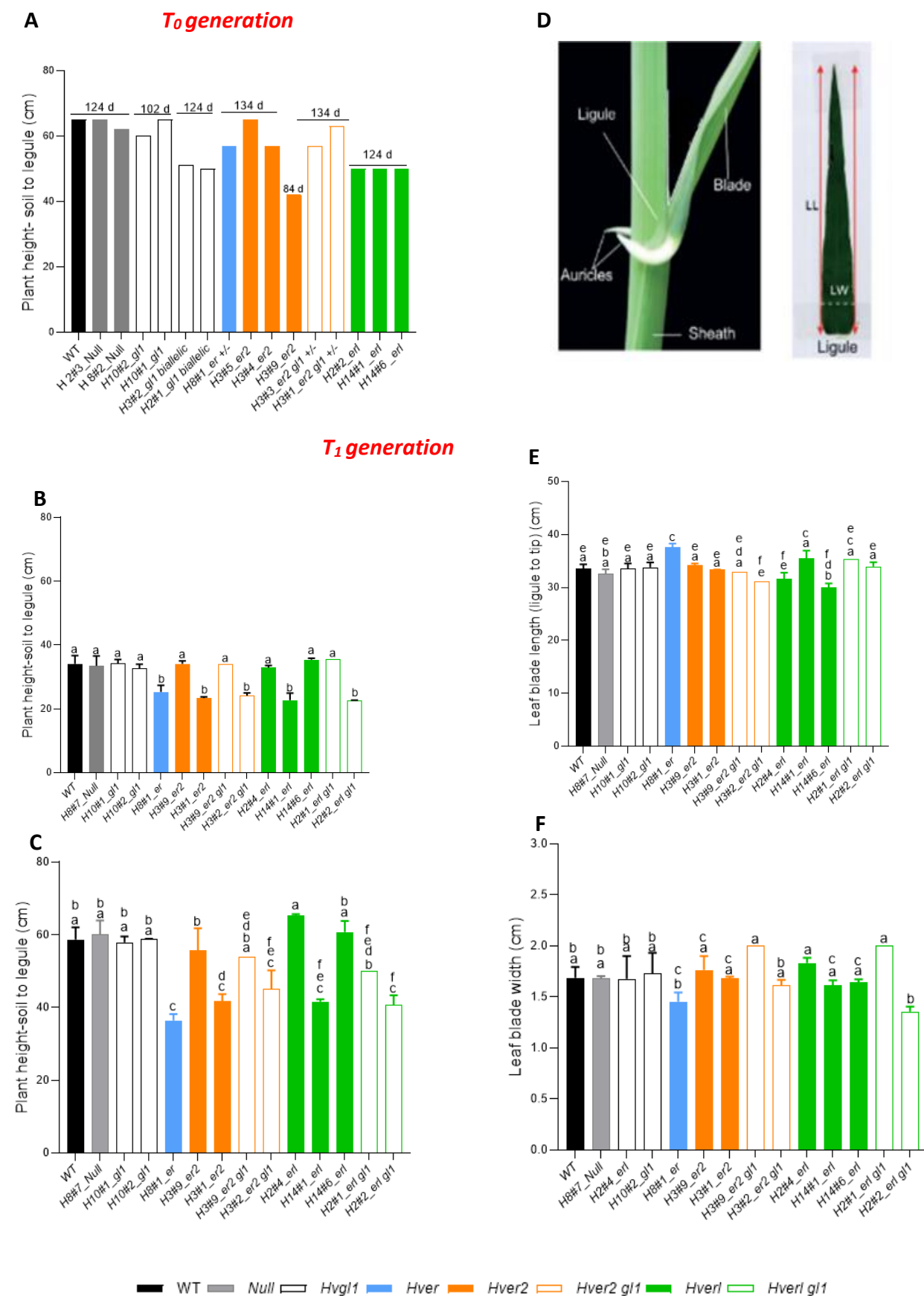
HvERf members are involved in regulating barley plant architecture

There are very few studies of the *ERECTA* family, apart from the Arabidopsis *ERf*. Most investigations have focused on the *ERECTA* member. They indicate wide conservation of *ERECTA* orthologues in promoting growth and organ elongation ([Zakhrabekova et al., 2015](#); [Y. Zhang et al., 2018](#); [Chen et al., 2024](#)) and provide some hallmark visual phenotypes typical of *ER* loss of function, in the first instance, reduced stature, more erect stem and leaf posture -two features which led to the *erecta* denomination given to the original Landsberg erecta discovered decades ago ([Redei, 1962](#); [Rédei, 1992](#)).

All *Hverf* and *Hvgl1* confirmed T₀ transformants and their derived T₁ progeny were phenotypically characterized for visual developmental and structural features associated with *erf* loss-of-function in Arabidopsis and other species and that could be scored without any damage or interference with plant function. It was indeed critical to maximize the chances that all transgenic lines survive and produce sufficient T₁ progeny for downstream characterization and propagation to T₂. It was also essential to obtain T₁ and derived T₂ progeny for minimizing the risks of confounding effects of tissue culture compromising reliable analysis of genotypic differences.

At the seedling stage (2.5 months old), there were only subtle genetic differences in general plant architecture and overall size, and in leaf posture among primary transformants. However, within a few weeks, some T₀ plants grew significantly shorter than WT and Null transgenics controls, such as *H 8#1_er+/-* and all lines T₀ edited in the *HvERL* gene (Figure 4-10 A; Supp figure 4-1; Table 4-2). These differences were conserved throughout vegetative growth up to maturity in the T₁ homozygous progeny, as illustrated by *H8#1.108*, *.110* & *.112* and *H14#1.153-155*, which carried the same editions in their *ER* and *ERL* receptor domain, respectively, than their T₀ mother plant (LRR repeat 11 and 2, respectively; Table 4-2 & Figure 4-10 B&C;G). Remarkably, knock-out of *HvER* alone, *H8#1* (T₀), *H8#1.108*, *.111*, *.112* (sister T₁s) induced severe plant dwarfism, and the quasi absence of stem elongation consistent (Figure 4-10 C&G) reminiscent of the phenotype induced by total arabidopsis KO mutant *er erl1 erl2* ([Torii et al., 2003](#); [Shpak et al., 2004](#)) and single *er* (*sler*) KO mutants in Tomato ([Chen et al., 2024](#)). However, *H 14#6_166*, *.169*, *.173* and *H2#4_32-34*, which carried different editions, and in LRR repeat 11 instead of LRR repeat 1 in *H8#1* lines (Table 4-2), were visually indistinguishable from WT (Figure 4-10 B&C). These results point to some functionally critical bases in the *ER* receptor, to be analysed in future studies. Moreover, they suggest that, contrary to Arabidopsis, *AtERL1* and *AtERL2*, the *HvERL* member is of significant functional importance on its own, as a positive regulator

of plant size, and hence might be functionally closer to the *AtERECTA* member than to its two *AtERL* paralogues.



G

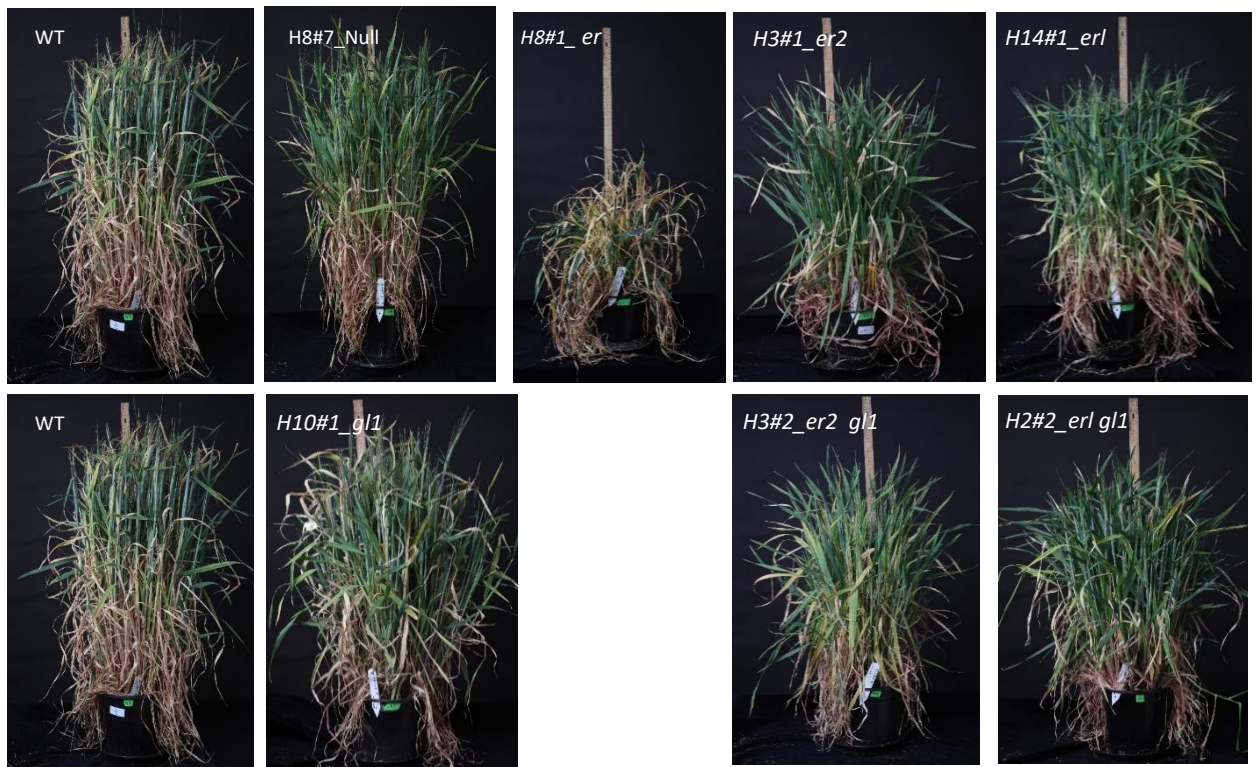


Figure 4-10. The barley *Erf* family regulates plant architecture (T0 and T1 generation). A, B, C. Average plant height (average distance between the soil surface and stem ligule for the main stem and two most advanced tillers cm) for the set of transgenic *Hver*, *Hver2*, *Hverl*, *Hvgl1*, *Hver2 gl1*, *Hverl gl1* in 2.5-months-old T0 (A), and T1 plants (B), and in mature 6-months-old T1 plants . (Each mutant lines were denoted by an identifier; for example, H2#1, H2 is the construct ID as in **Table 4-2**, and #1 denoted the independent transformant number in the T0 generation). (C). Plant height for each plant was taken as the average value of the distance from the top of the soil to the ligule of the youngest leaf, measured on the three most advanced tillers of $n \geq 2$ T1 plants from independent transformation events (mutated at the target site), and on segregating NULL transgenic plants with the same tissue culture history as the compared mutant lines, but WT genotype at *ERf* and *GL1* loci. (D), Depiction of a barley leaf, showing; sheath, blade, ligule and the two auricles. E-F. Blade length (LL), taken as the distance from ligule to blade tip (red arrow), and blade width (LW) measured at the widest point of the blade (dashed line) (illustration adapted and modified from (Shaaf et al., 2019)). Shown are averages of dimensions measured on adult leaves of 2.5 months old barley T1 transgenic *Hverf* and *Hvgl1*, WT and NULL lines, $n = 3$ leaves per plant. G, Representative images of T1 mature barley transgenic plants (6 months old) edited in *HvERf* and *HvGL1*. Statistically significant differences between the samples were determined by a one-way ANOVA using Fisher's LSD test, and are indicated by different letters. Error bars represent standard errors of the mean (s.e.m).

Homozygous *er2* T₀ mutant *H3#9* and its T₁ progeny were visually indistinguishable from Null and WT plants (Figure 4-10 B&C). Double mutant *H3#1_er2 er2+/- gl1+/-1* (Table 4-2) also looked similar to WT and NULL transgenic controls (Figure 4-10 A). However, in the next generation, derived T₁ homozygous *H3#1.38* and *H3#1.39* were found to be homozygous *er2* (Table 4-2), and both exhibited a significantly reduced height compared to controls. These observations indicate that *HvER2* also participate to the control of plant architecture, along with *HvERL*. No double *erl er2* mutants were obtained, so that a likely interaction between the *HvER2* and *HvERL* genes in that function remains an open question.

Homozygous editions of the *HvGL1* *H10#1* and *H10#2* (Table 4-2) had no detectable effect on plant size, stature and morphology, in either T₀ or T₁ generation. However, the biallelic *HvGL1* editions in *H3#2* and *H2#1* (Table 4-2) significantly reduced plant height in T₀ (Figure 4-10 A). These lines continued to be edited in T₁ for the other target genes, resulting in additional “sister” double mutants, homozygous *HvER2* and *HvGL1* mutants (*H3#2.46 & .47-er2 gl1*; Table 4-2). The *er2 gl1* double mutants *H3#2.46* and *H3#2.47* both showed a significantly reduced plant height compared to NULL controls, consistent with the already reduced stature of the *er2* single mutants (see above), while the *H3#9.99* double mutant *er2 er2+/- gl1+/-*, [biallelic *er2* (homozygous 1bp substitution in one allele, and heterozygous 1bp insertion on the other) and heterozygous *gl1+/-* mutation in *HvGL1*], were indistinguishable from WT and Null plants. These results indicate gene dosage effects of *GL1* and suggest a negative interaction between *HvER2* and *HvGL1* in the control of plant height and organ elongation, offsetting the positive regulation exerted by *HvER2*. Future experiments are required to test these hypotheses.

The *erl gl1* double KO mutants (Table 4-2) consistently showed significantly shorter plants compared to WT and Null (Figure 4-10 B&C), and also to a further reduction to the plant height in *H2#2_erlgl1* in T₁ compared to the parental *H2#2 erl* single mutant, indicating synergistic effects of *HvGL1* and *HvERL* in setting the plant architecture.

In summary, these data indicate that all three members of the *HvERf* family are involved in regulating plant stature and stem elongation, through partially overlapping / interactive mechanisms, similar to the well-documented such combined function of the Arabidopsis three *ERf* members. There was a consistent trend for all mutants with shortened or stunted stature to exhibit smaller leaves than GP and NULL controls (Figure 4-10 E&F), similar to Arabidopsis *erf* or *erf gl1* mutants, although the differences did not show statistical significance.

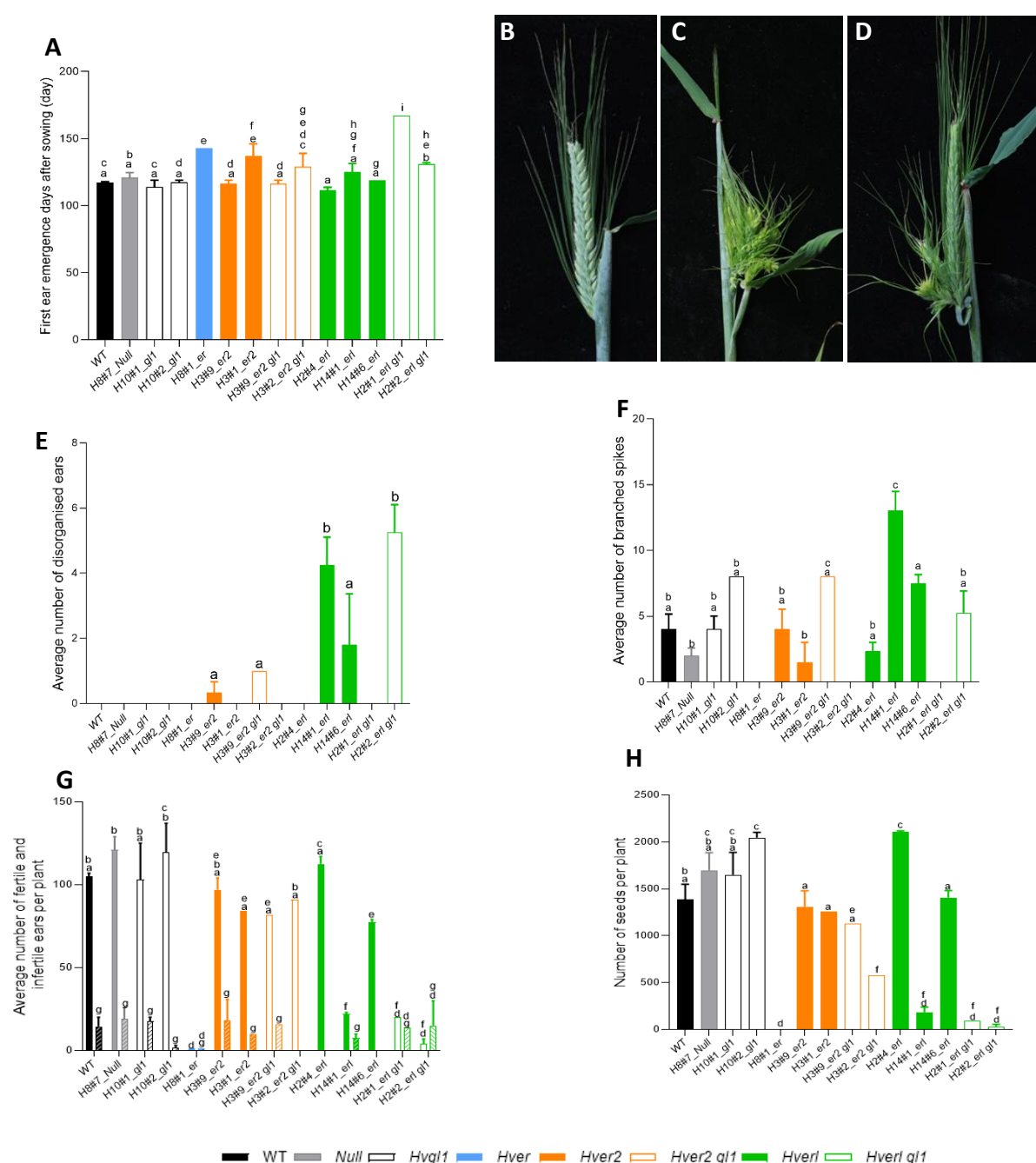
T₁ generation

Figure 4-11. The *Hverf* controls the timing of ear emergence, and ear architecture, in interaction with *HvGL1*.

A. Significant delay of ear emergence of one of the *Hver*, *Hver2* and *Hver1 gl1* editions (T_1 generation) from sowing compared to Null and WT. **B.** image of a fully formed ear of WT **C.** A representative image of a disorganised spike arrangement and twisted rachis of *Hverf* +/-GL1 (Image from line H14#1_er1). **D.** A representative image of a twisted and branched spike of *Hverf* +/-GL1 mutants (Image from line H14#1_er1). **E.** Average number of disorganised ears per plant **F.** Average number per plant of stems with non-canonical spike branching and twisted rachis. **G.** Total number of fertile (solid for *GL1* and empty for *gl1*) and non-fertile ears (dashed) per plant. **H.** Average total number of seeds per plant. Statistically significant differences between the samples were determined by a one-way ANOVA using Fisher's LSD test and are indicated by different letters. Error bars represent standard errors of the mean (s.e.m).

HvERf mutants show impaired reproductive development.

The Golden Promise (GP), used as WT in this study, is a two-row barley genotype. Its inflorescence is indeterminate (i.e., it doesn't have a terminal spikelet) and is characterized by the formation of three spikelets at each rachis node, each bearing a single floret. Only the central floret is fertile in two-row barley, while the two lateral florets are sterile ([Everard Hulton, 1919](#)). It is well established that the Arabidopsis *ERECTA* family is involved in the control of floral development and inflorescence architecture ([Shpak et al., 2004](#); [Woodward et al., 2005](#); [Pillitteri, 2007](#); [Bemis, 2013](#); [Shpak, 2013](#); [Chen et al., 2019](#)) and the *triple erf* mutant is sterile. In contrast, *gl1* alone or in combination with loss of *AtER* and /or *AtERL* signalling in the mutants generated in our lab did not show additional or more severe floral development defects than their *erf GL1* counterparts.

I therefore next examined differences in reproductive development among the above recovered set of T₁ *Hverf/gl1* mutant lines. All three T₀ independent *Hverl* mutants (*H14#1*), and the two *erl erf* +/- mutants *H2#2*, *H14#6*, both carrying 2 independent mutations (Table 4-2) consistently showed a non-canonical branching spike ([Stein & Muehlbauer, 2018](#)) (Figure 4-11 D) and a twisted rachis. The disorganized ear architecture in *H14#1_erl* led to a significant reduced number of ears per plant (66% reduction), ear and whole plant fertility (80% and 87% reduction in grain number per ear and per plant, respectively) (Supp figure 4-2 A& B). The *Hver2* T₀ lines produced WT-like ears, but in significantly reduced number (- 44%) leading to a similar, 50%, reduction in the number of grains per plant (Supp figure 4-2 C), indicating similar floret fertility as in WT/NULL plants in contrast with the partial sterility observed in *Hverl* mutants as described above. Homozygous *gl1* T₀ plants, *H10#1* and *H10#2* (Table 4-2), also showed normal ear morphology and fertility, comparable to WT and Null T₀ controls. Interestingly, however, the two independent primary transformants with a bi-allelic *gl1* mutation, *H3#2* and *H2#1* (Table 4-2), produced ears with a twisted rachis.

A significant delay of first ear emergence was observed in *er* and *er2* T₁ mutants *H8#1.108*, *.111* & *.112* and *H3#1.38* & *.39*, respectively (Figure 4-11 A) and in *erl gl1* double mutant *H2#1.4* compared to other lines and WT or Null plants (Figure 4-11A). Disorganized spikelet arrangement, twisted spike, and rachis were observed in *H3#9_er2*, *H3#9_er2 gl1*, *H14#1_erl* and *H14#6_erl*, *H2#2_erl gl1* mutants (Figure 4-11 E), in addition to some non-canonical spike branching (Figure 4-11 B-D, F). Partial to very severe sterility was observed in one *er2 gl1* mutant line (*H3#2.46* & *.47*) and the two independent *erl gl1* lines (*H2#1.4* and *H2#2.11*, *.12*, *.13*, & *.14*) as well as the *H14#1.153*, *.154*, & *.155_erl* mutant, respectively, due to a combination of a reduced proportion of tillers reaching flowering and of abnormal organization and reduced fertility of those tillers (Figure 4-11 E-H). The other two *erl* lines

(H2#4, H14#6), and the two independent *gl1* (H10#1, H10#2) or *er2* (H3#9, H3#1) lines were not significantly different in their reproductive development from WT/NULL controls. At the other extreme, the *er* mutant plants H8#1.108, 111, and 112 were all totally sterile, producing very few tillers, with none that produced any seed, and only 1 among those tillers that produced one single ear, of totally abnormal morphology and diminutive size.

