The Australian National University | Research School of Biology | Division of Plant Sciences

Analysis of *Fusarium oxysporum* effectors shared between strains that infect cape gooseberry and tomato

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I declare that this thesis is my own account of my work and contains as its main content that has not been previously submitted for any degree at any tertiary education institution. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given. Materials obtained for use in this study that were generated by others have been acknowledged accordingly in the text.

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Abstract

Physalis peruviana, known as cape gooseberry, is a solanaceous plant native to tropical South America, typically growing in the Andes at 2000 m. Its economic value has grown due to its nutritional and medicinal properties. However, a vascular wilt disease caused by a newly discovered *forma specialis* of *Fusarium oxysporum,* (here designated f. sp. *physali* [*Foph*]), has become one of the limiting factors in cape gooseberry production, with losses up to 90%.

The *F. oxysporum* species complex incudes numerous *formae speciales* (special forms), which are the causal agents of vascular wilt disease in a broad range of plants, including economically important crops such as banana, cotton, melon, tomato and recently, cape gooseberry. *F. oxysporum* f. sp. *lycopersici* (*Fol*) causes wilt disease on tomato. At least fourteen small secreted in xylem (SIX) proteins have been identified from the xylem sap of *Fol*-infected tomato plants. Five are associated with virulence and three are recognised by resistance proteins in the host. However, the function of most of these SIX proteins remains unclear.

In this project, six homologues of *Fol SIX* genes (*SIX1a*, *SIX1b*, *SIX7*, *SIX10, SIX12* and *SIX15*) and a homologue of Ave1 (an avirulence gene present in the broad-host-range wilt-pathogen *Verticillium dahliae,* with homologues in many other phytopathogens including *Fol*), were identified in *Foph*. These and other candidate effector genes were identified by mapping *Foph* RNAseq data against the *Fol* lineage-specific transcriptome and candidate *F. oxysporum* effector genes identified in other *formae speciales* in other studies. The *Foph SIX* gene and *Ave1* homologues were found to encode proteins with 70 to 100% identity to their *Fol* counterparts, with the latter suggesting recent horizontal transfer of a cluster of *SIX* genes comprising *SIX7*, *SIX10*, *SIX12* and *SIX15*.

The *Foph* SIX1a and SIX1b proteins are 74% and 80% identical, respectively, to their *Fol* counterpart. Both homologues were tested in a Δ*SIX1* strain of *Fol* to see if they could complement the virulence function of *Fol SIX1* in tomato. The results showed no restoration of virulence for ten *SIX1a* and six *SIX1b* transformants tested, suggesting that their function might be restricted to cape gooseberry pathogenicity. *Foph SIX1a* and *SIX1b* transformants were also tested to see if they might be recognised by tomato plants carrying the *I-3* resistance gene, which enables recognition of *Fol* SIX1 (i.e. Avr3). The results indicated that SIX1a was not recognised while SIX1b was recognised, suggesting that *Foph*-SIX1b may be recognised by I-3 as an avirulence factor and that the *I-3* resistance gene could potentially be used in cape gooseberry plants to mediate *Foph* resistance.

To investigate the function of *SIX7*, *SIX10* and *SIX12*, a triple gene knockout strategy was initiated to assess their role in *Fol* virulence. This strategy included the use of the HSV*tk* (Herpes Simplex Virus thymidine kinase) gene as a counter selection marker against the ectopic insertion of transfer DNA (T-DNA) during fungal transformation by *Agrobacterium tumefaciens*. However, after several transformation attempts no gene knockouts were obtained. Attempts to produce single (*SIX10*) and double (*SIX7* and *SIX12*) knockouts also failed.

The *Fol* Rapid Alkalinisation Factor (RALF) gene was also subjected to gene disruption using this approach. Four RALF knockouts were obtained out of 44 transformants thereby validating the gene deletion strategy described above. Pathogenicity tests in tomato showed that these four mutants all developed disease symptoms that were not significantly different from those of wild type *Fol* under the assay conditions used.

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Chapter 1.

Introduction

1.1 The cape gooseberry as an important crop species

1.1.1 Description, distribution and importance

Physalis peruviana, known as cape gooseberry, from the family *Solanaceae*, is a tropical native fruit of South America found typically growing in the Andes at an altitude of 1,500 to 3,000 meters above sea level (Figure 1.1). The cape gooseberry was known to the Incas and after European contact the species was introduced into Africa, India and other parts of the world including South East Asia (Popenoe et al., 1990). In Colombia, over the last three decades, the cape gooseberry has been transformed from a wild and under-utilized species to an important exotic fruit for national and international markets (Simbaqueba et al., 2011). The commercial interest in this fruit has grown due to its nutritional value related to high vitamin, mineral and antioxidant content, as well as its anti-inflammatory, anti-cancer and other medicinal properties (Martinez et al., 2010, Yen et al., 2010, El-Gengaihi et al., 2013).