These data indicate that the barley *ERf* plays a significant role in ear formation, spike and rachis structure, and floral development, and demonstrate specificity between the three family members in these functions, with a crucial role of *HvER* and a dominant role of *HvERL* compared to *HvER2*. Remarkably, knock-out of *HvER* is, alone, sufficient to not only severely restrict vegetative development (Figure 4-10), but also to completely abolish reproductive development and fertility in barley. The function of *HvER* in reproductive organ development is therefore much stronger than that of any of the three *AtERf* members in Arabidopsis, where extreme dwarfism, severely impaired floral development and total sterility such as in, is only observed in the total triple knock-out, *er erl1 erl2* ([Shpak et al., 2004](#)). The trend for *gl1* mutations to aggravate the negative effects of *er2* or *erl* mutations, suggest that both *HvERL* and *HvERL2* functions in reproductive development involve some synergistic interaction with *HvGL1*.

Altogether, the morphological and developmental characteristics of the above set of T₁ *Hverf* CRISPR-Cas9 edited mutants shared similarity with the hallmark features of documented *erf* mutants, but also specificity, and the interactive role of *HvERL*, and especially *HvERL* with *HvGL1*, strengthen the case for orthologous functions of that barley MYB transcription factor with *AtGL1*. The T₂ seed progeny of all T₁ mutants were collected, along co-segregating NULL lines, and WT to next probe the function of *HvERf* members and *HvGL1* in seed germination.

4.3.4 Barley *HvERf* is controlling seed germination sensitivity to NaCl

In barley, similar to Arabidopsis, germination is a two-step process. However, of a different kind: the coleorhiza, the sheath covering the radicle ([Bewley et al., 1994](#)), emerges first through the husk, breaking the palea-lemma at the micropylar end; in a second step, the radicle ruptures the coleorhiza and becomes directly exposed to the external environment and prevalent environmental conditions, ahead of the coleoptile and the enclosed first leaf. I thereafter refer to the first germination step as Coleorhiza Emergence (CE), and the second step as Coleorhiza Break (CB) (Figure 4-12 A).

An involvement of the HvERf in constitutive germination rate

Germination assays were carried out using T₂ seeds from two independent transformant events, with co-segregating NULL control lines and WT Golden Promise seeds harvested from plants grown together with the set of T₁, in the same growth chambers. The lines used in the first assay were WT, Null, *H3#9_er2*, *H3#9_er2 gl1*, *H2#4_eri*, *H2#2_eri gl1*, and *H10#2_gl1*. The two germination steps, CE and CB, were recorded (Figure 4-12A). On control media (0mM NaCl), the first seeds with Coleorhiza Emergence (CE) were observed 16h after sowing for all the *Hv erf* KO lines, WT-GP and Null lines (1-7% seeds with CE at that time, in all the lines). CB was observed 8 to 12h later in all lines, i.e., 24-28h from sowing. However, *H3#9_er2* took a significantly longer time (‘40h-CE and ‘65h -CB) to reach 50% of seeds with CE and CB, respectively, compared to ‘25h and ‘35h in WT, Null, and *H2#4_eri*, respectively (Figure 4-12F&G), and the maximum germination rate was 30% lower than in controls. In addition, while initially progressing at similar rates as in controls, germination of *eri* and *eri gl1* was markedly slower than in WT and NULL lines, and plateaued about 30h later, however, reaching 90 and 80-85% respectively, i.e., 10 -15% lower values than in WT and NULL controls. Similar results were obtained with a second set of independent lines (Supp figure 4-3 A& B). Overall, these data indicate that *HvER2* acts as a positive regulator of constitutive germination rate, and suggest perhaps some role of *HvERL* too (Supp figure 4-3 A-B), but weak and only in a *gl1* background. *HvGL1* had no effect on its own on final germination rates, although tending to slightly reduce germination speed. The importance of the third *HvERf* member, *HvER*, remains unknown due to the quasi-total sterility of *Hver* mutants (only 3 seeds available in T₂).

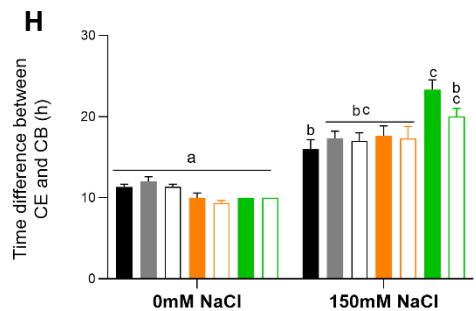
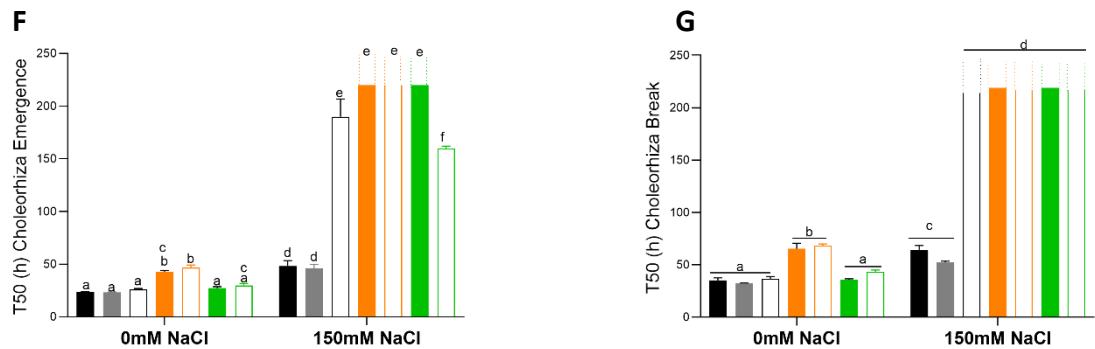
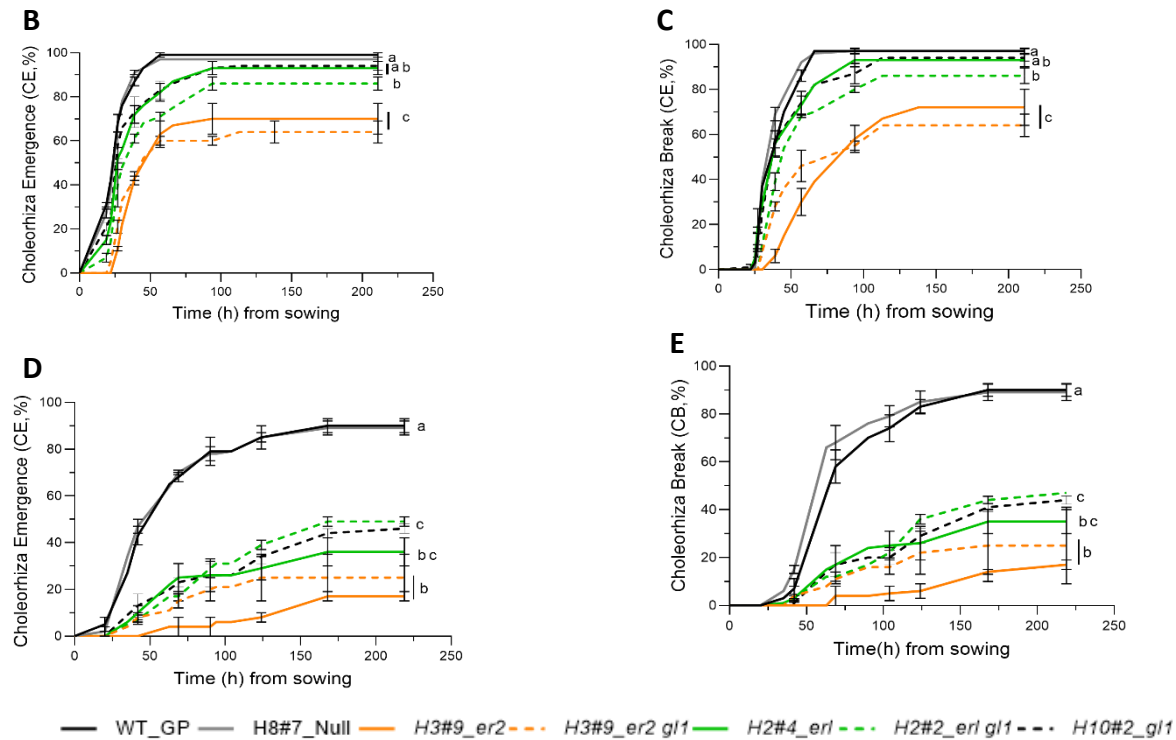
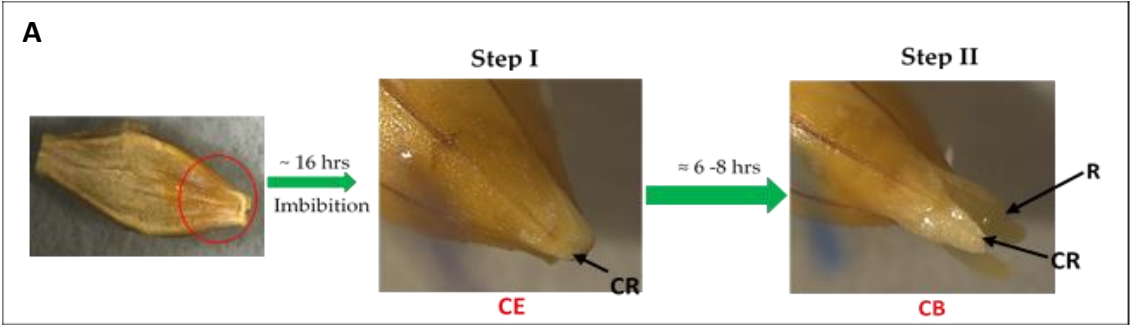


Figure 4-12. : Barley *HvERf* and *HvGL1* necessary to promote germination under salinity stress **A.** Two germination steps, Coleorhiza Emergence (CE) and Coleorhiza Break (CB), CR-Coleorhiza, R- Radicle. **B & C.** Coleorhiza emergence (B) and coleorhiza break (C) of *Hverf* +/- *GL1*, WT and Null under optimal conditions (0mM NaCl) showing the constitutive effect of *Hver2* and *Hver2 gl1*. **D & E.** Coleorhiza emergence (D) and coleorhiza break (E) of *Hverf* +/- *GL1*, WT and Null showing salt hypersensitive germination phenotype of *Hverf* +/- *GL1* that significantly reduces the germination rate and significantly increased the germination delay compared to WT and Null. Significant differences were calculated only at the maximum germination rate. Statistically significant differences between the samples were determined by a one-way ANOVA using Fisher's LSD test and are indicated by different letters. Error bars represent standard error of the mean (s.e.m) **F&G.** T₅₀ values (time(h) taken to reach 50% of CE or CB) for CE (F) and CB(G). **H.** Time interval between the CE and CB. The time interval between the two event was follow individually in each sample and each replicate and average was taken. The experiment was repeated with two independent transformant events and similar results were obtained. Data are means(+/-SE) from n=3 plates with 30 seeds per genotype per plate. Statistically significant differences between the samples were determined by a two-way ANOVA using Fisher's LSD test and are indicated by different letters. Error bars represent standard error of the mean (s.e.m).

HvERf family and the HvGL1 MYB transcription factor are necessary to promote barley seed germination under salinity.

In preliminary experiments, I had examined WT and Null germination responses on 100mM, 150mM, 200mM and 250mM NaCl media along with control 0 mM NaCl in order to select the ascertain the most appropriate NaCl concentration to apply in future germination instead of blindly the same concentration as in the germination assays conducted with Arabidopsis seeds (Chapter 2; and Nada et al. 2019). Germination was scored every 6 hours over a 10-day period. Seed sowed in 0mM, and 100mM NaCl media showed a 100% final germination rates. However, in the presence of 150mM NaCl, germination was significantly delayed, and 10 Days post-sowing (DPS) about 20% of the seeds had still failed to germinate (Supp figure 4-4). On 200mM and 250mM NaCl media, this percentage raised to 40% and 60%, respectively (Supp figure 4-4). I then chose 150mM NaCl concentration to probe the *Hverf/gl1* mutant seeds' germination sensitivity to salinity, to maximize the chances that enough would germinate for reliable tests of statistical significance of results.

WT, Null and *Hverf* +/- *HvGL1* seeds were germinated on the Whatman 1 filter imbibed with 150mM NaCl, concurrently with the seeds described above on 0mM NaCl (Figure 4-12A&B; Supp figure 4-3 A&B), and were individually scored every 3h, starting from 12 hours after sowing. A significant delay in germination under salt stress was observed in all the lines, including WT and Null. WT and Null seeds shared similar germination kinetics and final germination rate, while the germination of all *Hverf* and *Hverf gl1* mutant seeds was drastically held back, with more than 40% seeds still not germinated at 10DPS (Figure 4-12D-H). *er2* and *er2 gl1* exhibited the lowest maximum germination rates (10-20%), below those of *er1* and *er1 gl1* seeds (40-50%), all well below the 90% reached by the WT and NULL cohorts (Figure 4-12D-H). *Hvgl1* alone also drastically reduced germination speed and final

germination rates, and as severely as when combined to *erl*. The hyper-sensitivity to salinity compared to WT was observed for the two germination steps (CE and then CB), in all mutants (Figure 4-12 F-G), but in for *gl1* and *erl gl1*, was amplified during the second phase leading to CB (coleorhiza breakage). Similar results were obtained with an independent set of lines (Supp figure 4-3 C-F), carrying different editions (Table 4-2), with however a lower sensitivity to the salt of *gl1* seeds in this second set compared to the first.

These results demonstrate that, as in Arabidopsis, the barley *HvER/ERL* family promotes seed germination under salt stress, with an involvement of both its *HvERL* and *HvER2* members. The implication of *HvER* too is still to be resolved as is the question of interactive effects between the 3 genes, as no *er* seeds and no double mutant lines were recovered other than *Hver2 gl1* and *Hverl gl1*. The significant promoting role of *HvGL1* even in presence of functional *ERf* members is remarkable, and contrasts with the similar germination kinetics of *gl1* and WT seeds observed in Arabidopsis (Chapter 2).

4.3.5 Total or partial resumption of germination upon lifting salinity stress

I next focused on the large proportion of *Hverf/gl1* mutant seeds that failed to germinate on saline medium, to determine whether prolonged exposure to salinity had irremediably compromised their viability. Those seeds were all transferred to a salt-free medium at 10DAS, well after germination rates had reached a plateau for all lines (Figure 4-12 D, E), along non-germinated WT and NULL seeds. WT and Null seeds all germinated within 12h of lifting salt stress; all *er2* and *gl1* seeds too, with an increased time-lag, of 36 and 56h, respectively (Figure 4-13 B). In the *erl* and *er2 gl1* lines, however, only 67% and 86% of seeds had germinated 48h after transfer to non-saline media, and these percentages plateaued thereafter (Figure 4-13 B). I could not obtain data for *Hverl gl1* due to limited seed availability. These results show that salinity delayed or halted germination, but in all or a large proportion of seeds depending on genotype, without damaging them to the point of causing loss of viability. They also indicate specificity between the 2 *ERf* members tested, in either the extent of damage or/and in the mechanisms underlying germination, with interactive effects of *GL1*. This is similar to the responses observed in the Arabidopsis *erf/erf gl1* salt-hypersensitive mutant (Chapter 2);(Nanda et al., 2019), with however a trend to an increased spread of germination resumption.

I next asked: Was the fraction of *Hverl* and *Hver2 gl1* seeds that failed to germinate even once transferred to non-saline medium not viable anymore or, if they were still viable, what prevented them from germinating? Secondly, in those seeds that did germinate after salinity stress release, how far had germination progressed, if at all, before being halted by salt?

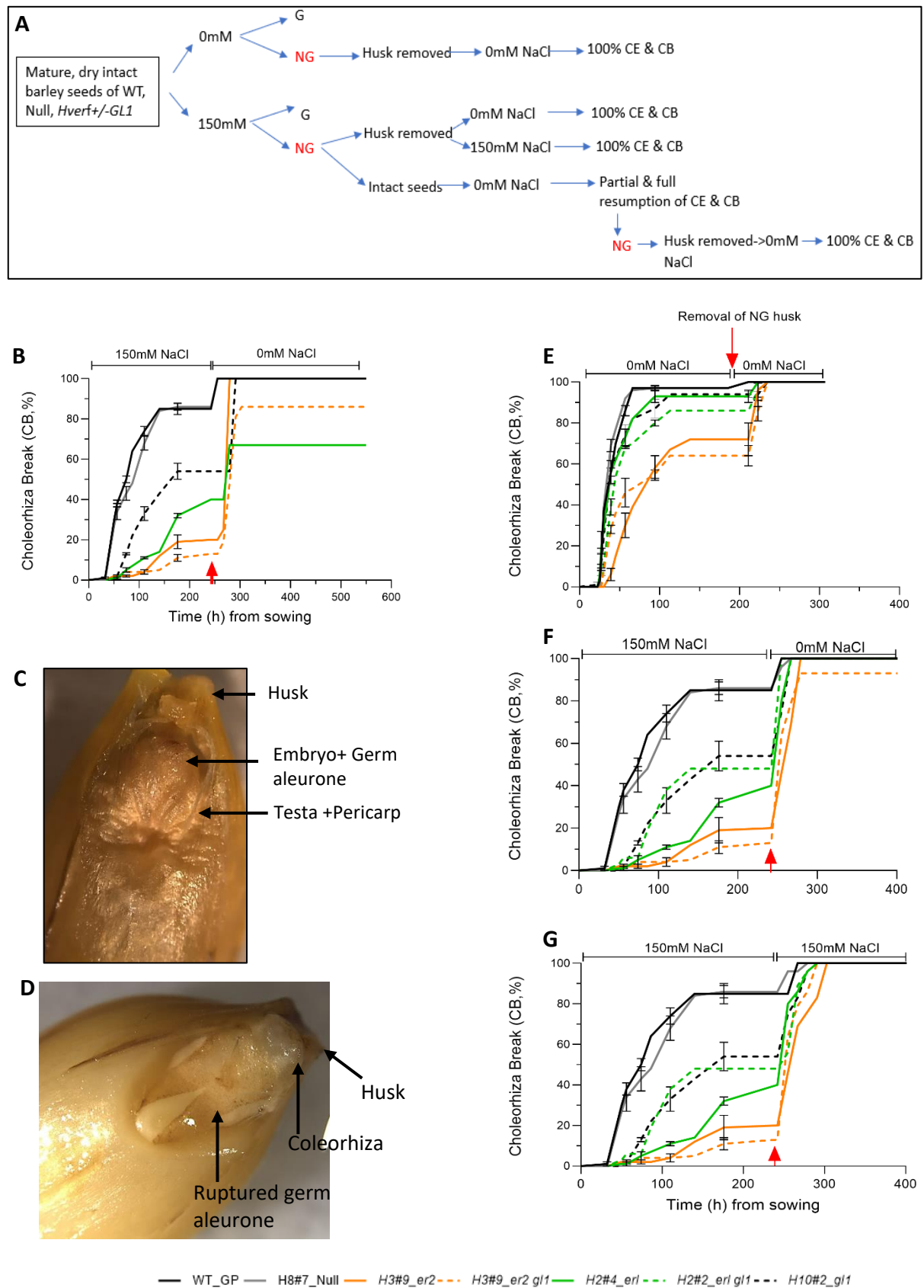


Figure 4-13. Germination arrest in *Hverf+/-gl1* seeds is primarily mediated by maternally derived husk. **A.** Decision tree illustrating the experimental design used to assess the impact of NaCl concentration on germination of *Hverf+/-gl1*, Null and WT seeds. NG-Non-Germinated, G-germinated, CE-Coleorhiza emergence, CB- Coleorhiza break. **B.** Partial and full resumption of the intact, NG seeds (in 150mM NaCl) when transfer to 0mM NaCl. **C.** Embryo of a dry mature seed (after 30mn imbibition), as a reference for dried embryo to compare with image D. **D.** A representative image of NG seeds at the time of husk removal (t₀), after 250h exposure to 150mM NaCl. The coleorhiza had elongated through all the inner tissues (germ aleurone, testa and pericarp) up to the lemma-palea binding junction. **E,F&G.** Resumption of germination of NG seeds in 0mM NaCl (**E**), AND 150mM (**F&G**) after removal of husk and transfer to 0mM (**E&F**) and 150mM (**G**) respectively. The red arrow indicates t₀ of husk removal of NG seeds. n=3 plates with 30 seeds per genotype per plate. All the NG seeds in each replicate were pooled to observed the resumption of germination due to low seed availability. *Hver1 gl1* were not included in graph B due to limited seed availability.

4.3.6 The maternally derived husk plays a key role in the regulation of *Hverf +/-GL1* seed germination under salinity stress

In Arabidopsis, the *ERF*-mediated function on seed germination is under maternal control, primarily through the testa/seed coat, a fully maternal tissue (Chapter 1). The seed mucilage, which is secreted from the outermost layer of the testa, was found to play a major role in this (Nanda et al. 2019; Chapter 1). The barley caryopsis is very different from the Arabidopsis seed, in its structure, organization and biochemistry, as previously described, and does not release mucilage upon imbibition as occurs in Arabidopsis and other species. Two endosperms, carrying two copies of the maternal genome and one copy of the paternal genome (♀♀♂), the starchy endosperm that takes up most of the seed cavity, and surrounding it and the embryo, the live thin aleurone layer. The testa/seed coat is thin two-cell layer of maternal origin (♀♀), is externally covered by the pericarp (♀♀) and highly suberized externally fused lemma and palea envelops (♀♀), collectively known as “husk” (Figure 4-1).

I developed a protocol to remove the husk, without damaging the adherent underlying tissues and carefully dehusked the full sets of WT/NULL and mutant, which had failed to germinate after transfer to non-saline conditions in the above experiments, and also of the non-germinated seeds that were kept on 150mM NaCl as controls. All dehusked seeds were carefully examined under the binocular at the time of dehusking to determine how far the germination process had progressed, if at all, before being halted and all were then transferred to a salt-free medium.

At the time of dehusking, 100% of WT, Null and *Hverf +/-HvGL1* seeds exhibited coleorhiza elongation (CElong) through all inner surrounding maternal tissues, germ aleurone, testa and pericarp, up to the outer-most husk, except for *Hver2gl*, which had about 80% CElong through all the inner tissues at the time of dehusking (t₀). (Compare Figure 4-13 C and D and Supp figure 4-5 A)

Coleoptile elongation (CleopE) within the husk through all underlying inner tissues at the time of dehusking was less advanced at t₀ for all mutant seeds, being observed in 65%, 40% and about 20% of

er2 gl1, *er2*, *erl* or *erl gl1*, and *gl1* seeds, respectively (Supp figure 4-5). Within 24h of transfer to a salt-free medium, 90-100% of dehusked seeds, whether mutant or WT/ Null, showed coleorhiza rupture by the radicle as well as primary and seminal roots elongated through the broken coleorhiza (Figure 4-13 F); and the coleoptile too had elongated through the inner testa and pericarp. These results show that prolonged exposure to salinity prior to seed transfer to salt-free medium had not caused irreversible damage to the inner living tissues of the seed, but rather that the husk was an impossible barrier to germination proceeding to completion, constitutively (Figure 4-13 E) and even more so under saline conditions, and through mechanisms involving the three genes tested, *Hv ER2*, *Hv ERL* and *Hv GL1*.

This led me to the question: what biophysical properties of the husk are involved in its *ERf-GL1*- and environment dependent roles in protecting the radicle from coming in direct contact with the external solution? Or do the above results reflect a “weaker” embryo in the mutant seeds, reducing the coleorhiza and enclosed radicle to develop sufficient turgor to break through the husk, regardless of any changes to the husk itself? Or could a combination of both factors be involved?

Several hypotheses could explain the slower germination of *Hverf +/-GL1* mutant lines, such as **a)** Water/solute availability for the embryo and within the endosperm during germination to carry out biochemical reactions. **b)** The properties of the coleorhiza or the shoot apical meristem (SAM) of primary and seminal roots may either increase or weaken the tissue. **c)** The properties of the pericarp and/or the cementing layer, where the thickness of the cementing layer can influence the proper adhesion of the husk to the caryopsis and/or its permeability. **d)** Changes in abscisic acid (ABA) and gibberellic acid (GA) within the coleorhiza. **e)** Low starch mobilization results in lower sugar availability for the growth of root and shoot primordia during germination due to reduced starch deposition during grain filling. **f)** changed molecular signalling between the husk and coleorhiza and root, or between the coleorhiza and root, aiding the latter to break through the coleorhiza and subsequently open the husk, perhaps without the need for differing interlocking strengths.

This highlights the complex interplay of factors affecting germination in the *Hverf +/-GL1* lines, providing a foundation for further investigation.

Considering the first question: Are the *ERf* and *GL1* interact to promote husk permeability to water and solutes during imbibition, especially in a saline environment?

4.3.7 The solute uptake during the imbibition and germination progress has a minimum/no role in controlling *HvERF1-GL1*-induced germination phenotype

Water/solute uptake curves during germination show an initial phase of rapid uptake during the first 10 hours of imbibition, followed by a period of gradually decreasing rate ([Baker, 1905](#)). Several studies of water movement during barley grain have demonstrated that water first moves to and within the maternal surrounding layers below the hull before redistribution to the internal tissues (aleurone layer, scutellum, and starchy endosperm) after about 1 hour of hydration ([Baker, 1905](#); [Brookes, 1976](#); [McEntyre et al., 1998](#)). The embryo itself is preferentially hydrated ahead of the surrounding tissues ([Brookes, 1976](#)) and, due to its rapid hydration, could be the first source of hydrolytic enzymes during the early stage of germination for reserve degradation and cell wall weakening in the maternal envelopes ([McEntyre et al., 1998](#)). The micropylar region, which appears as a brown stretch along the ventral furrow (crease) ([Briggs, 1978](#); [Collins, 2003](#); [Briggs, 2012](#)) where the cementing layer does not deposit (around the embryo), and the modified testa there are thought to allow the passage of most water during necessary to the hydration of the barley caryopsis ([Brookes, 1976](#)).

To compare water uptake kinetics among WT and mutant lines under optimal and salinity stress, I considered 4 phases: phase I (0-5h, rapid water uptake phase I), phase II (5-10h, rapid water uptake phase II ([Brookes, 1976](#))), Phase III (10-20h, Baker-Dick break ([Brookes, 1976](#))), Phase IV (beyond 20h, steep-ripe plateau ([Brookes, 1976](#))) (Supp figure 4-6). Results showed average water contents of 17mg and 20 mg at CE and CB, respectively, in WT and Null seeds. The average seed water content relative to seed initial dry weight (see methods) (mg, H₂O) in mutants seeds was not significantly different to WT and Null, at either CE or CB, suggesting that *Hverf* or *gl1* mutations did not significantly alter the seed total holding capacity for water at either of the two-germination step. However, uptake kinetics showed some genotypic effects (Figure 4-14 A). Statistical analysis of rates of water uptake over 0-1h, 3-5h, 7-10h and 12-16h time windows showed a higher initial water uptake in *gl1*, and consistently lower uptake rates in *erl* and *er gl1* compared to other lines, apart from the 8-10 first hours (Figure 4-14 A). However, even when statistically significant, these differences were small and no more significant when considered relative to initial seed weight (Figure 4-14 C&D), i.e., accounting for genetic variation in seed sizes (Figure 4-15 A, B), the reduced average size *er*, *er2* and *er2 gl1* compared to other lines), and also, shows no different in the water content at a given time between the germinated and non-germinated seeds.

On 150mM NaCl media, rates of solute uptake tended to be higher during the initial the 0-1h time window than under 0mM NaCl, for all lines, but the difference only reached statistical significance for *Hver2* and *Hver2 gl1*, and remained small. There was no consistent genetic effect, except for the lower

relative water content at the 48h and 72h time points, of WT and NULL seeds that still had not germinated (Figure 4-15 E-F). Overall, these results suggest that *HvER2*, *ERL* and *HvGL1* had no significant impact on water or saline solution uptake rates and that differential rates of water or saline solution between the salt hypersensitive mutant seeds and WT/NULL were not the cause of their drastically reduced germination rates on 150mM NaCl.

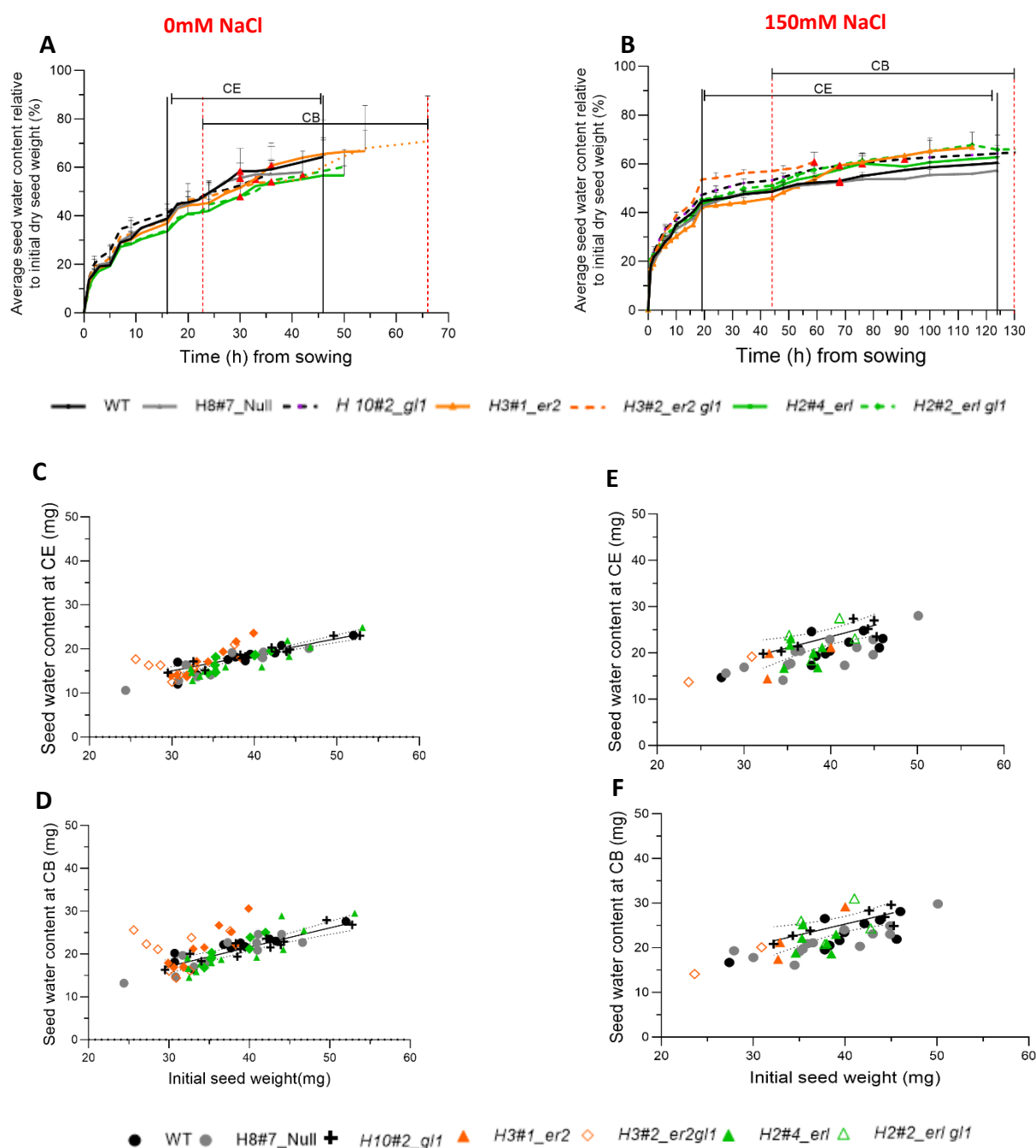


Figure 4-14. Water uptake kinetics of *Hverf+/-GL1* mutants under control (A) and 150mM salt medium (B). The data shown is only for the germinated seeds, and the red symbol represents the final average water content of all the germinated seeds within that replicate. **C&D.** seed water content at coleorhiza Emergence-CE (C) and Coleorhiza break-CB (D) of the seed imbibed in 0mM NaCl as a function of initial seed weight **E&F.** seed water content at CE (E) and CB (F) of the seed imbibed in 150mM NaCl as a function of initial seed weight. Experiment was repeated two time. n=15.

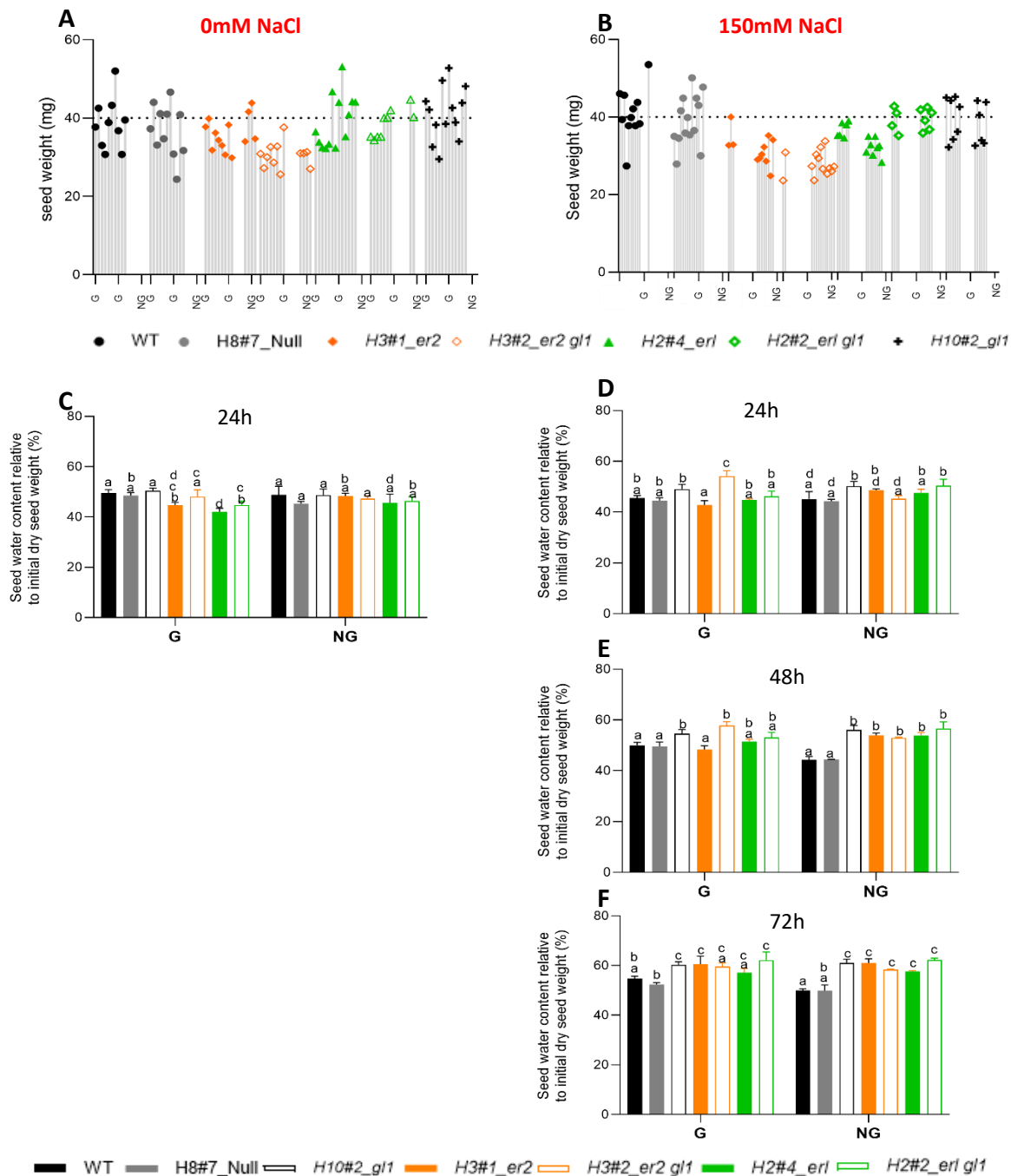


Figure 4-15. No genetic effect in water content of germinated (G) and non-germinated (NG) seeds under both control and 150mM NaCl stress. A & B. Initial seed weight of G and NG seeds used in control medium (A) and 150mM NaCl medium (B). **C.** Seed water content relative to initial dry seed weight (%) of G and NG seeds after 24h imbibition in 0mM NaCl (control) medium. **D-F.** Seed water content relative to initial dry seed weight (%) of Germinated (G) and non-germinated (NG) seeds after 24h (D), 48h(E), 72h(F) from imbibition in 150mM NaCl medium. Experiment was repeated two time. $n=15$. Statistically significant differences between the samples were determined by a two-way ANOVA using Fisher's LSD test and are indicated by different letters. Error bars represent standard error of mean (s.e.m).

4.3.8 The *HvERf*- alters the stomata and trichome density gradients in the husk epidermises

Results in Chapters 2 and 3 demonstrated that in *Arabidopsis*, the *ERf* and *GL1* functions on seed germination were maternally determined and indicated that both the *ERf* and *GL1* exerted wide-transcriptional control on genes involved in the synthesis, deposition and lysis of cell wall components and mucilage pectins, as well as of the seed coat-derived endosperm-associated cuticle. The critical role of the barley husk in the *HvERf-GL1* mediated functions on the germination of the enclosed seed shown above in this Chapter also pointed to predominant maternal effects. I thus next speculated that *HvERf* and *GL1* might promote germination via modifications of the anatomical and structural characteristics of the husk and underlying maternal pericarp and testa. WT (GP) and one of the salt-hypersensitive mutant *Hver2* were chosen to investigate that hypothesis.

The patterning of the husk outer epidermis was consistent with descriptions in the literature (Figure 4-16 A&C; Figure 4-17 A&B) in both WT and *Hver2* seeds, showing rows of long epidermal cells, running parallel to the long axis of husk, and separated by deep ridges. Two types of microstructures were visible on the epidermis of both the lemma and palea, as reported ([Boateng et al., 2007](#)), and referred to as papillae trichomes and “stomata” although there is no evidence of any role of these in gas exchange during seed development, and in the mature husk, their central “pore” is blocked by a plate-like structure that has never been further described (Figure 4-16& Figure 4-17; ([Boateng et al., 2007](#))). The silicious bases of these two types of micro-structures, so-called “papillae trichomes “ and “stomata”(Boateng et al., 2007), and silicious depositions at the junctions of the epidermal cells, are globally referred to in the literature as phytoliths or “plant-rocks” ([Lentfer et al., 1997](#); [Ball et al., 1999](#); [Boateng et al., 2007](#)).

The papillae trichomes presented a large solid base with a small outward pointing swollen peak, apart exceptionally where instead of that peak, an elongated trichome was visible near the brush end of the lemma and palea in both genotypes. The stomata-like structures had rounded bases and a central pore, which in many cases had lost its obstructing “plate” ([Boateng et al., 2007](#)) during sample preparation (Figure 4-16 C & D and Figure 4-17 B & C). The two types of microstructures were randomly distributed along the epidermal row but with a density gradient along the seed axis, from the embryo end to the brush end. The stomata-like structures were highly abundant near the embryo end, and gradually becoming less and less frequent toward the brush end, whereas the papilla trichomes density along epidermal rows showed an inverse gradient (rare near the embryo end and gradually more and more frequent towards the brush end) (Figure 4-17 E-J). In addition, 2-3 files of stomata with two dumbbell-shaped guard cells typical of grass stomata were visible on the ventral rows of the palea (Figure 4-17 D) in both genotypes.

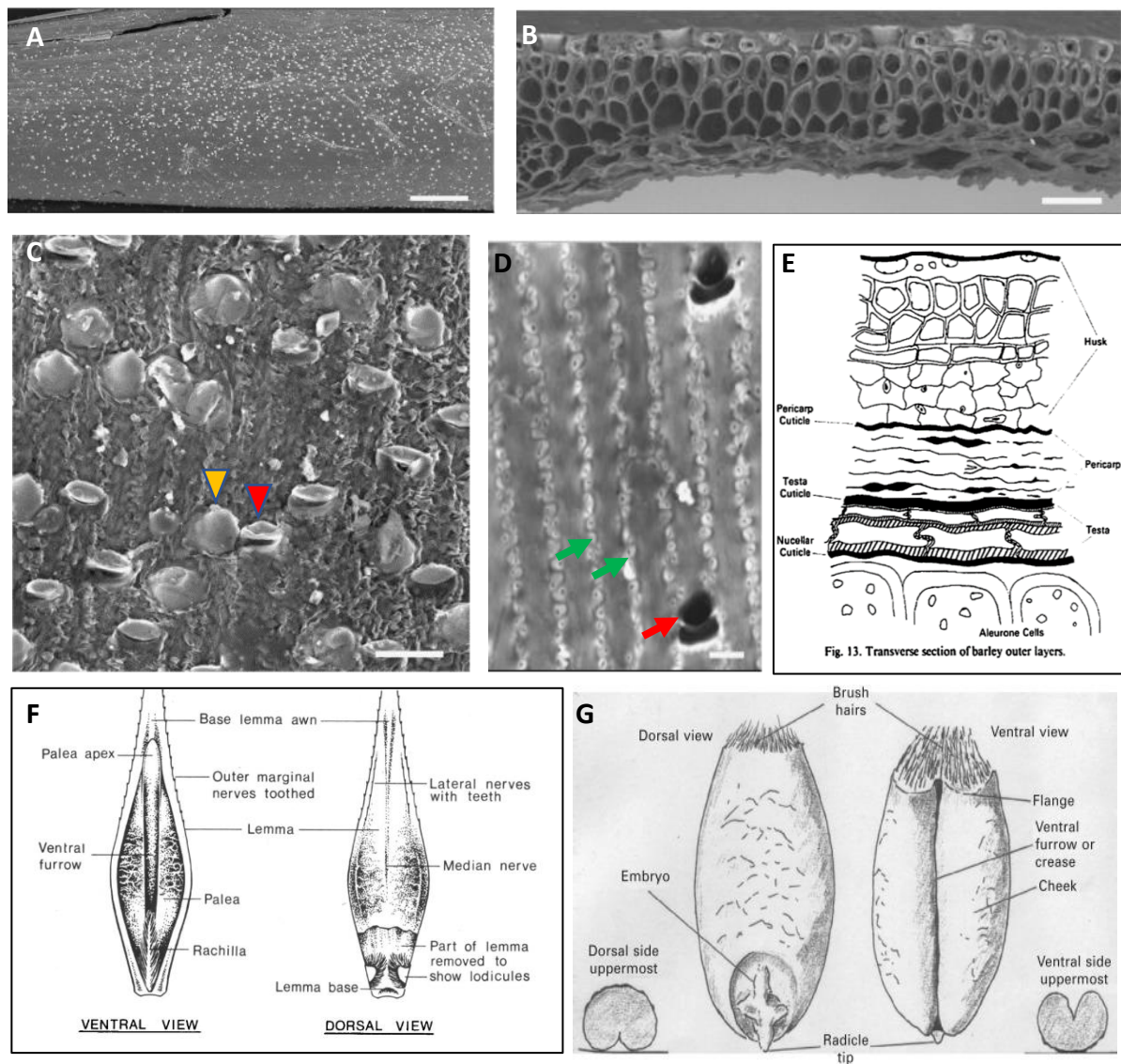


Figure 4-16. Documented WT barley grain and maternal tissue anatomy in the literature. **A.** SEM image of distribution of micro structures (papillae trichomes and stomata) in barley husk. scale bar=0.25mm. **B.** Cross section of the barley husk showing sclerenchyma and parenchyma cells. Scale bar 25 μ m. **C.** An enlarge top view of surface of barley husk showing papillae trichomes and stomata (yellow and red arrow respectively) and long epidermal cells. scale bar 25 μ m. **D.** Intercellular borders in optical image barley husk epidermis showing locations of silica-depositions in the epidermal cell ridges (green arrow) and stomata (red arrow) (scale bar = 10 μ m). Images were adopted from ([Boateng et al., 2007](#)). **E.** Illustration of transverse section of barley seed covering layers. Image from ([Freeman & Palmer, 1984](#)). **F.** Grain characters of barley showing the lemma, palea, awn and ventral furrow (crease). Image from ([Sparks & Malcolm, 1978](#)). **G.** Barley grain after removing the husk showing the embryo and brush end. Image from ([Wrigley, 2017](#)).