A. B. C.

Figure 1.1. Cape gooseberry fruit. A. A fruiting cape gooseberry plant growing in the Colombian Andean region. B. Dessert presentation of the fruit (left) and a close-up of the characteristic fruit calyx (right). C. Ripe fruit used as a fresh product. Photographs courtesy of Luz Stella Barrero, Corpoica, Colombia.

Colombia is the main producer of cape gooseberry in the world followed by Zimbabwe, Malaysia, China, Kenya and South Africa (Barrero et al., 2012). The economic value of the cape gooseberry in Colombia has grown annually since 1991, and in 2013 exports brought USD 27.6 million into the country from about 7000 tons and, after banana, the cape gooseberry represents the second most exported fruit in Colombia (Barrero et al., 2012).

Despite its increasing value on the international market, the production of cape gooseberry has faced a major decrease in Colombia, from 1087 ha with a yield of 17.8 t in 2009, to 749 ha with a yield of 15 t in 2013 (Barrero et al., 2012). This decline in production could be associated with two important aspects of cape gooseberry as a crop species. One is the low genetic diversity observed as a consequence of the use of few cultivars. The second is the low technology nature of cape gooseberry cultivation and the resulting absence of adequate phytosanitary measures (Barrero et al., 2012).

1.1.2 Vascular wilt disease as a limiting factor for cape gooseberry production

One of the most important disease problems in cape gooseberry is the vascular wilt disease caused by a newly discovered forma specialis of the fungus *Fusarium oxysporum,* here designated *F. oxysporum* f. sp. *physali* (*Foph*). This disease was first described in 2005 and has become one of the limiting factors for cape gooseberry production and export. Field observations indicated typical symptoms of a vascular wilt disease (Figure 1.2) with an incidence ranging from 10 to 50% (Barrero et al., 2012). In 2011, losses in production of 90% were reported (unofficially) in the Cundinamarca central region of Colombia. As a consequence, producers moved to other places in the same region, spreading contaminated plant material and seeds.

A. B.

Figure 1.2. Cape gooseberry plantations at the production stage located in Cundinamarca (central region of Colombia). **A**. Cape gooseberry plants showing wilting, browning and death of stems, leaves and fruit calyces caused by *Foph* infection. **B**. Healthy cape gooseberry plants showing green stems, leaves and fruit calyces. Photographs courtesy of Edwin Rodriguez, Corpoica, Colombia, were taken in 2014 on different farms.

In 2012, samples of wilted cape gooseberry plants were collected from the central region of Colombia. A total of 205 fungal isolates were obtained and described as *Fusarium oxysporum*, using Koch postulates and molecular markers for intergenic spacers (IGS) specific to *F.oxysporum*. From the 205 isolates, one was found to be highly virulent on a commercial variety of cape gooseberry (Edwin Rodriguez, Corpoica, pers. comm.). An RNAseq analysis was performed to study differential gene expression in susceptible and resistant cape gooseberry plants inoculated with this isolate of *Foph* (Gina Garzon, 2013, Corpoica, pers. comm.). Although, the genome of *Foph* has been recently sequenced using the Illumina HiSeq 2500 platform (San Diego, California, USA) (Carolina Gonzalez, 2017, Corpoica, pers. comm.), the genome assembly of *Foph* was still not available at the time of writing this thesis. Despite the identification of a highly virulent strain of *Foph* and the genomic resources available for this isolate, little is known about the interaction between *Foph* and cape gooseberry.

1.2 Vascular wilt disease in plants caused by *Fusarium oxysporum*

1.2.1 *Fusarium oxysporum* is a species complex

Fusarium oxysporum is a cosmopolitan ascomycete fungus that commonly inhabits agricultural soils. Rather than a single species, it is a species complex of nonpathogenic, plant pathogenic, and human pathogenic strains, termed the *Fusarium oxysporum* species complex (FOSC) (Di Pietro et al., 2003; Michielse and Rep 2009; O'Donnell et al. 2009; Ma et al., 2013, 2014). Several hundred different members of the FOSC are able to penetrate plant roots, colonise xylem vessels and produce vascular wilt diseases in a broad range of host plants, including economically important crops such as banana (plantain), cotton, date palm, onion, brassicas, cucurbits, legumes and solanaceous species, such as tomato, eggplant, chilli and cape gooseberry, but not grasses (Michielse and Rep, 2009).