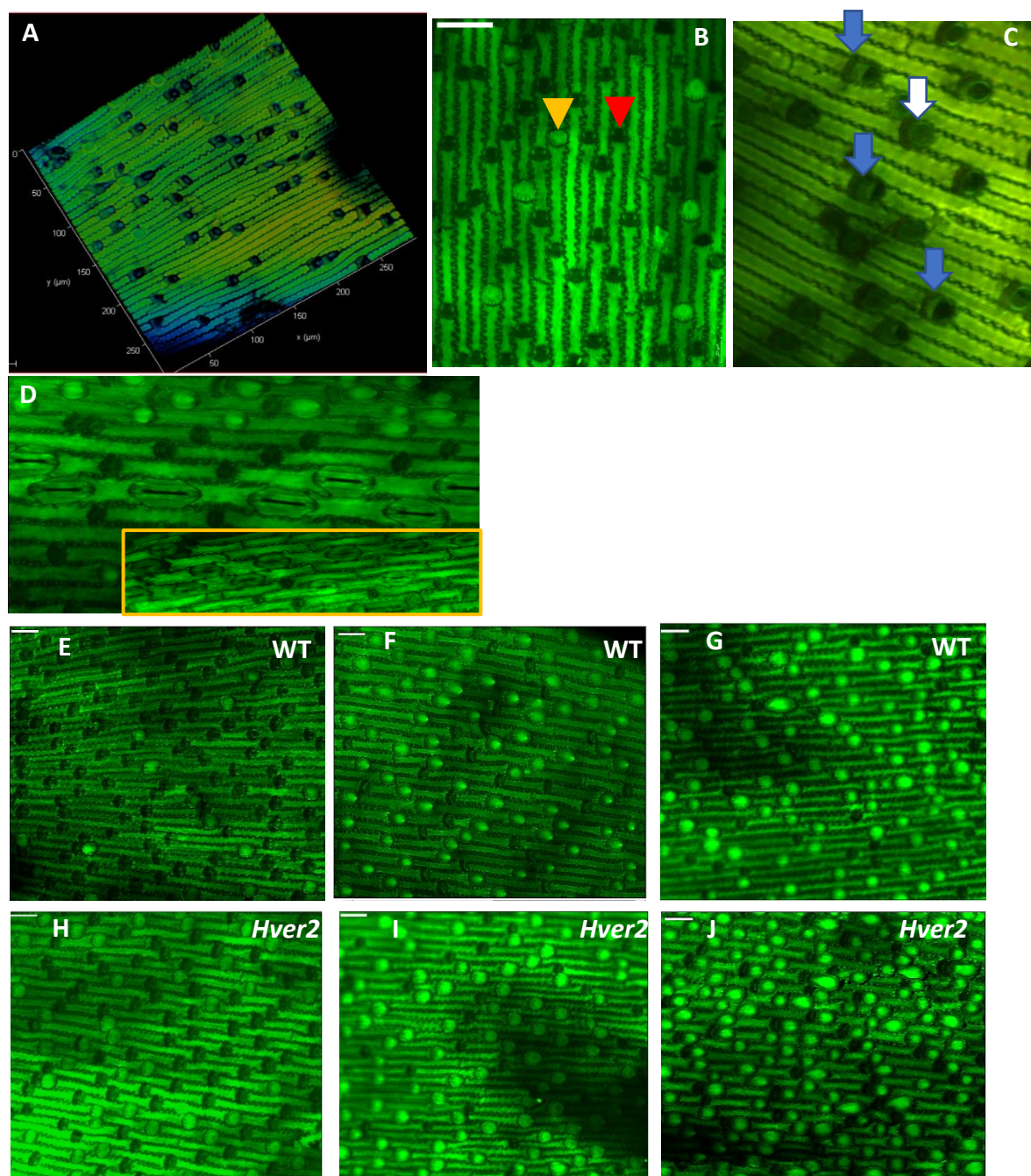


Figure 4-17. Husk upper epidermis anatomy of barley WT and *Hver2* seed. **A.** 3D reconstruction of barley WT lemma upper surface showing distribution of stomata. **B.** Upper surface view (upper epidermis) of barley WT lemma showing distribution of papillae trichomes (yellow arrow) and stomata-like (red arrow). **C.** Stomata-like are plugged (white arrow) with plate like structure, and that has been detached from most of them during tissue clearing (blue arrow). **D.** 2-3 rows of grass stomata are aligning along the ventral furrow of the palea upper surface (WT seed were shown) **E-J.** Papillae trichomes and stomata-like distribution gradient from embryo end (E,H), middle of the seed(F,I) and near the brush end (G,J) of WT (E-G) and *Hver2* (H-J) palea upper surface.

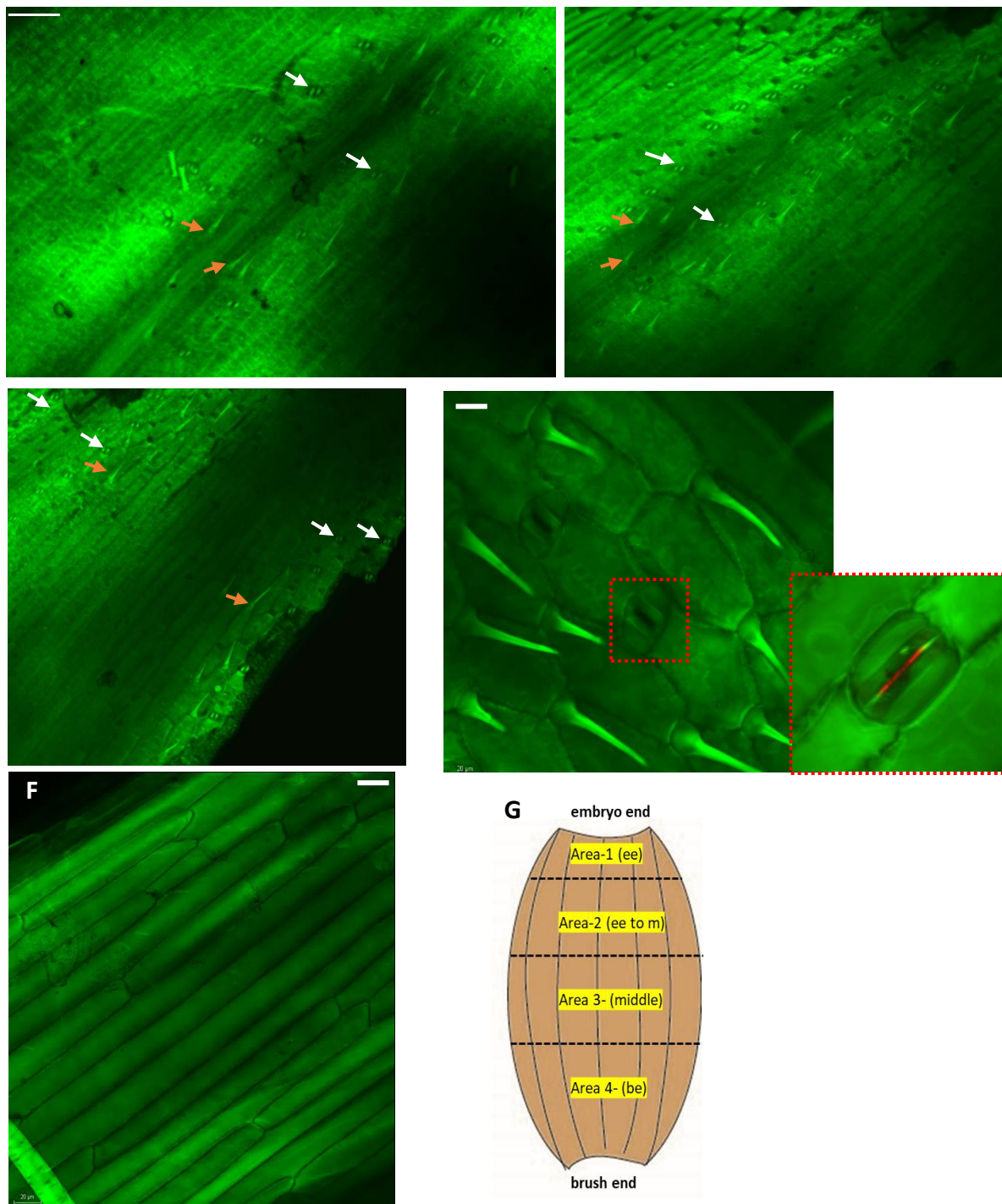


Figure 4-18. Anatomy of lower epidermis of the husk of WT barley seed. **A & B & C.** The trichomes (orange arrow) and stomata (white arrow) distribution along the mid veins in three locations of lower epidermis of the husk, scale bar 100 μm. **D & E.** Enlarged view of the trichomes and grass stomata (two dumbbell shape guard cells), scale bar 50 μm. **F.** Long epidermal cells running parallel to the longitudinal axis of the husk and located in between the veins, scale bar 50 μm. **G.** A cartoon of lemma/palea illustrating the areas used for quantitative measurements. ee- embryo end, e to m- embryo end to middle of the seed, middle- middle of the seed, be- brush end.

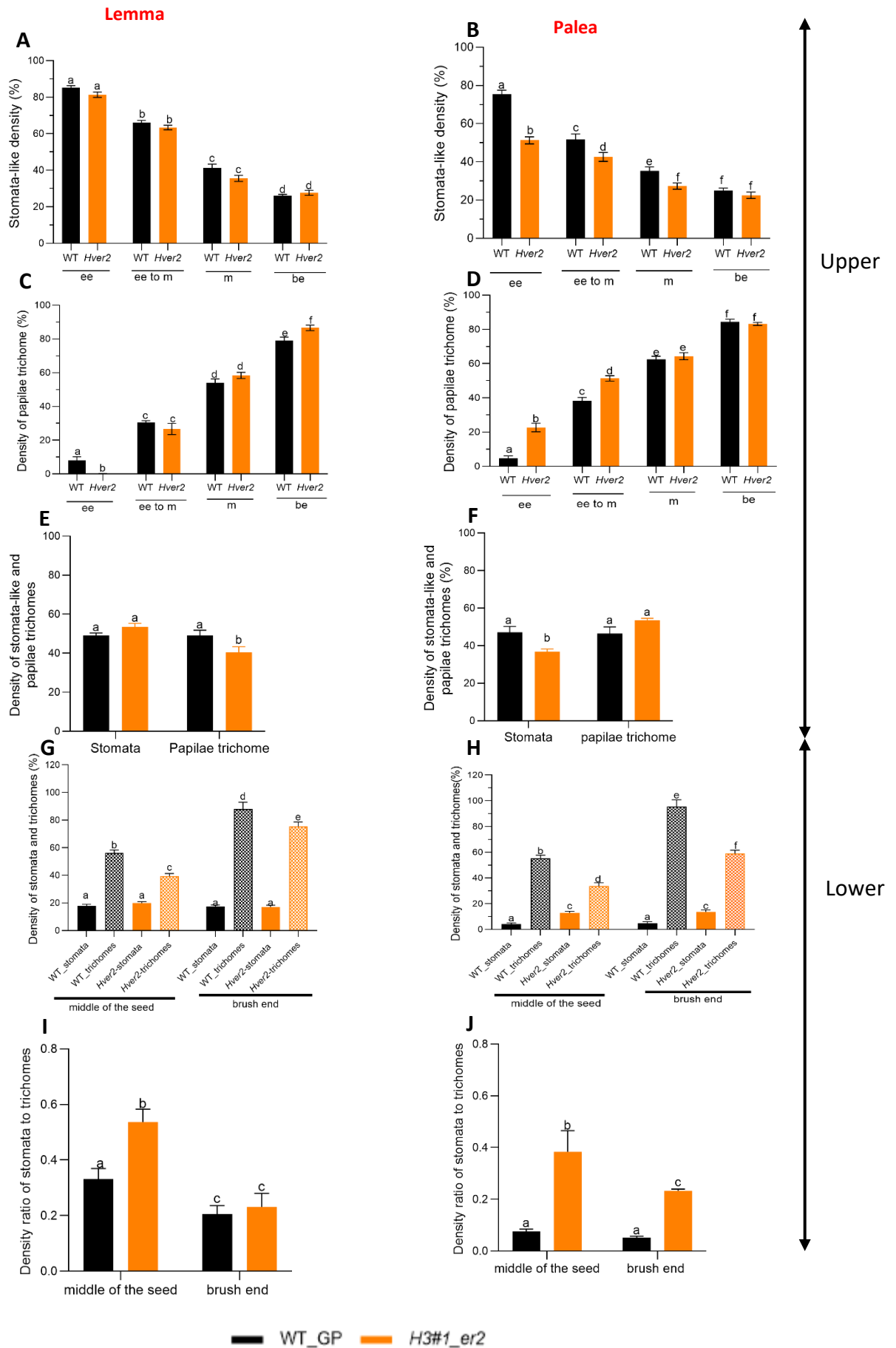


Figure 4-19. *HvER2* signalling is involved in the patterning of papillae trichomes and “stomata -like” microstructures on the upper and lower epidermis of the barley seed husk. A- F. Upper epidermis, G-I- Lower epidermis. **A-D.** Average density along WT and *Hver2* lemma and palea of stomata-like structures (A&B, respectively) and papillae trichomes (C&D, respectively). ee- embryo end, ee to m- in between embryo end to middle of the seed, m- middle of the seed, be- near the brush end. **E&F.** Total density of stomata and papillae trichomes across the whole lemma (E) and palea (F) upper epidermis. **G &H.** Density of stomata and trichome along lemma (G) and palea (H) lower epidermis in two locations. **I&J.** Ratio of stomata/trichome numbers on the lower epidermis of lemma (I) and palea(J). n=3 seeds per genotype, each examined on 5 frames for each of the 4 regions spanning the whole length of the husk. Statistically significant differences were determined by a two-way ANOVA using Fisher’s LSD test and are indicated by different letters. Error bars represent standard error of the mean (s.e.m).

In the two genotypes, the lower husk epidermis showed parallel files of short barrel-shaped epidermal cell files along veins (Figure 4-18 A-D), separated by files of more elongated pavement cells with smooth margins, all parallel to the longitudinal axis of the husk (Figure 4-18 F). Two (rarely 3) rows presenting typical grass-stomata (two dumbbell-shape guard cells and two subsidiary cells) (Figure 4-18 A- E), and trichomes, in highest density at the embryo end, gradually decreasing along the axis towards the brush end. There were also some trichomes interspersed among the short epidermal cell files adjacent to veins (Figure 4-18 A-C).

The husk's upper epidermal surface was divided into four regions from embryo to brush end (Figure 4-18 G), and the density of each type of microstructure within each region was quantified (Figure 4-19). The longitudinal spatial gradient of papillae trichomes density was opposite to that of the stomata-like structures for both the lemma and palea in each of the two epidermises, and in the two genotypes (Figure 4-19 A-D). The density of stomata-like micro-structures along the *lemma upper surface* and its longitudinal gradient were not significantly affected by the loss of function of *HvER2* (Figure 4-19 A), but the density of papillae trichomes was drastically reduced, around the embryo (Figure 4-19 C). In contrast, the *palea upper epidermis* showed consistently lower densities of stomata-like structures in *Hver2* than in WT (Figure 4-19 B) and, in contrast to lemma, significantly increased the number of papillae trichomes at the embryo-end region (Figure 4-19 D). These effects were reflected in the opposite rankings between WT and *Hver2*, of the total numbers of each type of micro-structures on the combined 4 locations examined along the husk (Figure 4-19 E-F). The opposite longitudinal gradients of stomata-like and papillae trichome densities along the husk outer surface, suggested that there might be some trade-off between the formation of the two types of specialized structures, involving *HvER2* signalling.

I next examined whether *HvER2* had any influence on the patterning of the lower epidermis of the lemma and palea (Figure 4-19 G-J). Several regions mid-husk, just below the veins and near the brush

end, were examined for stomata and trichome counts. Stomatal densities were similar in the lemma but showed increased density in palea at both locations (Figure 4-19 G&H), whereas trichome densities were systematically lower in *Hver2* than WT in both the lemma and palea. Density ratios of the stomata to trichome show that *Hver2* have a significantly higher ratio in the middle of the seed for both lemma and Palea and at the brush end in only the palea lower epidermis of the husk (Figure 4-19 I&J).

4.4 Discussion

Over the last two decades, *ERECTA* family LRR-RLKs have emerged as major regulators of multiple aspects of plant development, intercellular signaling, and the integration of developmental and stress responses pathways, such as in the control of transpiration efficiency, thermotolerance, or of some immune responses ([Torii et al., 1996](#); [Godiard et al., 2003](#); [Torii et al., 2003](#); [Masle et al., 2005](#); [Shpak et al., 2005](#); [Pillitteri, 2007](#); [Sanchez-Rodriguez et al., 2009](#); [Bemis, 2013](#); [Shen et al., 2015](#); [Jorda et al., 2016](#); [X. Qu et al., 2017](#); [Y. Zhang et al., 2018](#); [Nanda et al., 2019](#); [Guo et al., 2020](#); [Berchembrock et al., 2021](#); [Chen et al., 2024](#)). These discoveries mostly stem from numerous studies, mostly in Arabidopsis. Despite their obvious agronomic and adaptive importance and the broad conservation of *ERECTA*/*ERECTA*-like genes across the plant kingdom, as a small family of generally 2-4 members with high structural homology ([Torii et al., 1996](#)) (e.g. number of Leucine-rich repeats in the receptor domain; number of introns and exons in the cytoplasmic domain), only a few *ERECTA* genes from other species have been analyzed, furthermore mostly limited to only one family member, selected on the basis of its closest homology to the *ERECTA* Arabidopsis gene ([Guo M, 2011](#); [Huang, 2013](#); [Shen et al., 2015](#); [Zakhrabekova et al., 2015](#); [Y. Zhang et al., 2018](#); [Berchembrock et al., 2021](#); [Chen et al., 2024](#)). Yet, Arabidopsis studies where the whole family has been considered all converge to the conclusion of some redundancy but also functional specificity among family members, with interactive, often synergistic roles of the three, that vary depending on biological process and involve diverse combinatorial configurations of the three members, and activating ligands ([Shpak et al., 2004](#); [Pillitteri, 2007](#); [Abrash et al., 2011](#); [Lee et al., 2012](#); [Bemis et al., 2013](#); [Nanda et al., 2019](#)). And the limited data available in other species indicates functional conservation but also differences across species, even when considering the single Arabidopsis *ERECTA* and its characterized orthologues to date in other eudicots ([Villagarcia et al., 2012b](#); [Shen et al., 2015](#); [Berchembrock et al., 2021](#); [Zhao et al., 2023](#); [Chen et al., 2024](#)).

The present study constitutes the first investigation of the barley *ERECTA* family, and test of functional conservation of the *AtERf* novel function in the regulation of seed germination, between the reference -eudicot- Arabidopsis *ERf* family as a whole, and a monocot whole *ERf* family, furthermore representing a major grain crop.

4.4.1 *HvERf* and *HvGL1* act to promote vegetative and reproductive development, but with some divergence compared to *Arabidopsis*

From Search homology in the barley genome draft with the three *Arabidopsis ERECTA* family members and other confirmed *ER/ERL* genes from other species, indicated that as wheat or rice, barley *Hordeum v.* has evolved 3 *Erf* genes. Based on the phylogram of *ER/ERL* genes, we named them *HvER*, *HvER2* and *HvERL*, respectively. *HvER* was the gene reported by Zakhrabekova et al. (2015) (*HvER- Ert-m/HORVU.MOREX.r3.7HG0662630*). There was no information on the other two putative members. CRISPR/Cas9 was used for gene editing, targeting the upstream repeats of the LRR- receptor, so as to maximize chances to obtain KO mutants, with truncated proteins not only lacking the intra-cellular kinase catalytic domain but also likely unable to potentially act as co-receptor with other membrane-localized proteins. A collection of mutants was successfully generated that provided powerful tools for functional genomic studies and constituted a unique resource for exploring *ERECTA* gene functions in cereals.

Several closely related MYB R2R3-like sequences to *Arabidopsis AtGL1* transcription factor-MYB0, were identified using homology search and phylogenetic analysis, all un-annotated when the present study was started. The sequence with the highest identity score among them, *HORVU.MOREX. r3.6HG0596350.1*, was selected to test for functional homology with *AtGL1* in its novel role in seeds, and synergistic roles with the *ERECTA* gene family in the regulation of seed germination sensitivity to salinity (Chapter 2). It was arbitrarily named *HvGL1*. Through CRISPR/cas9 gene editing targeting the MYB DNA binding domain of the translated *HvGL1* protein, several independent mutant lines were identified with successful editions introducing a premature stop codon causing loss of gene product (Table 4-2; and no gene amplification in PCR using gene-specific primers).

The initial characterization here of the set of *Hver*, *gl1* or double mutant *erf gl1* lines, validates that approach and provides evidence of functional homology between the selected barley putative *GL1* and *ER/ERL* family genes, with however only partial conservation and significant, fascinating divergence between the two species.

The range of *Hver/erf* mutants obtained show hallmark characteristics of reported KO mutants in confirmed *ER/ERL* genes, to varying degrees among both the set of single or double mutants that could be recovered - reduced stature at vegetative and/or reproductive stages, smaller more erect leaves, impaired inflorescence morphogenesis and development of reproductive organs, reduced or complete sterility, and as in *Arabidopsis* altered seed development and sensitivity to salinity during germination. The observed variation in these effects among allelic mutants, for example, the several *Hverl* or *gl1* mutants that were recovered was to be expected. It is well established that some point mutations that

result in one amino acid substitution/change often induce only subtle phenotypic differences with WT, or as totally neutral, as was here the case of the point mutation in line *H3#9-er2*.

To account for this and establish causality rather than correlation between target gene editions and phenotypic differences, all positive transgenic T₀ plants were carried forward to T₁ and T₂ generations, to recover at least two independent lines, carrying different editions in the target *ERf* members, alone or in combinations. This was successfully achieved for each of *ER2* and *ERL* but not *ER*.

Only one *er* positive primary transformant was generated despite several rounds of transformation. Its extreme dwarfism, and erectness, together with the failure to recover any double mutant of either *ER2* or *ERL* in combination with *ER*, with any of the several independent constructs targeting an edition of both *ER* and *ER2* or *ERL*, or the 3 genes (Table 4-2), indicate that *HvER* is indispensable to plant function and reproduction in barley. In most of the Arabidopsis *ERf* functions, the *ERECTA* member *AtER*, does assume a dominant role over its two paralogues, but it is not necessary to survival, and its loss can be largely compensated by *AtERL1* and *AtERL2* or even by only one of them (in *er erl2* or *erl1* mutants) ([Shpak et al., 2004](#); [Shpak et al., 2005](#); [Pillitteri, 2007](#)). This difference between the two species indicates that the strength of the well-established functions of *AtER*, such as its role as a master positive regulator of cell proliferation and cell fate organ growth and elongation, stomatal cell fate and water use efficiency, is much increased in barley to the point of vital physiological functions being totally abolished in KO mutations of *HvER*. This could be due to sequence differences between the two genes and translated proteins, and/or to differences in the family signaling cascade components. Both possibilities are supported by the reported diversification of the function of *SPCH*, one of the key *BHLH* *ER* partners, in stomatal development, or co-receptors of the *ERf* (such as TMM or some SERKs in Arabidopsis) ([Doll et al., 2021](#); [Doll et al., 2023](#)).

Similarly, while Arabidopsis *Aterl1* or *Aterl2* have no reported phenotype, and even the double *erl1 erl2* is mostly undistinguishable from WT ([Shpak et al., 2004](#)) apart from in seed size ([Nanda et al., 2019](#); [Wu et al., 2022](#)), the barley *Hver2* and *Hverl* mutants both presented not only a reduced stature compared to WT but also a smaller proportion of fertile tillers, with moreover disorganized ears, with twisted rachis, and deferred, slower or delayed germination under salinity (*Hver2* and *Hverl*), or even constitutively (*Hver2*) (Figure 4-12).

Although not representing all the *Hver*, *erl* and *gl1* single and multiple mutants necessary to an exhaustive exploration of functional redundancy, specificity, and interactions between the four targeted genes, the CRISPR/Cas9 edited lines that were generated and be successfully propagated, included a sufficient number of independent lines with truncated target proteins, and of sister lines

with promising distinctive phenotypes, and sufficient progeny for reliable germination studies, to next probe my central question of interspecific similarity or divergence in the mechanisms underlying the *ERf*-mediated regulation of seed germination discovered in Arabidopsis.

4.4.2 Conservation and divergence in the *ERECTA* family- and *HvGL1* - mediated regulations of both constitutive seed germination and germination sensitivity to salinity, and their interactions

In Arabidopsis, *ERf* signalling acts to dampen the sensitivity of the seed germination to salinity and high osmotic potential in the surrounding solution (Chapter 2), and this function involves the three *ERf* members, with specificity. This is also the case of the barley *ER* family (Figure 4-12); and furthermore, even single mutants *Hver1* and *Hver2*-with still 2 remaining functional *HvERf* members, while in Arabidopsis, loss of *ERL1* or *ERL2* was neutral, and loss of *ER* had very mild effects. In barley, as in Arabidopsis, the *HvERf*-mediated regulation of seed germination is involved in the control of its two steps, and most predominantly the second, germination-completion step, with unequal importance of individual family members (*HvER2* vs *HvERL*, *AtERL1/AtERL2* vs *AtER* in Arabidopsis). However, my results in this study indicate a constitutive promoting role of the barley *ERECTA* genes in the regulation of constitutive seed germination, which was not detected in Arabidopsis. In the range of germination assays conducted in Arabidopsis in our lab, there has not been signs of a role of any *AtERf* member on constitutive germination, other than a trend for a subtle, non-statistically significant, but consistently reproduced delay in *er erl1* seed coat rupture (1st germination step), but with no carry effects on the timing nor frequency of germination completion by endosperm rupture (Chapter, Figure 2-2) ([Nanda et al., 2019](#)).

Loss of *HvGL1* function in the *ERf* background had no apparent effect on vegetative or reproductive, as was the case of *AtGL1* in Arabidopsis. However, when introduced in *Hver2* or *Hver1*, *Hvgl1* KO mutations significantly decreased, increasing the proportion of tillers that remained vegetative, and decreasing the fertility of those that progressed through reproductive development and produced an ear. In Arabidopsis, introducing *gl1* in *er*, *erl1* or *erl2* backgrounds was neutral, and still appeared inconsequential in *er erl1* or *er erl2* double mutant backgrounds.

Moreover, loss of *HvGL1* function alone in WT seeds induced a hypersensitivity of seed germination to salinity (Figure 4-12 D-E), contrary to Arabidopsis where *gl1* KO seeds germination kinetics mirrored those of WT with functional *GL1*. *HvGL1* seems to act as a positive regulator of constitutive germination too, speeding up its progress to completion (Figure 4-12 B-C). Consistent with this, is the trend caused

by its knock out in *Hver2* or *Hver1* background to further repress germination, This, and the strong synergy observed in *Arabidopsis* between *AtERf* genes and *AtGL1* in reducing germination sensitivity to salinity, led to expect a similar synergy of *HvGL1* with *HvERf*, of even greater strength, i.e., amplifying the germination delay and rate of failure caused by reduced *ERf* signaling (Chapter 2, Figure 2-2). Yet, this was not the case, and in fact I observed the opposite: *HvGL1* loss of function tended to consistently moderate the drastic reduction of germination rates induced by the *Hver2* or *Hver1* KO mutations. Hence, indicating antagonistic rather than synergistic interactions between *Hver2* or *Hver1* and *HvGL1*, in the regulation of seed germination under salinity.

Altogether these results: a) demonstrate conservation of the *ERECTA* genes germination-promoting function across the *Arabidopsis* seed and the barley caryopsis, but with several points of divergence: in *Arabidopsis* that function is only expressed under adverse conditions, salinity or hyper-osmotic stress; in barley *ERf* function is involved in the control of constitutive germination rate; b) demonstrate a contribution of each of the two *HvERf* members that could be tested, and the absolute requirement of them both to maintain maximum germination speed and success rate, and even more critically limit their drop under salinity, contrary to *Arabidopsis* where *AtERL1* or *AtERL2* are individually and collectively dispensable, even under saline conditions, provided the *AtER* member is functional.

4.4.3 The barley *ERECTA* family and *HvGL1* regulatory functions in the timing and pace of seed germination are primarily maternally controlled via properties of the husk and inner cementing layer lining

In *Arabidopsis* the *erf*- or *erf gl1*-induced inhibitions of germination under salinity are readily lifted upon stress release ([Nanda et al., 2019](#)); Figure 2-2, even after prolonged exposure to adverse conditions. My data here give evidence for a similar feature in barley too. However, contrary to the *Arabidopsis* salt hypersensitive *Aterf*, or *Aterf gl1*, a significant fraction of ungerminated *Hver1* and *Hver2 gl1* seeds still failed to germinate once transferred from saline to non-saline conditions (Figure 4-13 B). However, manual removal of their husk (including the inseparable “cementing layer” secreted from the pericarp and facilitate tight attachment of the husk to the pericarp) ([Harlan, 1920](#); [Gaines, 1985](#); [Brennan, Hedley, et al., 2019](#); [Brennan, McDonald, et al., 2019](#)) to test the possibility of irretrievably damaged embryos, revealed the coleorhiza tip, intact, just visible out of the ruptured pericarp, with the 3 radicles tips enclosed and sitting just below; and once transferred to non-saline conditions, all de-husked caryopses exhibited swift coleorhiza rupture, by a normal, healthy radicle that thereafter developed normally (Figure 4-13 D, Figure 4-20). Therefore, in barley as in *Arabidopsis*,

the *er2*- or *erl*-mediated inhibition of germination under salinity is not due to stress-induced loss of seed viability, but to a halt of the germination process, whether in a *GL1* or *gl1* background.



Figure 4-20. Longitudinal section of a non-germinated *Hver2* seed exposed to salt for 250 hours at the time of dehusking (t0) before transfer to the control medium-0mM NaCl. The seed was cut along the longitudinal axis of the coleorhiza. The image shows the radicle elongation up to the coleorhiza (blue arrow), with the black dashed line indicating the inner lining of the coleorhiza. R-radicle.

Further, the facts that the elongation of the coleorhiza and enclosed radicle had similarly progressed up to the lower surface of the husk and derived cementing layer, regardless of the salinity level in the surrounding solution, stopped there, and readily resumed as the husk was removed, with the radicle quickly piercing through the coleorhiza, as a whole demonstrate that: **a)** The embryo had undergone normal development and the coleorhiza and radicle had been able to elongate and make their way through all the covering maternal structures, testa, pericarp and associated cuticles, up to the husk lower epidermis; **b)** Their arrested elongation at that point was caused by the impossibility to break through the husk; **c)** This impediment is *ERf*- and *-GL1* dependent, in strong interaction with the ionic and osmotic conditions around the seed, given the much increased frequency of germination failure observed for intact (husked) mutant caryopses carrying *erl*, *er2*, *er2 gl1*, or *erl gl1* KO mutations than in WT, and under 150mM vs 0 mM NaCl; **d)** Hence, as in *Arabidopsis*, the *HvERf*- and *HvGL1*-mediated hypersensitivity of seed germination in barley is maternally controlled; **e)** However, not through the seed coat as in *Arabidopsis*, but through the husk, **f)** Not apparent contribution of the underlying other maternal tissues.

It is fascinating that although formed from different cell lineages, the Arabidopsis seed coat and barley grain husk, both constitutes the outer-most structure of the reproductive unit, and the most complex, highly differentiated structure forming a mechanically strong, low permeability structure, pigmented with anti-oxidative compounds- altogether making it a highly protective structure for the vital inner embryo from mechanical or biophysical aggressions. In the two species, the *ERf* and *GL1/GL1*like genes act to control biophysical properties of this crucial outermost protective structure of their reproductive unit, which condition the modifications it undergoes during imbibition and germination, to facilitate its rupture by the inner embryo. The nature of these properties -anatomical, structural, architectural and biochemical- and of the controlling mechanisms at the cellular and molecular levels and, importantly, whole tissue level, remain to be resolved, keeping in mind the core functions of *ERECTA*-*LRR-RLK*inases documented in aerial organs in inter-cellular and inter-tissue signaling, transduction of endogenous and exogenous stimuli.

Altogether, these results demonstrate that, as a whole, the *AtERf* and *HvERf* families control similar physiological functions and plant attributes, but that contrary to Arabidopsis where *ERL1* and *ERL2* are dispensable, provided *ER* remains functional, this not the case in barley where the functionality of each member is necessary to maintain the integrity of plant function, reproductive and germination potential. Collectively they suggest reduced functional redundancy within the barley *ERf* family, and indicate functional specialization of *ERECTA* genes during evolution of monocots and dicots, and the evolution of the huge diversity of seed types seed and protective structures found across plant species. In practice, this study also flags *ERECTA* genes and interaction MYB transcription factor as novel prime candidates for genetic improvement of germination, crop establishment and yield sustainability of barley in saline environments. In addition, the germination promoting role of *HvER2* in the absence of salinity, points to perhaps the potential of that *HvERf* member for crop establishment and yield potential in the absence of salinity.

From an evolutionary and adaptive perspective, it is fascinating to discover that two species, as different phylogenetically, anatomically and with respect to domestication, have evolved and conserved a small family of membrane localized receptor kinases, that sensitises germination to salinity, delays it or frequently arrests it before completion and allow it to readily resume upon stress release and progress to completion as fast as in seeds never exposed to stress, with the emergence of a healthy fully viable seedling.

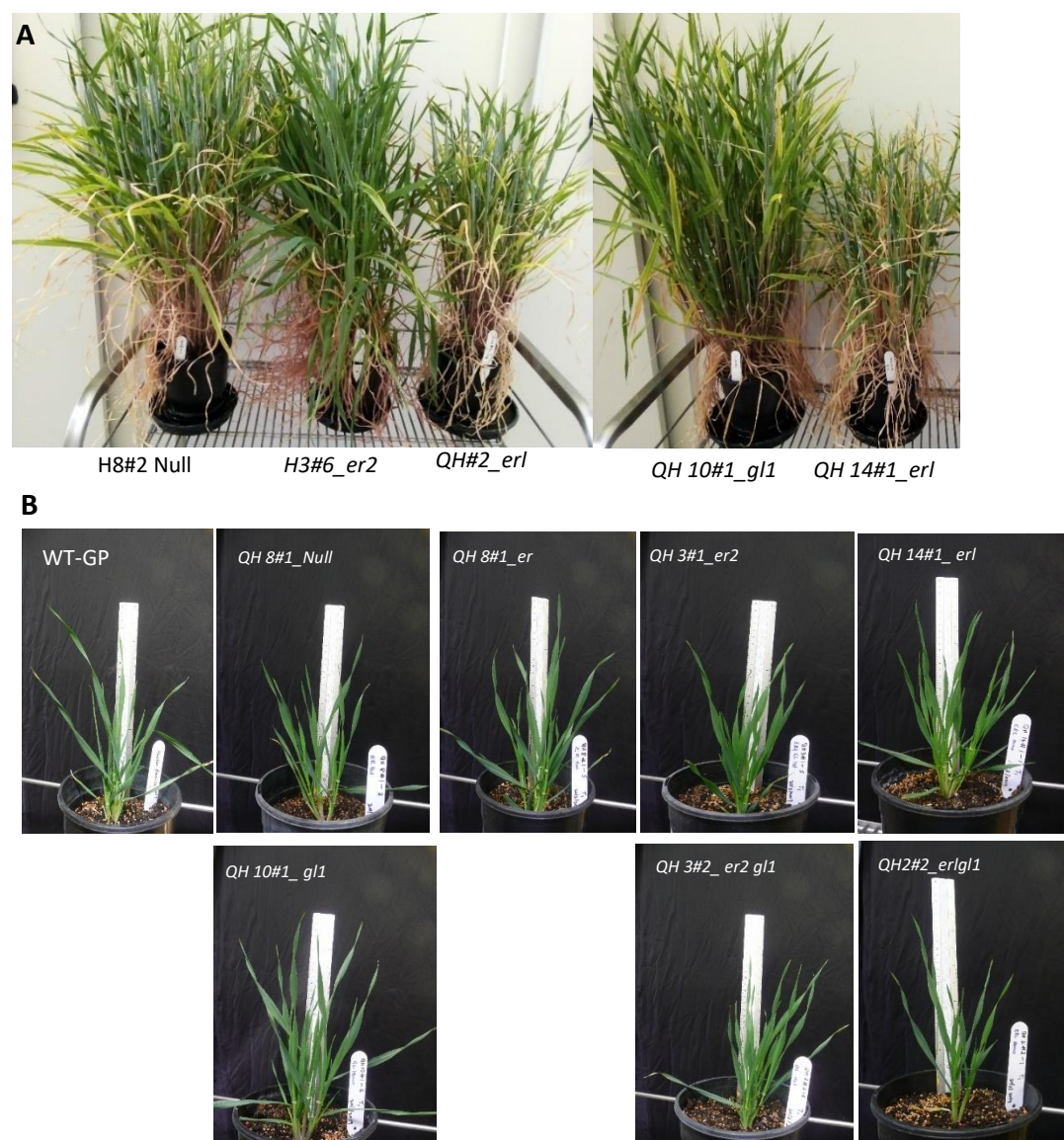
That finding support the proposition that rather than penalizing, such a function can be seen as an exquisite strategy to safeguard the embryo and new seedling from high risks of death upon sudden exposure to adverse surrounding conditions, more over before even having had the time to acquire

autotrophy. It is interesting that, in the two species, some seeds do germinate at the same time as WT even under high salinity stress, while others do so with increasing delay, and others are arrested until stress release. That mixed response within a seed cohort, might balance the risks of death with those of severe loss of fitness associated with the inability to complete the life cycle in time for reproduction and yield.

It will be exciting to test natural functional diversity within barley in *ERECTA* family and cooperating MYB transcription factor in the regulation of germination and crop establishment, and the physiological and molecular mechanisms. The large natural diversity present in barley, impressive collection of preserved natural mutants of diverse ancestry, and availability of “hullness” cultivars, provides an especially fascinating model for that exploration, especially given the maternal control exerted by the husk and its amenability to targeted mechanistic studies.

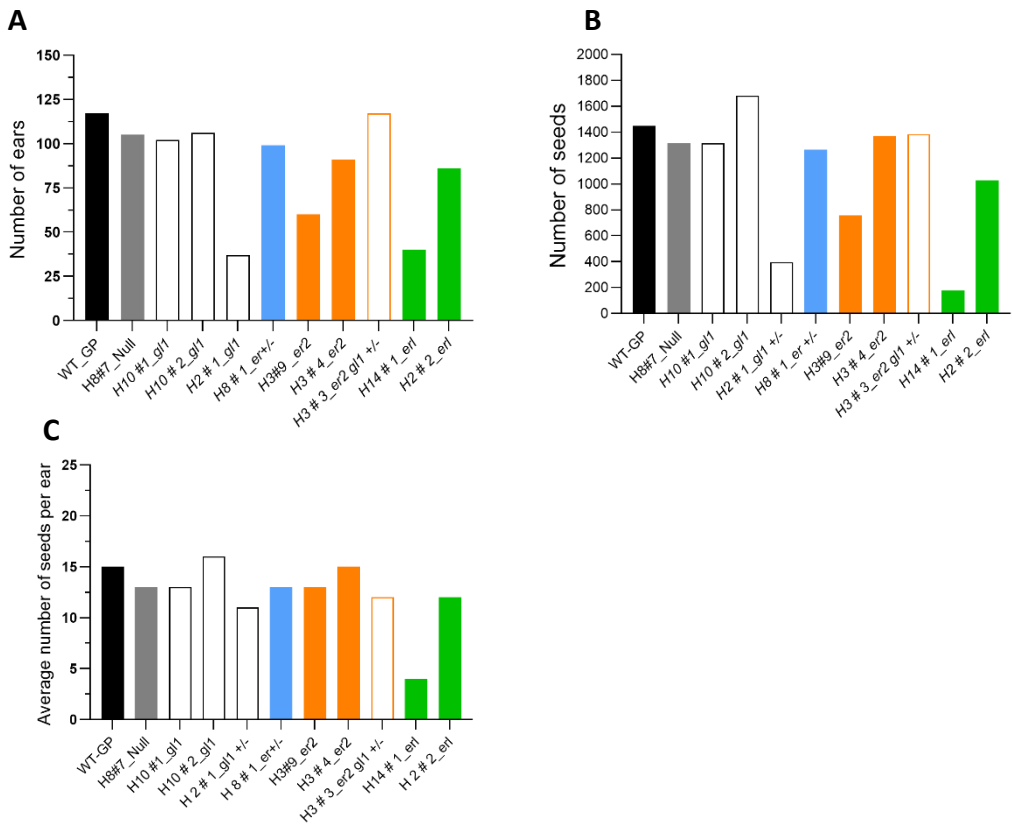
4.5 Supplementary Figures

4.5.1 Supplementary Figure 1



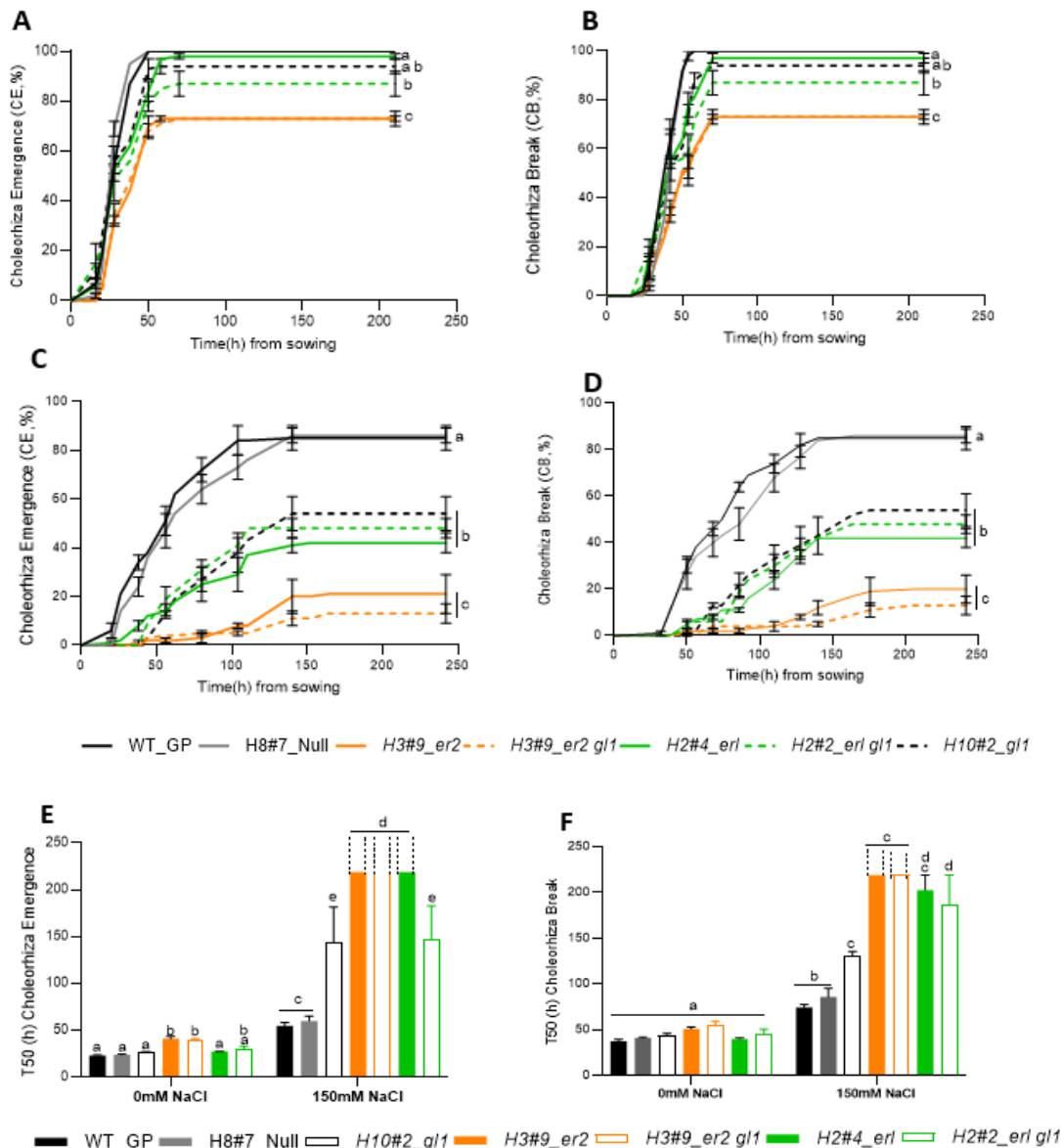
Supp figure 4-1. A. Vegetative growth of To plant after 101 days of potting. **B.** Vegetative development of T₁ plants edited for *HvER*, *HvER2*, *HvERL*, *HvGL1*, *HvER2 GL1* and *HvERL GL1*, five weeks- old plants.