However, individual pathogenic isolates of *Fusarium oxysporum* are highly host specific and have therefore been classified into different *formae speciales* (ff. spp.) according to the host they infect e.g. strains that infect banana can not infect tomato plants and vice versa (Armstrong and Armstrong, 1981; Lievens et al., 2008; Michielse and Rep 2009; Ma, 2014). *F. oxysporum* has no known sexual stage and the mechanism for species diversification has been associated with the parasexual cycle through heterokaryon formation, which enables a mitotic genetic exchange between different nuclei (Glass et al., 2000; Di Pietro et al., 2003).

1.2.2 *Fusarium oxysporum*-host interaction

The two best-studied interactions between *Fusarium oxysporum* and a plant host are the interaction between *Arabidopsis thaliana* and *F. oxysporum* f. sp. *conglutinans* (*Foc*), which is model pathosystem for ff. spp. that infect the *Brassicaceae* (Ospina-Giraldo et al., 2003; Berrocal-Lobo and Molina, 2004; Thatcher et al., 2012) and the interaction between *F. oxysporum* f. sp. *lycopersici* (*Fol*) and tomato (*Solanum lycopersicum*), which is one of the best-studied *F. oxysporum* pathosystems. Although *Fol* does not have a high impact on tomato production (due to the effective deployment of disease resistance genes) compared with the severe damage to cape gooseberry production caused by *Foph*, it is currently one of the best models to study the molecular basis of disease resistance and susceptibility in response to *F. oxysporum* infection (Rep et al., 2004; Houterman et al., 2008, 2009). It is also a model pathosystem for ff. spp. that infect the *Solanaceae,* of which cape gooseberry is also a member.

Several resistance genes against *Fol* (*I*, *I-2, I-3* and *I-7*) have been introgressed into tomato from wild relatives (Ori et al., 1997; Simons et al., 1998; Catanzariti et al., 2015, 2017; Gonzalez-Cendales et al., 2016). Three races of *Fol* have been described and named 1, 2 and 3 (Scott and Gardner, 2006) according to their pattern of virulence on tomato cultivars carrying *I, I-2* and *I-3*. The *I* gene, introgressed from *S. pimpinellifolium*, confers resistance to *Fol* race 1 (Bohn and Tucker, 1939). *I* mediated resistance was overcome by *Fol* race 2, but the *I-2* gene introgressed from a hybrid between *S. pimpinellifolium* and *S. lycopersicum* confers resistance to *Fol* race 2 (Stall and Walter 1965). *I-2* mediated resistance was overcome by *Fol* race 3, but the *I-3* gene introgressed from *S. pennellii* confers resistance to *Fol* race 3 (Scott and Jones, 1989; Lim et al., 2006).

The *I-7* gene, also introgressed from *S. pennelli* (McGrath et al. 1987; Lim et al., 2006), confers resistance to all three races (Gonzalez-Cendales et al., 2016). The *I* and *I-7* genes encode leucine-rich-repeat receptor-like proteins (Gonzalez-Cendales et al., 2016; Catanzariti et al., 2017); the *I-2* gene encodes a cytosolic coiled-coil nucleotide-binding leucine-rich-repeat protein (Simons et al., 1998); and the *I-3* gene encodes an S-receptor-like kinase (Catanzariti et al., 2015). These receptors recognise specific pathogenicity proteins or effectors, which are secreted by *Fol* into the xylem during host infection, known as SIX proteins. The SIX4 (Avr1) protein is recognised by I, SIX3 (Avr2) is recognised by I-2 and SIX1 (Avr3) is recognised by I-3, while the effector recognised by I-7 has not yet been established (Rep et al., 2004; Houterman et al., 2008, 2009).

1.3 *Fusarium oxysporum* infection

1.3.1 Adhesion and colonisation of the host roots

F. oxysporum can survive in the soil in the form of conidia, microconidia and chlamydospores, which are specialised thick-walled spores able to survive for long periods without a host and tolerate the effects of fungicides applied to the soil. Therefore, the use of resistant varieties is the most effective alternative to control the disease (Armstrong and Armstrong, 1981; Agrios, 1997).

The close proximity of host roots induces the dormant chlamydospores to germinate and initiate infection, penetrating the roots through natural openings at the junctions of epidermal cells (Bishop and Cooper, 1983; Rodriguez-Gálvez and Mendgen, 1995). Invasive hyphal growth and adhesion to host roots depend on the cell wall integrity (CWI) MAPK cascade, which is regulated by the MAPK genes *Fmk1* and *Msb2* (Di Pietro et al., 2001; Rispail and Di Pietro, 2009; Perez-Nadales and Di Pietro, 2011). Recent evidence suggests that the CWI-MAPK cascade is involved in chemotropic sensing of root exudates to promote spore germination and hyphal adhesion to host roots. Gene deletion mutants of downstream components of the CWI-MAPK cascade showed impaired growth towards root exudates (Turra et al., 2015). *Fol* chemotropism is also mediated by the host plant type III peroxidases secreted in root exudates, which generate a peroxidase gradient in the rhizosphere that is sensed by the α-pheromone receptor Ste2 in *Fol.* A *Ste2* deletion mutant showed reduced disease symptoms on tomato plants, indicating that chemotropic sensing of plant exudates has a role in host infection (Turra et al., 2015, 2016).