4.5.2 Supplementary Figure 2



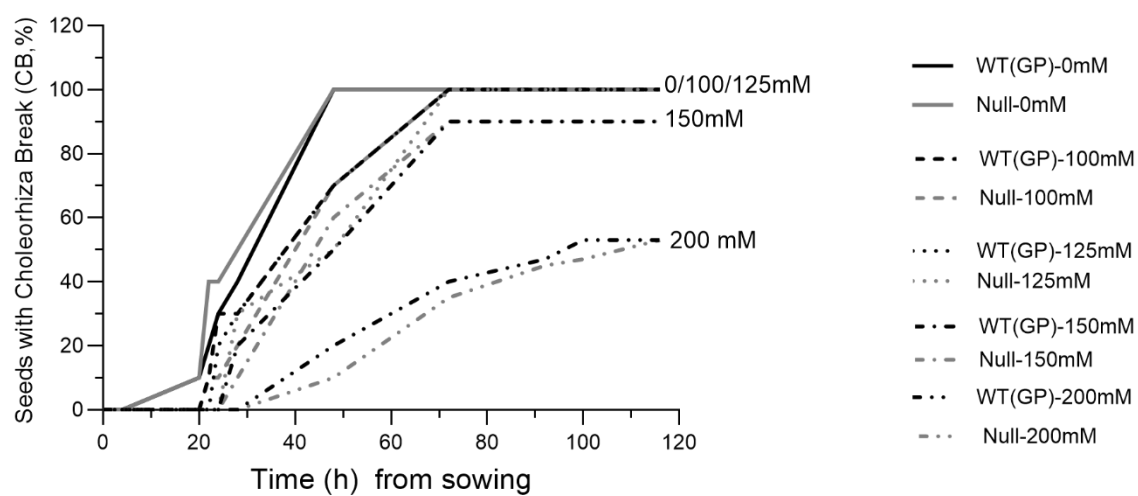
Supp figure 4-2. Reduced fertility of *T₀* *Hverf* mutants. **A.** Total number of ears in each plant. **B.** Number of seeds obtained from each plant **C.** Average number of seeds per ear.

4.5.3 Supplementary Figure 3



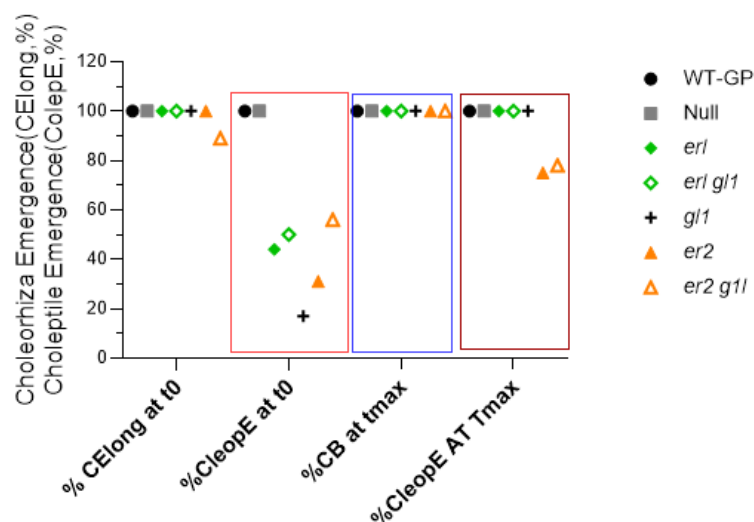
Supp figure 4-3. Barley seed germination assays of *HvERf* +/- *GL1* under control and 150mM NaCl media- 2nd independent lines. **A&B.** Coleorhiza emergence (B) and coleorhiza break (C) of *Hverf* +/- *GL1*, WT and Null under optimal conditions showing the constitutive effect of *Hver2* and *Hver2 gl1*. **C&D.** Coleorhiza emergence (D) and coleorhiza break (E) of *Hverf* +/- *GL1*, WT and Null showing salt hypersensitive germination phenotype of *Hv erf* +/- *GL1* that significantly reduces the germination rate and significantly increased the germination delay compared to WT and Null. Significant differences were calculated only at the maximum germination rate. Statistically significant differences between the samples were determined by a one-way ANOVA using Fisher's LSD test and are indicated by different letters. Error bars represent standard errors of mean (s.e.m) **E&F.** T₅₀ values (time(h) taken to reach 50% of CE or CB) for CE (F) and CB(G). Data are means(+/-SE) from n=3 plates with 30 seeds per genotype per plate. Statistically significant differences between the samples were determined by a two-way ANOVA using Fisher's LSD test and are indicated by different letters. Error bars represent standard errors of mean (s.e.m).

4.5.4 Supplementary Figure 4



Supp figure 4-4. Germination kinetics of WT and Null seed under different NaCl concentrations.

4.5.5 Supplementary Figure 5



Supp figure 4-5. Left two panel of the graph showing Coleorhiza elongation (**CElong**) through all the inner embryo surrounding layers (germ aleurone, testa and pericarp) up to the inner lining of the husk and Coleoptile Emergence (**CleopE**) through all the inner tissues (germ aleurone, testa and pericarp), within the husk, at the time of husk removal of **non-germinated seed**, that exposure to salt for 250h, to transfer to the control medium. Right two panel of the graph showing the 100% Coleorhiza Break (CB) and CleopE of the dehusk seeds when the germination scoring stop on the control medium (0mM NaCl).

4.5.6 Supplementary Figure 6

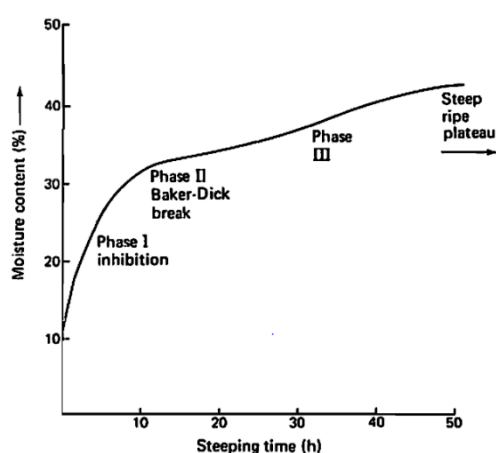


Fig. 1. Water uptake during traditional steeping of barley (55°F).

Supp figure 4-6. The water uptake kinetics reported in (Brookes, 1976), and used as the reference WT water uptake curve in this study to define the various water uptake phases.

4.6 References

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Chapter 5

Thesis overview and some future perspectives

5.1 Main findings

The *ERECTA* family (*ERf*), a well-known subgroup of LRR-RLKs, includes *ER* and its close homologs *ERL1* and *ERL2* (Shpak et al., 2004). These kinases have significant roles in various plant developmental processes, such as organ shape, stomatal patterning, leaf initiation rate, ovule integument growth and responses to environmental stimuli (Masle et al 2005., Pillitteri et al 2007., Hord et al 2008., Shpak et al 2004., Xu et al 2003.). *ERECTA* signalling is initiated by ligand binding to the extracellular domain of *ER* family proteins, triggering downstream MAPK phosphorylation cascades that regulate cellular functions. Notably, the *ERf* interacts with multiple hormonal pathways, including auxin, gibberellins, cytokinin, and brassinosteroids, to coordinate complex growth responses (Uchida et al., 2012., Sarnowska et al., 2023., Chen et al 2013). A recent study of the *ERECTA* gene family (*ERf*) in Arabidopsis revealed a significant breakthrough in understanding seed germination under high salinity and osmotic stress ([Nanda et al., 2019](#)) demonstrated that Arabidopsis *ERECTA* plays a crucial role, significantly delaying or inhibiting germination under adverse conditions while allowing for normal germination when conditions improve. This underscores the *ERECTA* gene family as a critical player in regulating seed germination, modulating this process through interactions with other genes and pathways.

The present study is an extended study of Nanda et al. (2019) aiming to understand better a) the physiological and molecular basis of the maternal inheritance of the *ERf*-mediated germination phenotype b) the conservation of the *ERf* function in regulating salinity signalling during germination in cereals.

One of the key findings of this study is the discovery of *GLABRA 1* (*GL1*), an R2R3 MYB Transcription factor, interacting with the *ERf* family, promoting germination delay and reducing germination percentage under stress conditions. Under control conditions, the functional specificity between *ERL1* and *ERL2* shows an antagonistic interaction dependent on *GL1*. This interaction controls cryptic genetic variation in germination speed, indicating a complex and intricate regulatory network involving *ERf* and *GL1* in seed germination under optimal and stressed conditions.

The maternal control exerted by *GL1* in regulating *ERf*-mediated germination under salinity stress is primarily determined by the maternal origin seed coat surrounding the embryo. This was demonstrated by *erf+/-gl1* mutants' ability to recapitulate the wild-type germination, except in the case of *er erf2 gl1* mutants., not up to the WT level but recapitulate significantly, under constitutive

expression of *ERg* in the epidermis. This evidence suggests that maternally derived *ERf* and *GL1* functions in the seed coat are critical for germination under salinity stress. The overexpression of *ERECTA* alone in the seed epidermis compensates for the loss of native *ER* and *ERL1* or *ER* and *ERL2* genes, emphasizing the dominant role of *ER* in the *ERf*-mediated control of seed germination and its interactions with *GL1*.

The data also indicate that loss of *ERf* and *GL1* functions leads to significant changes in the seed coat's structural properties, such as modulating the seed cavity expansion by regulating cell expansion and proliferation, secondary wall depositions i.e., loss of functions results thicker anticlinal secondary cell walls and broader columellae with early differentiation of *oi2* mucilage secretory cells, prominently in salt hypersensitive *erf* seeds. These structural modifications suggest that *ERf* and *GL1* play interactive roles in depositing secondary cell wall material in the seed coat epidermis. Moreover, this study reveals the involvement of the *GL1* transcription factor in the synergistic *ERf-GL1* regulation of the mucilage's modulatory role under salinity. Differences in the auto-fluorescence spectra of wild-type and salinity hypersensitive *erf* $\pm$ *gl1* mature seed coats suggest biochemical differences in wall material and mucilage deposition, further indicating *ERf-GL1* role in regulating seed coat formation and architecture. Additionally, the promoting roles of *ERf* and *GL1* in maternally origin endosperm cuticle formation were uncovered. A thicker endosperm cuticle might be a mechanism delaying germination under salinity stress, maintaining seed viability during prolonged exposure to saline environments.

This study provides evidence that the *ERf-GL1* synergy in reducing germination sensitivity to salinity is partially ABA-dependent, marking the first instance where *GL1* is shown to be involved in an ABA-GA signalling loop. DELLA proteins GAI, RGA1, and RGL2, RGL3, which repress GA responses, have been demonstrated to physically interact with *GL1*, repressing its transcriptional function in trichome initiation in aerial organs (Qi et al., 2014; Maes et al., 2008; Perazza et al., 1998). GA-responsive GIS transcription factors function upstream of the trichome development MYB-bHLH-WD40 (MBW) complex, consistent with the upregulation of *GL1* by GA. It is speculated that *ERf* and *GL1* might interact during germination through roles in the transcriptional control of GA receptors and DELLA proteins, hence influencing GA signalling and MBW formation, SWI3B phosphorylation, and binding to *GID1a*, and reducing *GID1a* transcription (Sarnowska et al., 2023). This hypothesis was supported by the differential expression of *GID1a* in the *gl1* developing seeds at the embryo torpedo stage reported in the study (chapter 2). *GL1* loss-of-function, while not influencing germination on its own, dramatically enhances the delay and spread of germination in *ERf* salt-hypersensitive mutants, indicating that *GL1* is not integral to the *ERf* signalling pathway but intersects downstream of *ERf*. The *ERf* proteins promote seed size in a combinatorial manner with *ERL1* and *ERL2*, a function not influenced by *GL1*, further underscoring the distinct yet overlapping pathways through which *GL1* and

ERf regulate seed germination and size (Nanda et al., 2019; Wu et al., 2022). These findings open new avenues for exploring the broader roles of *ERf* and *GL1* in regulating seed germination and development through complex hormonal and genetic interactions.

Next, this study extended to understand the transcriptional networks regulated by the *ERf* and *GL1* pathways during early seed development, the critical 0-6 DAFO developmental time window that modulates the seed coat characteristics explained above to get insights into the molecular basis of the maternal control germination phenotype. The differential expression patterns observed across various developmental stages highlight significant shifts in transcriptional activity associated with *ERf* and *GL1* function and identified significant overlap and wide-spread interactions between the *ERf* and *GL1* transcriptional networks during seed development in the regulation of genes involved in abiotic stress responses, metabolic processes, hormonal signalling, and also, specifically, of genes involved in photosynthesis, cell wall organization, and cell wall component biosynthesis. In addition, this study underscores the pivotal roles of *ERf* and *GL1* in regulating transcriptional networks during early seed development. The study identified 104 differentially expressed transcription factors spanning multiple families, such as ERF, MYB, NAC, and others, with distinct expression patterns across different genotypes. The data reveal a complex interplay between *ERf* and *GL1*, influencing the transcriptional regulation of genes involved in key biological processes, including hormonal signalling and secondary cell wall biogenesis. The unique expression profiles observed in each genotype highlight the differential roles of these transcription factors and suggest that *ERf* and *GL1* may have both overlapping and distinct functions in seed development. Particularly, the study points to an important role for *GL1* in regulating NAC TFs and suggests potential new regulatory interactions in the absence of *GL1*.

Further, this study elucidates the intricate roles of the *ERf* and *GL1* in controlling seed development, mainly through their synergistic regulation of hormonal gene networks crucial for determining seed size. Very early in seed development, as soon as ovule fertilization occurs (0 DAFO), *ERf* influences a range of auxin-related genes. This action is primarily transient, not observed again until 6 DAFO, where the *ERf* effect is less extensive but involves different sets of auxin genes. Across both stages, the *ERf* loss of function consistently results in the transcriptional repression of key auxin-related genes, including *PAI2* and various Gretchen Hagen (*GH3*) genes, involved in auxin conjugation and synthesis. *ERf* also represses auxin carriers such as *AUX1* and *PIN4* and several IAA transcriptional repressors and members of the Small Auxin upregulated RNAs (*SAUR*) family implicated in cell elongation. *GL1*, on the other hand, primarily comes into play around 6 DAFO, the onset of seed coat differentiation and the transition from morphogenesis to maturation in seed development. Unlike *ERf*, *GL1* significantly

influences the SAUR family, particularly members of phylogenetic clades II and III, which are associated with cell elongation and stress responses, respectively. This regulation by *GL1* occurs with a delay and involves different subsets of SAUR genes compared to *ERf*. The differential expression patterns observed in *erf* and *gl1* mutants reveal a complex interplay between these regulators, with the loss of function of *ERf* acting as a repressor and *GL1* as an activator of auxin signalling pathway explaining the reduced size of *erf* seeds and the similar size of *gl1* seeds to wild type observed (Nanda et al. 2019 and Chapter 1). Further, *ERf-GL1* shows the synergistic interaction in the auxin signalling pathway, expressing some SAUR family genes (SAUR21-24, SAUR66-67) and IAA genes development stage-specific manner aligning with its counterparts.

In addition to auxin, the loss-of-function mutations in *GL1* revealed significant repression of the ABA signalling pathway during early embryogenesis, particularly around six days after fertilization (DAFO). The data showed differential expression in key components of the ABA receptor complex, including the upregulation of *PYL4* and downregulation of *PYL7* and *PYL9*, as well as the downregulation of *ABI1*, *SnRK2.2*, and *ABF1*, which are critical for ABA signalling and response, compared to the repression of two ABA receptors in *erf* (*PYL4*, *PYL9*)., only the ABA signalling *SNRK2.2* in *erf gl1*. This suggests that *GL1* plays an activating role in ABA signalling during this crucial developmental stage and interacts with *ERf*, thus balancing the expression.

Recently, the cross-talk between the phytohormones and cellulose and hemicellulose synthesis was substantially reviewed (see reviewed in ([Wang et al., 2020](#))), indicating the altered localization of the auxin and other phytohormones could affect the synthesis of cell wall component. The data of this study showed that *ERf* plays a relatively limited role compared to *GL1* in the transcriptional regulation involved in secondary cell wall biosynthesis. Significant upregulation of *CESAs* regulating cellulose synthesis and large family of genes involved in hemicellulose -xylan and xyloglucan biosynthesis particularly in *gl1* and to a lesser extent in *erf* and even more so in the combined *erf gl1* mutants. This explains the *GL1* and *ERf-GL1* mediated increased secondary cell wall thickness, including the broader columella observed in Chapter 1 through an intersecting pathway.

In addition, this study identified significant differences in the transcriptional regulation of genes involved in outer ovule integument development, seed coat differentiation, and cell wall biogenesis across the three mutant lines (*erf*, *gl1*, and *erf gl1*) at various developmental stages. Notably, the downregulation of *KAN1* and *NARS1/2* in the *gl1* and *erf gl1* mutants suggests that *GL1* is crucial for proper outer integument development and differentiation.

Further, key genes involved in mucilage modification and extrusion, such as *MYB61*, *KNAT7*, and *SBT1.7* in the *erf* mutant and *BXL1* and *PMEI6* in the *gl1* and *erf gl1* mutants, showed altered expression patterns. These findings indicate that *GL1*, together with HD-Zip and WD40 family transcription factors like *GL2* and *TTG1* that were identified to be regulating the above gene expression, may have a distinctive role in this transcriptional network. They seem to operate independently of *ERF* signalling, particularly during the crucial developmental transition to differentiation, to regulate gene expression associated with mucilage modification and extrusion. Furthermore, the induction of *XTHs*, *PMEs*, *PMEIs*, and *EXPs* by gibberellin (GA) signalling suggests that these genes are part of a broader regulatory network influenced by GA. The observed downregulation of *GID1a*, an upstream regulator of GA signalling, in the *gl1* mutant at 6 DAFO, supports the hypothesis that *GL1* acts as an upstream transcriptional controller of *GID1a*. This regulatory role of *GL1* appears crucial in modulating the expression of genes involved in mucilage modification and extrusion, highlighting its significant role in the developmental processes.

The observed promoting roles of *ERf* and *GL1* in maternally derived endosperm cuticle formation, as reported in Chapter 1, suggest that these factors play significant roles in regulating lipid biosynthesis. However, the precise mechanisms through which *ERf* and *GL1* mediate alterations in cuticle structure and composition require further investigation. The loss of *ERf* function led to gene repression in cutin, wax, and suberin biosynthesis, including GPATs, CYPs, and LACSs, at the earliest developmental stage (0 DAFO). Conversely, the loss of *GL1* function, either alone or in combination with *ERf*, resulted in the activation of these genes around the torpedo stage. Notably, the quadruple mutant exhibited a gene expression profile similar to that of the *gl1* mutant but with increased expression levels. These findings underscore the importance of *ERf* and *GL1* in regulating cuticle development and provide a robust foundation for future research to elucidate the *ERf-GL1*-mediated pathways governing cuticle formation. Further studies are needed to fully characterize how these factors influence cuticle lipid biosynthesis and determine how their interactions contribute to cuticle integrity and function.

Extensive research has highlighted the roles of the *ERf* in regulating various aspects of plant development, including stomatal and inflorescence architecture. Upon ligands binding (mostly ERFs/EPFLs), *ERECTA* transphosphorylates SERKs and activates downstream YODA-MKK4/MKK5-MPK3/MPK6 cascade, which modulates the gene expression regulating stomatal development. In inflorescence development, *ERECTA* utilizes similar immediate signalling components, including BR-SIGNALING KINASE1/2 (BSK1/2) and MAPKs. The regulation of *ERECTA* signalling in both stomatal and inflorescence development is modulated by U-box ubiquitin E3 ligases *PUB30* and *PUB31*, which

attenuate *ERECTA* activity (ref). In the context of early seed development (0 DAFO), loss of *ERf* function results in the deregulation of components such as *MPK3*, *MKK9*, and *FLS2* (which coreceptor with *BAK1* and interact *ERECTA* to mediate MAPK cascade signalling). Notably, the expression patterns of these components are stage-specific, with *MPK3* and *FLS2* being significantly upregulated by 6 DAFO. This indicated that these components are conserved in the *ERf*-mediated seed development but are still regulated via a molecular network specific to the seed development process, as discussed previously. On the other hand, *GL1* regulate the trichome development via WD40-TTG1-bHLH (*GL3*)/(EGL3), and none of the components seems to be conserved in the *GL1*-mediated hormonal signalling, cell wall biosynthesis and cuticle formation. However, the *ERf-GL1* interaction abolished the expression of *MPK3* and *MKK9* and only conserved the *FLS2*. These results indicate a combination of specificity and strong negative interactions of the *ERf* and *GL1* in the transcriptional regulation of MAPK signalling components during early seed development.

In conclusion, this study provides a comprehensive analysis of the roles of *ERf* and *GL1* in seed development, revealing their intricate involvement in transcriptional regulation, hormonal signalling, seed coat development, differentiation and cuticle formation. The differential expression patterns of genes regulated by *ERf* and *GL1* across various developmental stages underscore their critical roles in modulating vital biological processes such as auxin signalling, secondary cell wall biosynthesis, and stress responses, thus potentially modulating the seed adaptability to stress and protecting the seed viability during prolonged exposure to salt. The findings highlight a complex interplay between these transcription factors, with *ERf* and *GL1* exhibiting overlapping and distinct functions in controlling seed development. Particularly, the observed regulatory effects on hormonal pathways, including auxin and ABA signalling, as well as their influence on cell wall component biosynthesis, underscore the multifaceted roles of *ERf* and *GL1* and further advance our understanding of *ERf-GL1* interaction with ABA-GA signalling cascade controlling germination.

Future research should focus on further elucidating the specific mechanisms through which these factors interact and regulate gene expression, particularly in relation to cuticle development and stress response pathways. This will enhance our understanding of seed biology and potentially contribute to agricultural practices aimed at improving seed quality and stress resilience.

5.1.1 Transitioning from model to crop: conservation of *ERECTA -GL1* function in cereals

As the first step of transitioning the novel findings to agronomical practices, I tested the conservation of *ERECTA* function in promoting seed germination under high salinity stress in barley, a crop with a significantly different seed structure from *Arabidopsis* and germination of great importance in the brewing industry. This study identified two close sequence orthologues of *Arabidopsis ERECTA*, one to *AtERL1/ERL2* and one to *AtGL1* in barley and named them as *HvER* (Ert-m/HORVU.MOREX.r3.7HG0662630) and *HvER2* (HORVU.MOREX.r3.6HG0620170.0), *HvERL* (HORVU.MOREX. r3.7HG06445 70) and *HvGL1* (HORVU.MOREX. r3.6HG0596350.1) respectively with similar gene structure to that of *Arabidopsis*. Using CRISPR/cas9, the mutation was introduced in the LRR domain for *Erf* and in the DNA binding domain for *GL1*. Phenotypic characterization of the *Erf +/- GL1* or *GL1* mutant plants revealed that loss-of-function mutations in *HvER* led to significant alterations in plant architecture, including increased tiller numbers and severe sterility, highlighting its conserved function in plant architecture. Although the single ear obtained from *HvER* plants limited our ability to assess inflorescence architecture, the study did reveal that *HvER2* and *HvERL* also influence plant height, the spike architecture, and sterility, with varying effects depending on the specific CRISPR/Cas9 edits and most importantly strong effect on sterility compared to *Arabidopsis* with only one gene - *HvER* KO causing complete or severe sterility suggesting its potential as a candidate for improving barley yield. Similar to *Arabidopsis gl1* in WT background does not show any effect in plant or spike architecture, whereas it showed positive synergy in the *ERL* background (*erl gl1*). The study also underscored the variable impact of point mutations, as evidenced by the QH 3#9 line, where a single amino acid change in *HvER2* resulted in no noticeable phenotypic difference from the wild-type plant. Overall, the data suggest that *HvER* and *HvERL* play a more substantial role in reproductive organ development and fertility compared to their *Arabidopsis* counterparts, *AtER* and *AtERL1/2*, and are critical regulators of barley yield potential. The findings provide valuable insights into the functional specificity of *HvER* family members and their significant contribution to barley plant architecture and yield, positioning them as key targets for enhancing cereal crop productivity.

The *Arabidopsis ERF-GL1* mediated salt hypersensitive germination phenotype was also found to be conserved but with specificity among the species. *HvER2* has a constitutive effect, whereas in *Arabidopsis* those are negligible. *HvER2* and *HvERL* were shown to promote germination rate and speed under salinity stress, as observed in the *AtERf*. Partial resumption when the conditions get favourable and constitutive effect indicate that the *HvERf* function in controlling germination is not completely conditional and this gene family is important in regulating germination even under optimal

conditions. This study also highlighted that salinity stress dramatically influences this timing of coleorhiza break after it emerges through the maternal tissues, particularly in *HvERL* mutants along with this persisting germination delay under salt to some extent in salt hypersensitive barley mutants in dehulled assays suggests that the effects of salt on germination are not solely due to the husk but also involve interactions between the husk and underlying tissues, especially the coleorhiza. Similar to *Arabidopsis GL1*, *HvGL1* plays a significant role in seed germination. The research reveals that *HvGL1* interacts with the *HvERf* pathway, influencing germination speed through a mechanism that intersects with *ERECTA* and salt signalling pathways. This interaction is evident even after husk removal, suggesting that *HvGL1* effects are not solely dependent on the husk but involve complex signalling between the husk and underlying tissues.

In addition, While *HvERf* mutants did not exhibit a direct relationship between seed weight or water uptake and the salt-hypersensitive germination phenotype, they demonstrated notable changes in the anatomical structure of the husk. The findings indicate that *HvER2* influences the formation and distribution of trichomes and stomata, with a distinct trade-off observed in the lower epidermis of the palea. This supports the notion that *HvER2* serves as a crucial node linking stomatal and trichome development. The observed alterations in trichome density and stomatal formation further suggest that these factors are intricately regulated, potentially affecting grain skinning, malting quality, and pathogen susceptibility. Notably, trichomes may play a role in the interaction between the husk and the cementing layer, impacting grain adhesion and potentially influencing germination timing. This study proposes two hypotheses regarding trichome origins and their interaction with the pericarp and cementing layer, highlighting the need for further investigation and extending the investigation to other *ERf +/-GL1* members.

Future research should focus on exploring the mechanical properties of the husk and coleorhiza in *erf +/-gl1* mutants, including their strength and stiffness under different conditions and the differential gene expression in these tissues during germination. Additionally, the expression patterns of *HvERf* and *HvGL1* in various seed tissues during imbibition and exploring their impact on trichome and stomatal patterning in both leaves and seed coverings will provide insights into their signalling pathway. Such research will provide deeper insights into the functional roles of *HvERf* and *HvGL1* orthologues, their interactions with other developmental pathways, and their potential impact on barley growth and yield.

5.2 Future Perspectives

Do *ERf+/-GL1*-mediated maternal seed coat properties affect the mechanical properties of the *Arabidopsis* seed coat and the force required to break open the endosperm and seed coat during germination under different salinity stress conditions?

To fully grasp this, a series of comprehensive studies is necessary.

1. Quantification of Cell Wall Compounds:

The initial focus is on quantifying key cell wall compounds in the *Arabidopsis* seed coat, particularly lignin and proanthocyanidin (PAs), using metabolomic approaches. These compounds play a crucial role in influencing the mechanical properties of the seed coat and may also affect signalling to inner compartments during germination. The differences in the autofluorescence spectrum data suggest variation in lignin and suberin content between the *erf+/-gl1* and WT. Therefore, the quantification of these compounds at different developmental stages (1 day after flowering (1 DAFO), 3 DAFO, and 6 DAFO) is a significant first step. Additionally, the cuticular waxes, including polyester cutin, need to be measured based on the cuticular thickness differences observed in *erf+/-gl1* mutants. Techniques such as Fourier Transform Infrared (FTIR) spectroscopy and mass spectrometry imaging could also be used to observe the spatial distribution of these biochemicals.

2. Measurement of mechanical force:

The mechanical force required to break open the seed coat, and if possible, the tannin cell walls and the endosperm at the micropylar area during imbibition, need to be measured using a nano indenter. If direct measurement is not feasible, we will create 3D structural models of *ERf* and wild-type (WT) seeds based on their quantitative data and then simulate the force required to break these hypothetical seed coats. This approach will help us understand the mechanical properties and the potential contribution to the salt-hypersensitive germination phenotype.

3. Cellular fibrils attachment to the columella in inner mucilage and pectin branching and swelling of mucilage during imbibition:

In the present study, I extended and established that mucilage plays a protective role against salt and osmotic stress, and the *ERf-GL1* signalling loop controls a large part of it. Through the time-lapse microscopy of *erf* and WT seed, I observed that the *erf* mutants had sunken the inner mucilage halo via a reduced length of the cellulose fibrils. This experiment needs to be further investigated using an increased sample size and including *erf gl1* mutants. It can be further extended by removing the outer mucilage and staining only the inner mucilage cellulose rays to study the attachment of cellular fibrils

to the columella and the branching and swelling of pectin during imbibition under both optimal and saline conditions. Once confirmed the effect we need to quantify the inner mucilage and analyse its composition between the *ERf +/-GL1* mutants and WT. This will provide insight s into the *ERf +/-GL1* involvement in regulating the mucilage architecture and swelling/extrusion properties and its influence on germination under stress.

4. Testing the lab model on mucilage and seed coat structure impacting germination

To test the hypothesis that changes in mucilage and seed coat structure impact germination in a salinity-dependent manner due to Na^+ - Ca^{2+} exchange in mucilage and cell walls, we will conduct germination assays in the presence of Ca^{2+} chelators such as EDTA. Microscopy observations of mucilage and seed coat structures will complement these assays.

Validation of RNA-seq identified key genes

The RNA-seq analysis conducted in this study has identified several *ERf*, *GL1* and *ERf-GL1* regulated genes potentially involved in key processes such as lipid biosynthesis, cuticle formation, cell wall and mucilage modification. While these findings provide a valuable foundation for understanding the genetic regulation of these pathways, further experimental validation is necessary to confirm these genes' roles and elucidate their functional mechanisms. Initial validation could be done by selecting a representative subset of genes from different functional categories, including those involved in wax, cutin, and mucilage and cell wall biosynthesis, and analysing their expression in quantitative real-time PCR (qRT-PCR) at the respective seed development stages. Next, functional characterization of the identified key genes could generate KO mutants of the interested genes in *ERf +/-GL1* and WT background using CRISPR/cas9 gene editing.

ERf +/-GL1 signalling in salinity stress signalling during germination?

1 Is there a direct *ERf* signalling role during germination itself?

For this, we need to separate *ERf* effects on seed germination via effects on seed development from independent post-germination *ERf* effects. This can only be done genetically by turning *ERf* expression ON /OFF at anthesis, at the start of imbibition or during appropriate germination stages.

- Seed coat bedding assay where the dead seed coat and the live thin endosperm can be separated and signalling of the endosperm can be examined

- Analysis of *ERf* expression (spatial pattern with reporter fusion proteins) during imbibition and germination.
 - Analysis of *ERf* inducible lines—silencing and induction—under constitutive promoter, seed coat or/and endosperm specific promoters. We already have DEX:ER inducible silencing lines from an earlier project in the Lab. Cross them to the *erl2* or *erl1* non-salt hypersensitive mutants and test for salt hypersensitivity induction upon induction of *ER* silencing by DEX application.
- 2 Molecular components of the *ERf*+/-GL1 pathway in the control of seed sensitivity to salinity
- is there a direct interaction between *ERf-GL1* through ChIPseq and yeast 2-hybrid experiments /CO-IP
 - genome-wide RNA seq analysis of germinating *erf* and WT seeds under optimal and salt conditions
 - germination sensitivity to salinity in mutants knocked out for putative components of the *ERf* signalling pathway according to the current knowledge of *ERf* signalling in other organs and developmental or defence processes ([Shpak, 2013](#)) ([Qi & Torii, 2018](#)) ([Sopena-Torres et al., 2018](#)): EPF/EPFL peptides (putative ligands?), TMM (putative conserved coreceptor?); MAPK cascade (putative conserved components YDA, MKK4, 5; MPK3, 6), in wild type and in cases where mutants are available in *erf* KO background.

Does *HvERf* regulate the short plant stature and disorganized spike architecture by regulating cell proliferation and cell expansion as reported in *Arabidopsis*?

The future research directions outlined below aim to deepen the understanding of the conservation of *ERf* function in barley regulating the plant and spike architecture.

- Investigation of *HvERf* effect on cell proliferation and expansion

As reported, *ERf* regulates the plant and inflorescence architecture by regulating the cell proliferation and expansion. The observed phenotypes of *Hverf* mutant plants suggest that the conservation of that function in barley, too. To further investigate that., future studies should focus on the expression of *HvERf* in the root and shoot apical meristem (SAM and RAM, respectively), and development, and should assess their roles in cell division, elongation and differentiation.

Does *HvERf* +/-GL1 change the mechanical strength of the MATERNAL TISSUES?

This study has reported that maternal tissues, mostly the husk, play a crucial role in regulating the constitutive and salt-hypersensitive germination phenotype in *erf +/-gl1* mutants. Future research should explore the expression of *HvERf* and *HvGL1* in these tissues and examine changes in their mechanical properties., thus the possibility of changing the force required to break open the embryo surrounding tissues contributing to the *Hver2*-mediated constitutive germination phenotype. This includes measuring the thickness of the suberized cell layers in the husk and analysing the composition of cell wall constituents like suberin, cellulose, and lignin using GC-MS. Additionally, quantifying the force required to break open the lemma and palea interlock, potentially using nano-indentation techniques, will provide insights into the mechanical strength of these tissues under various conditions, including salinity stress.

Does *ERf* alter the anatomical and /or compositional changes of coleorhiza during germination?

The coleorhiza, a sheath protecting the emerging root, undergoes significant anatomical and compositional changes under stress conditions. Future research should focus on the differences in coleorhiza anatomy and composition before and after imbibition, particularly under salt stress. Techniques such as Scanning Electron Microscopy (SEM) and proteomics/GC-MS can be employed to visualize and analyze the configuration of interlocking structures and compositional changes that might affect the seed's *ERf +/-GL1* mediated germination phenotype.

Is *ERf +/-GL1* involved in temporal and spatial gene expression during germination?

Understanding the temporal and spatial expression of *HvERf* and related genes during germination, particularly at critical stages like imbibition, Coleorhiza Emerging (CE), and Coleorhiza break (CB), is essential to understanding the primary molecular network involved in *ERf +/-GL1* mediated germination phenotype. Special attention should be given to genes potentially involved in hormonal signalling, such as those regulated by ABA and GA, cell expansion and wall modifications/degradation. As a first step, this could initiate by taking the reference from ([Barrero et al., 2009](#))., monitoring the expression of genes like *HvABA8'OH-1*, *HvABA1*, *HvAPI2*, *NCED1*, *HvPP26*, *HvLPP1 and 2*, *HAV22*, and *HvVIP1* in both coleorhiza and roots under salinity and control conditions.

Translating laboratory findings to agronomic practices

To fully elucidate the role of *HvERf* in germination and plant development, it is crucial to extend the study beyond controlled laboratory conditions and into the field, particularly in areas prone to salinity. The laboratory findings indicate that *HvERf* significantly influences both the speed and final percentage

of germination under optimal and high salinity conditions. Investigating *HvERf* function in field trials using overexpressing lines in saline-prone and normal conditions will be a critical step in validating these laboratory results. Such field studies will help to confirm the practical implications of *HvERf* overexpression in agronomically important barley cultivars. By controlling germination timing through modulating *ERf/GL1* expression levels, it may be possible to achieve more uniform harvesting times and accelerate germination under favourable conditions, thus improving overall crop yield. Moreover, fine-tuning the developmental cycle can optimize the timing of seed set and maturation, reducing the risks associated with plant exposure to adverse environmental conditions.

The insights gained from these field studies will provide a valuable foundation for developing barley varieties that are better adapted to saline environments, thereby enhancing food security and agricultural sustainability in areas affected by soil salinity. This extension of the research represents a significant step towards translating genetic and molecular findings into practical, field-level applications.

5.3 References

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Chapter 6

Appendices

6.1 Appendix 1

Genotyping primers used for sequencing and PCR in the screening of CRISPR/cas9 edited *HvERf*⁺/⁻*GL1* mutants.

Gene	Primer	
HvER 631 F	CCACGGCCCTGTTTTCTTC	PCR
HvER 1205 R	CGTCCGTACATGGAGACGAG	PCR
HvER 672 F	TTCTTGTTCCGACACCCGAG	Seq
HvER 1159 R	AGATTTGCGCCTCGAGGTTG	Seq
HvER2 676 F	GGGGTCAGCAAGCAAGTTTG	Seq
HvER2 628 F	AACTGGGTGTTTTGGGGT	PCR
HvER2 1139 R	GACGCTCTTCAAATCCCCGA	PCR
HvERL F1	AGGGGGAAGACCGAGGAGG	PCR
HvERL R3	CCAGATATGCAGAGCAAGCG	PCR
HvGL F1	ATGGGTAGGTCTCCGTGCT	Seq
HvGL R1	GCTTTCGCTTAGGCAGAG	PCR
HvGL R2	ACGTTCTTGATCTCGTTGTCCG	Seq
HvGL F2	ATCCATCACACTCGACCG	Seq
HvGL R3	GAGCATGCTCACTGCCACA	Seq
HvGL F3	AGCCATTTCCCTCCGACAG	PCR
HvGL R4	CATGGAGAGGCACTGCTCTG	PCR
HvER qPCR F1	ACAGCTTCTTGGTAATCCTGG	qRTPCR
HvER qPCR R1	TCAGAAGGATAACAAGCCCACC	qRTPCR
HvER qPCR F2	TACATGGAAAAATGGCAGCCTCT	qRTPCR
HvER qPCR R2	AGTCATGGTGAAAGGTAGGCAAG	qRTPCR
HvER2 qPCR F1	ACAGCTTCTTGGTAATCCTGG	qRTPCR
HvER2 qPCR R1	AGAATGACAGCCCTTGAGACAG	qRTPCR
HvER2 qPCR F2	AGAAGGAAGCCTCTCTCCAGAT	qRTPCR
HvER2 qPCR R2	TCCAAGACCTGAAAGCTCGTAC	qRTPCR
HvERL qPCR F1	CTCCAGCTGTGGTAACTCACAT	qRTPCR
HvERL qPCR R1	GGCTGTGGTCGCTTGTGTTTAT	qRTPCR
HvERL qPCR F2	TGGCGCTTCAAGTACTGTGTAT	qRTPCR
HvERL qPCR R2	CTGTCTCCAGTTCCGTCTCAA	qRTPCR
HvGL qPCR F1	GGAGGTGTTCCAGATTGACGAT	qRTPCR
HvGL qPCR R1	GCACTATCTGCGCCTAAGGTAT	qRTPCR
HvGL qPCR F2	GATGGAAACCGGCGATACCTTA	qRTPCR
HvGL qPCR R2	GTGACAACTCTGCATGTCACC	qRTPCR
TaUBQ10-rev	CGCAGACGGAGCACCAAGTGAA	qRTPCR
TaUBQ10-for	CGGACCAGCAGCGTCTCATCTTT	qRTPCR
GAPDH-R	CCTACCCCACGGGATCT	qRTPCR
GAPDH-F	TTCGGCGAGAAGCCAGTTA	qRTPCR
UBI-R	CCTTCCTGAGCCTGGTTACCT	qRTPCR
UBI-F	TCGCCGTCTCCAGTTCTAC	qRTPCR
ADP-R	CCGCATTATCGCATTAGG	qRTPCR
ADP-F	CGTGACGCTGTGTTGCTTGT	qRTPCR

6.2 Appendix 2-conformation of the absence of target gene of CRISPR/cas9 edited plants by qPCR.

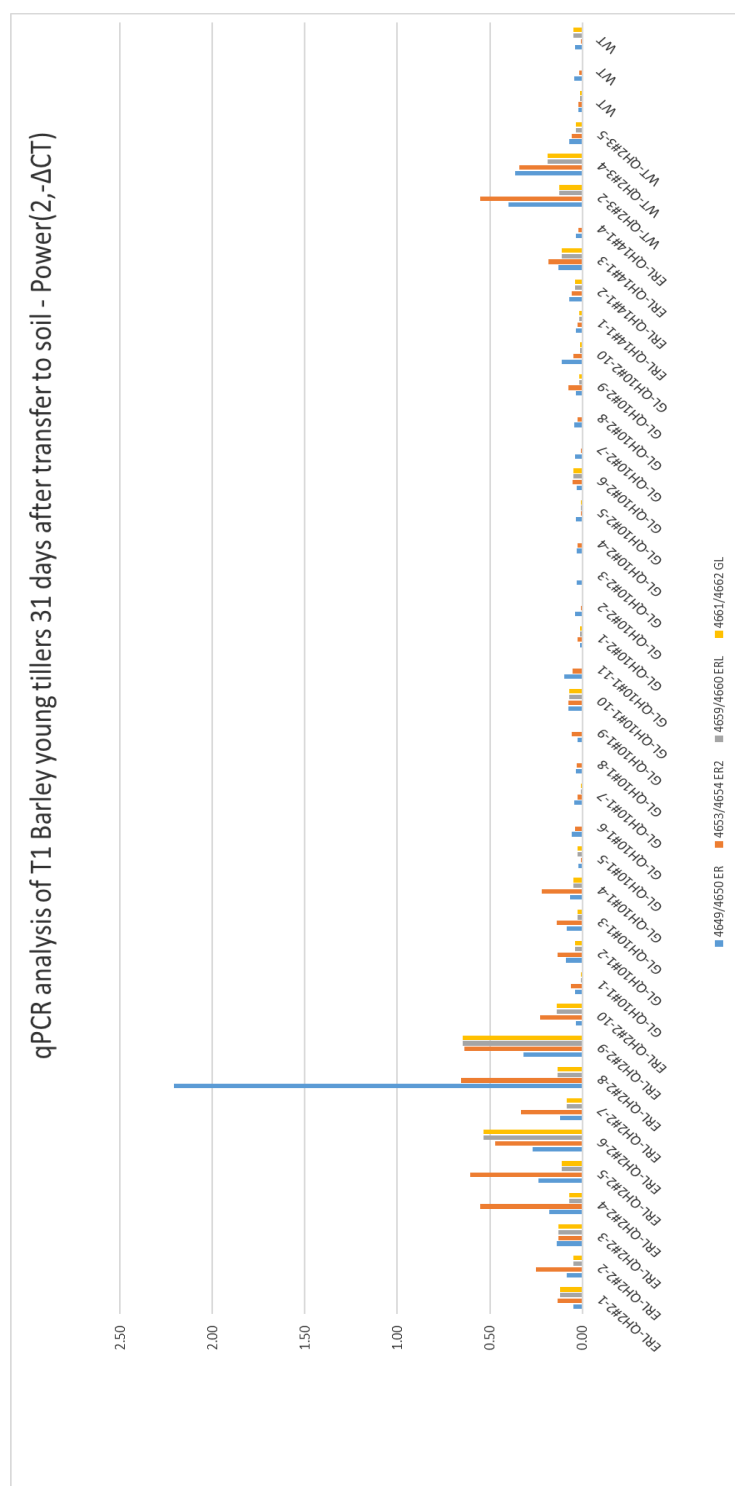


Figure 6-1. Relative gene expression *HvERf* and *HvGL1* of CRISPR/cas9 edited mutant plants

6.3 Appendix 3

RNAseq-Differentially expressed gene ID and names

Gene ID	Gene Symbol	Gene name
AT3G02610	AAD2	Plant stearyl-acyl-carrier desaturase family protein
AT3G25585	AAPT2	Choline/ethanolaminephosphotransferase 2
AT1G49720	ABF1	Absciscic acid responsive element-binding factor 1
AT4G26080	ABI1	ABA insensitive 1
AT3G57510	ADPG1	Polygalacturonase ADPG1
AT2G41850	ADPG2	Polygalacturonase ADPG2
AT3G44720	ADT4	Arogenate dehydratase 4
AT5G08380	AGAL1	Alpha-galactosidase 1
AT5G08370	AGAL2	Alpha-galactosidase 2
AT1G27930	AGM1	Probable methyltransferase At1g27930
AT3G02020	AK3	Aspartokinase 3
AT4G39940	AKN2	Adenylyl-sulfate kinase 2
AT4G13040	APD1	Integrase-type DNA-binding superfamily protein
AT4G13040	APD1	Integrase-type DNA-binding superfamily protein
AT2G14750	APK1	Adenylyl-sulfate kinase
AT5G67520	APK4	Adenylyl-sulfate kinase 4
AT4G16130	ARA1	L-arabinokinase
AT4G14716	ARD2	Acireductone dioxygenase 1
AT4G23980	ARF9	Auxin response factor 9
AT1G74890	ARR15	Two-component response regulator ARR15
AT5G58080	ARR18	Two-component response regulator ARR18
AT5G07210	ARR21	response regulator 21
AT1G25220	ASB1	Anthranilate synthase beta subunit 1
AT5G41040	ASFT	aliphatic suberin feruloyl-transferase
AT3G47340	ASN1	Asparagine synthase 1
AT5G65010	ASN2	Asparagine synthetase 2
AT5G10240	ASN3	Asparagine synthetase 3
AT4G04610	ATAPR1	5'-adenylylsulfate reductase 1
AT1G62180	ATAPR2	5'-adenylylsulfate reductase 2
AT4G21990	ATAPR3	5'-adenylylsulfate reductase 3
AT4G24260	ATGH9A3/KOR3	Glycosyl hydrolase 9A3
AT2G44570	AtGH9B12	Glycosyl hydrolase 9B12
AT4G39000	AtGH9B17	Glycosyl hydrolase 9B17
AT4G39010	AtGH9B18	Glycosyl hydrolase 9B18
AT2G44540	AtGH9B9	Glycosyl hydrolase 9B9
AT1G64390	AtGH9C2	Glycosyl hydrolase 9C2
AT4G11050	AtGH9C3	Glycosyl hydrolase 9C3
AT3G01640	ATGLCAK	Glucuronokinase G

AT2G38010	ATNCER2	-
AT1G11580	ATPMEPCRA	methylesterase PCR A
AT2G01850	ATXTH27	Probable xyloglucan endotransglucosylase/hydrolase protein 27
AT3G44990	ATXTH31	Xyloglucan endotransglucosylase/hydrolase protein 31
AT2G38120	AUX1	Auxin transporter protein 1
AT3G46110	AUXRP3	DUF966 domain containing protein
AT2G27860	AXS1	UDP-D-apiiose/UDP-D-xylose synthase 1
AT3G23920	BAM1	Beta-amylase
AT4G17090	BAM3	Beta-amylase 3
AT4G15210	BAM5	Beta-amylase 5
AT2G32290	BAM6	Beta-amylase 6
AT1G10060	BCAT-1	branched-chain amino acid transaminase 1
AT1G50110	BCAT6	Branched-chain-amino-acid aminotransferase 6
AT3G50750	BEH1	BZR1/BES1 family protein
AT5G12050	BG1	Protein BIG GRAIN 1-like D
AT5G49360	BXL1	Beta-D-xylosidase 1
AT2G45150	CDS4	cytidinediphosphate diacylglycerol synthase 4
AT1G70710	CEL1	cellulase 1
AT1G02800	CEL2	cellulase 2
AT4G39350	CESA2	Cellulose synthase A2
AT5G44030	CESA4	Cellulose synthase A4
AT5G17420	CESA7	Cellulose synthase catalytic subunit 7
AT4G18780	CESA8	Cellulose synthase 8
AT2G21770	CESA9	Probable cellulose synthase A catalytic subunit 9
AT2G43570	CHI	Chitinase
AT3G12500	CHI-B	Basic endochitinase B
AT4G04870	CLS	Cardiolipin synthase (CMP-forming)%2C mitochondrial
AT1G69370	CM3	Chorismate mutase 3
AT5G15630	COBL4	COBRA-like protein 4
AT4G11140	CRF1	Ethylene-responsive transcription factor CRF1
AT3G61630	CRF6	cytokinin response factor 6
AT3G03630	CS26	Probable S-sulfocysteine synthase
AT5G22740	CSLA2	Glycosyltransferase (Fragment)
AT3G43670	CUAOGAMMA2	copper amine oxidase gamma 2
AT2G34490	CYP710A2	Cytochrome P450 710A2
AT3G10570	CYP77A6	Cytochrome P450 77A6
AT5G04630	CYP77A9	Cytochrome P450 77A9
AT1G01600	CYP86A4	Cytochrome P450 86A4
AT1G63710	CYP86A7	Cytochrome P450 86A7
AT2G45970	CYP86A8	Cytochrome P450 86A8
AT5G23190	CYP86B1	Cytochrome P450 86B1
AT1G57750	CYP96A15	Cytochrome P450 96A15
AT5G28020	CYSD2	Bifunctional L-3-cyanoalanine synthase/cysteine synthase D2
AT3G59890	DAPB2	Dihydrodipicolinate reductase

AT5G23940	DCR	BAHD acyltransferase DCR
AT2G23340	DEAR3	dreb and ear motif protein 3
AT5G28030	DES1	Bifunctional cystathionine gamma-lyase/cysteine synthase
AT1G48300	DGAT3	Diacylglycerol acyltransferase 3
AT5G20250	DIN10	Raffinose synthase family protein
AT3G44460	DPBF2/BZIP67	Basic-leucine zipper (bZIP) transcription factor family protein
AT5G64860	DPE1	4-alpha-glucanotransferase DPE1
AT2G40840	DPE2	4-alpha-glucanotransferase DPE2
AT5G44480	DUR	Putative UDP-arabinose 4-epimerase 4
AT2G25490	EBF1	EIN3-binding F-box protein 1
AT5G25350	EBF2	EIN3-binding F-box protein 2
AT2G41070	EEL	Transcription factor homologous to ABI5
AT2G27050	EIL1	ETHYLENE INSENSITIVE 3-like 1 protein
AT5G25190	ERF003	Ethylene-responsive transcription factor ERF003
AT1G28360	ERF012	Ethylene-responsive transcription factor 12
AT5G47220	ERF02	Ethylene-responsive transcription factor 2
AT1G12630	ERF027	Ethylene-responsive transcription factor ERF027
AT2G44940	ERF034	Ethylene-responsive transcription factor ERF034
AT2G35700	ERF038	ERF family protein 38
AT4G16750	ERF039	Ethylene-responsive transcription factor ERF039
AT5G47230	ERF05	Ethylene-responsive transcription factor ERF05
AT5G65130	ERF057	Integrase-type DNA-binding superfamily protein
AT1G64380	ERF061	Ethylene-responsive transcription factor ERF061
AT1G53170	ERF08	Ethylene-responsive transcription factor 8
AT5G44210	ERF09	Ethylene-responsive transcription factor 9
AT5G67190	ERF10	Ethylene-responsive transcription factor ERF010
AT1G28370	ERF11	ERF domain protein 11
AT5G50080	ERF110	Ethylene response factor 110
AT5G61890	ERF114	Ethylene-responsive transcription factor ERF114
AT4G17500	ERF1A	Ethylene-responsive transcription factor 1A
AT3G23240	ERF1B	Ethylene-responsive transcription factor 1B
AT4G39780	ERF60	Ethylene-responsive transcription factor ERF060
AT1G04310	ERS2	Ethylene response sensor 2
AT3G23150	ETR2	Ethylene receptor 2
AT3G12120	FAD2	fatty acid desaturase 2
AT5G22500	FAR1	Putative fatty acyl-CoA reductase 1
AT5G22420	FAR7	Putative fatty acyl-CoA reductase 7
AT5G60490	FLA12	Fasciclin-like arabinogalactan protein 12
AT4G28370	FLY1	Transmembrane E3 ubiquitin-protein ligase FLY1
AT5G47770	FPS1	Farnesyl pyrophosphate synthase 1
AT4G12250	GAE5	UDP-D-glucuronate 4-epimerase 5
AT3G10700	GAE6	Galacturonic acid kinase
AT3G10700	GALAK	Galacturonic acid kinase
AT1G02720	GATLS5	Probable galacturonosyltransferase-like 5

AT2G30575	GAUT5	Probable galacturonosyltransferase 5
AT2G14960	GH3.1	indole-3-acetic acid-amido synthetase GH3.1
AT1G28130	GH3.17	indole-3-acetic acid-amido synthetase GH3.17
AT2G23170	GH3.3	Indole-3-acetic acid-amido synthetase GH3.3
AT2G47750	GH3.9	indole-3-acetic acid-amido synthetase GH3.9
AT3G05120	GID1A	GA insensitive dwarf1A
AT5G14470	GLCAK2	Probable glucuronokinase 2
AT3G14130	GLO4	Peroxisomal (S)-2-hydroxy-acid oxidase GLO4
AT1G56600	GOLS2	Galactinol synthase 2
AT4G26250	GOLS6	Galactinol synthase 6
AT1G06520	GPAT1	Glycerol-3-phosphate acyltransferase 1
AT4G01950	GPAT3	glycerol-3-phosphate acyltransferase 3
AT1G01610	GPAT4	Glycerol-3-phosphate acyltransferase 4
AT3G18660	GUX1	UDP-glucuronate:xylan alpha-glucuronosyltransferase 1
AT3G18660	GUX1	UDP-glucuronate:xylan alpha-glucuronosyltransferase 1
AT1G33800	GXMT1	Glucuronoxylan methyltransferase 1
AT1G58080	HISN1A	ATP phosphoribosyl transferase 1
AT5G48930	HST	Shikimate O-hydroxycinnamoyltransferase
AT4G14560	IAA1	indole-3-acetic acid inducible 1
AT4G14550	IAA14	indole-3-acetic acid inducible 14
AT1G04250	IAA17	indole-3-acetic acid inducible 17
AT3G15540	IAA19	indole-3-acetic acid inducible 19
AT3G23030	IAA2	indole-3-acetic acid inducible 2
AT5G25890	IAA28	indole-3-acetic acid inducible 28
AT3G62100	IAA30	indole-3-acetic acid inducible 30
AT3G17600	IAA31	indole-3-acetic acid inducible 31
AT1G52830	IAA6	indole-3-acetic acid 6
AT3G23050	IAA7	indole-3-acetic acid 7
AT4G13430	IIL1	3-isopropylmalate dehydratase large subunit
AT5G14200	IMDH3	3-isopropylmalate dehydrogenase
AT3G58990	IPMI1	Isopropylmalate isomerase 1
AT2G43100	IPMI2	Isopropylmalate isomerase 2
AT1G18500	IPMS1	Isopropylmalate synthase 1
AT4G39800	IPS1	Inositol-3-phosphate synthase isozyme 1
AT2G22240	IPS2	Inositol-3-phosphate synthase isozyme 2
AT5G10170	IPS3	Probable inositol 3-phosphate synthase isozyme 3
AT1G03310	ISA2	Isoamylase 2
AT4G09020	ISA3	Isoamylase 3
AT1G17380	JAS5	Jasmonate-zim-domain protein 5
AT1G72450	JAS6	Jasmonate-zim-domain protein 6
AT5G16560	KAN1	Transcription repressor KAN1
AT1G04710	KAT1	ketoacyl-coa thiolase 4
AT1G53000	KDSB	Nucleotide-diphospho-sugar transferases superfamily protein
AT5G03770	KDTA	Probable 3-deoxy-D-manno-octulosonic acid transferase

AT1G62990	KNAT7	Homeobox protein knotted-1-like 7
AT2G38080	LAC4	Laccase-4
AT2G47240	LACS1	Long chain acyl-CoA synthetase 1
AT1G64400	LACS3	Long chain acyl-CoA synthetase 3
AT1G77690	LAX3	auxin influx carrier LAX3
AT3G15356	LEC	Lectin-like protein LEC
AT1G04640	LIP2	lipoyltransferase 2
AT3G45140	LOX2	Lipoxygenase 2
AT1G67560	LOX6	Lipoxygenase 6
AT1G12640	LPLAT1	Lysophospholipid acyltransferase 1
AT4G30270	MERI-5	Xyloglucan endotransglucosylase/hydrolase protein 24
AT3G03780	MS2	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 2
AT1G09540	MYB61	MYB61
AT1G52880	NARS2/ANAC018	NAC domain containing protein 18
AT5G45110	NPR3	NPR1-like protein 3
AT1G76690	OPR2	12-oxophytodienoate reductase 2
AT3G55610	P5CSB	Delta-1-pyrroline-5-carboxylate synthase
AT3G52430	PAD4	Lipase-like PAD4
AT5G05590	PAI2	N-(5'-phosphoribosyl)anthranilate isomerase 2
AT5G17990	PAT1	Anthranilate phosphoribosyltransferase
AT5G13640	PDAT1	Phospholipid:diacylglycerol acyltransferase 1
AT2G33150	PED1	peroxisome defective 1
AT1G59870	PEN3	penetration 3
AT3G50990	PER36	Peroxidase 36
AT5G51820	PGMP	Phosphoglucomutase
AT3G29320	PHS1	Alpha-glucan phosphorylase 1
AT3G46970	PHS2	Alpha-glucan phosphorylase 2
AT2G01420	PIN4	Auxin efflux carrier component
AT4G38530	PLC1	Phosphoinositide phospholipase C 1
AT5G58690	PLC5	Phosphoinositide phospholipase C 5
AT3G55940	PLC7	Phosphoinositide phospholipase C 7
AT4G11830	PLDGAMMA2	Phospholipase D gamma 2
AT1G04680	PLL26	Pectate lyase
AT1G53840	PME1	Pectinesterase
AT2G36710	PME15	Probable pectinesterase 15
AT2G43050	PME16	Probable pectinesterase/pectinesterase inhibitor 16
AT3G14310	PME3	Pectinesterase/pectinesterase inhibitor 3
AT4G02330	PME41	Probable pectinesterase/pectinesterase inhibitor 41
AT1G23200	PME6	Probable pectinesterase/pectinesterase inhibitor 6
AT2G47280	PME66	Probable pectinesterase 66
AT5G46960	PMEI12	Pectinesterase inhibitor 12
AT2G47670	PMEI6	Pectinesterase inhibitor 6
AT2G47550	PMEI-PME20	Probable pectinesterase/pectinesterase inhibitor 20

AT3G10720	PMEI-PME25	Probable pectinesterase/pectinesterase inhibitor 25
AT3G60730	PMEI-PME65	Pectinesterase
AT5G14800	PROC1	Pyrroline-5-carboxylate reductase
AT4G35630	PSAT1	Phosphoserine aminotransferase 1
AT2G38310	PYL4	Absciscic acid receptor PYL4
AT4G01026	PYL7	regulatory components of ABA receptor 2
AT1G01360	PYL9	regulatory components of ABA receptor 1
AT3G47390	PYRR	Riboflavin biosynthesis protein PYRR
AT3G07970	QRT2	Polygalacturonase QRT2
AT4G36900	RAP2-10	Ethylene-responsive transcription factor RAP2-10
AT1G13260	RAV1	AP2/ERF and B3 domain-containing transcription factor RAV1
AT1G68840	RAV2	Ethylene response DNA binding factor2
AT3G57520	RFS2	Probable galactinol--sucrose galactosyltransferase 2
AT5G17490	RGL3	DELLA protein RGL3
AT5G15650	RGP2	UDP-arabinopyranose mutase 2
AT5G50750	RGP4	Probable UDP-arabinopyranose mutase 4
AT5G46340	RWA1	Reduced wall acetylation 1
AT1G29890	RWA4	O-acetyltransferase family protein
AT3G02630	S-ACP-DES5	Stearoyl-acyl-carrier-protein
AT1G55920	SAT1	Serine acetyltransferase 1
AT4G35640	SAT4	serine acetyltransferase 3;2
AT5G18010	SAUR19	small auxin up RNA 19
AT2G28085	SAUR2	small auxin up RNA 2
AT5G18020	SAUR20	small auxin up RNA 20
AT5G18030	SAUR21	small auxin up RNA 21
AT5G18050	SAUR22	small auxin up RNA 22
AT5G18060	SAUR23	small auxin up RNA 23
AT5G18080	SAUR24	small auxin up RNA 24
AT3G03850	SAUR26	small auxin up RNA 26
AT3G03840	SAUR27	small auxin up RNA 27
AT3G03820	SAUR29	small auxin up RNA 29
AT2G46690	SAUR32	small auxin up RNA 32
AT3G61900	SAUR33	small auxin up RNA 33
AT4G34800	SAUR4	small auxin up RNA 4
AT1G19830	SAUR54	small auxin up RNA 54
AT1G29430	SAUR62	small auxin up RNA 62
AT1G29450	SAUR64	small auxin up RNA 64
AT1G29500	SAUR66	small auxin up RNA 66
AT1G29510	SAUR67	small auxin up RNA 67
AT1G29490	SAUR68	small auxin up RNA 68
AT3G12830	SAUR72	small auxin up RNA 72
AT4G36110	SAUR9	small auxin upregulated RNA 9
AT5G67360	SBT1.7	Subtilisin-like protease SBT1.7
AT5G11190	SHN2	Ethylene-responsive transcription factor SHINE 2

AT5G25390	SHN3	Ethylene-responsive transcription factor SHINE 3
AT3G50500	SNRK2.2	SNF1-related protein kinase 2.2
AT4G01970	STS	stachyose synthase
AT1G70230	TBL27	Trichome birefringence-like 27
AT3G55990	TBL29/ESK1	Trichome birefringence-like 29
AT5G01360	TBL3	Trichome birefringence-like 3
AT2G40320	TBL33	Trichome birefringence-like 33
AT1G25560	TEM1	AP2/ERF and B3 domain-containing transcription repressor TEM1
AT4G27070	TSB2	Tryptophan synthase
AT5G19200	TSC10B	3-dehydrosphinganine reductase TSC10B
AT4G23920	UGE2	UDP-glucose 4-epimerase 2
AT1G63180	UGE3	UDP-D-glucuronate 4-epimerase 3
AT1G21070	URGT2	UDP-rhamnose/UDP-galactose transporter 2
AT2G28315	UXT1	UDP-xylose transporter 1
AT1G53700	WAG1	Serine/threonine-protein kinase WAG1
AT1G75500	WAT1	Protein WALLS ARE THIN 1
AT2G46400	WRKY46	Probable WRKY transcription factor 46
AT3G56400	WRKY70	Probable WRKY transcription factor 70
AT5G37300	WSD1	O-acyltransferase (WSD1-like) family protein
AT5G12420	WSD7	O-acyltransferase (WSD1-like) family protein
AT3G48580	XTH11	Probable xyloglucan endotransglucosylase/hydrolase protein 11
AT5G57550	XTH25	Probable xyloglucan endotransglucosylase/hydrolase protein 25
AT4G18990	XTH29	Probable xyloglucan endotransglucosylase/hydrolase protein 29
AT1G32170	XTH30	Probable xyloglucan endotransglucosylase/hydrolase protein 30
AT2G06850	XTH4	Xyloglucan endotransglucosylase/hydrolase protein 4
AT5G65730	XTH6	Xyloglucan endotransglucosylase/hydrolase
AT4G37800	XTH7	Probable xyloglucan endotransglucosylase/hydrolase protein 7
AT4G03210	XTH9	Xyloglucan endotransglucosylase/hydrolase (Fragment)
AT5G57655	XYLA	Xylose isomerase
AT4G09760	_	Probable choline kinase 3
AT4G16800	_	3-methylglutaconyl-CoA hydratase
AT5G08250	_	Cytochrome P450 superfamily protein
AT2G31810	_	Acetolactate synthase small subunit 2
AT1G14810	_	Aspartate-semialdehyde dehydrogenase
AT2G45300	_	3-phosphoshikimate 1-carboxyvinyltransferase
AT4G24910	_	Glucuronoxylan 4-O-methyltransferase-like protein (DUF579)
AT3G17940	_	Aldose 1-epimerase
AT3G47800	_	Aldose 1-epimerase
AT5G18200	_	ADP-glucose phosphorylase
AT2G43620	_	_
AT5G61680	_	Pectin lyase-like superfamily protein
AT3G15720	_	Probable polygalacturonase At3g15720