After penetration, the hyphae colonise the cortical cells and form an interconnected network of mycelium that advances between host cells to reach the root cortex and invade the xylem vessels, where the fungus proliferates through the production of microcondia (Di Pietro et al., 2003). A correlation between virulence and rapid colonisation of the host vascular system was found when a highly virulent strain (HV) of *F. oxysporum* f. sp. *phaseoli* (specific to common bean) was compared with a weakly virulent (WV) strain (Nino-Sanchez et al., 2015). Furthermore, the HV strain showed differential up-regulation of the FTF1 transcription factor and the effector genes *SIX1* and *SIX6*, suggesting that they may be involved in rapid colonisation of xylem vessels (Nino-Sanchez et al., 2015). In *Fol*, *SIX1* is expressed upon penetration of the host root cortex (van der Does et al., 2008). *SIX1* expression is strongly upregulated during host colonisation and in the presence of living but not dead tissue, suggesting that *Fol* might

detect an undescribed host signal, allowing it to distinguish dead (plant) material from living plants (van der Does et al., 2008).

1.3.2 Proliferation through the xylem vessels

The target tissues of *Fol* are the xylem vessels, which allow the transport of water and solutes from the roots to upper plant parts and are characterised by low nutrient content. Although this tissue represents a harsh environment for a pathogen to succeed, the major part of the *F. oxysporum* lifecycle is in the xylem, where the fungus is able to obtain all the factors required for growth, reproduction, and survival (Yadeta and Thomma, 2013). Once in the xylem, the hyphae of *Fol* advance through the vessels and disseminate microconidia with xylem sap movement, which eventually germinate to form hypahe that colonise the upper parts of the plant. Wilting symptoms appear during this stage as a result of water stress due to vessel clogging by the accumulation of fungal biomass, and tyloses, pectin-rich gels and gums produced by the host to prevent the pathogen spreading to adjacent xylem vessels (Rahman et al., 1999; Di Pietro et al., 2003; Yadeta and Thomma, 2013).

Fol uses nutrients such as secondary nitrogen sources available in the xylem for its growth. Specifically, the *Gap1*, *Mtd1*, and *Uricase* genes, involved in nitrogen aquisition, are induced during host infection (Divon et al., 2006). Furthermore, the expression of these genes is controled by the global nitrogen regulator transcription factor FNR1, which also mediates nitrogen uptake by the fungus (Di Pietro et al., 2003; Michielse and Rep, 2009; Yadeta and Thomma, 2013).

Fol secretes enzymes that degrade the cell wall of the xylem vessls in tomato, which thereby provides a nutrient source to facilitate hyphal colonisation of the adjacent parenchyma cells, where the fungus is able to manipulate host immunity and obtain additional nutrients to complete its lifecycle (Di Pietro et al., 2003; Agrios, 2005). Although *F. oxysporum* secretes an array of cell wall degrading enzymes, such as polygalacturonases, pectate lyases, xylanases and proteases, during root penetration and colonisation, their specific roles in pathogenicity have not been determined due to functional redundancy (Di Pietro et al., 2003; Michielse and Rep, 2009). The pathogenic cycle of *F. oxysporum* ends once the host tissue collapses and dies as a result of continued cell wall degradation and severe water stress due to vessel clogging. The fungus then sporulates from the dead host tissue, releasing conidia onto the soil where eventually resting structures are produced (Di Pietro et al., 2003; Agrios, 2005; Fradin and Thomma, 2006; Michielse and Rep, 2009; Yadeta and Thomma, 2013).

1.4 *F. oxysporum* genome structure and function in pathogenicity

Among the *Fusarium* species whose genomes have been sequenced, *F. oxysporum* has the largest at 61 Mb compared to *F. graminearum* 36 Mb*, F. verticillioides* 42 Mb and *F. solani* 51 Mb. This increase in genome size of *F. oxysporum* might be a consequence of genome duplications or horizontal acquisition of supernumerary lineage-specific (LS) chromosomes or chromosome regions from foreign genomes (Ma et al., 2010, 2013; Vlaardingerbroek et al., 2016a, b). Horizontal transfer of *Fol* LS chromosome 14 was demonstrated when a pathogenic strain of *Fol* was co-cultivated *in vitro* with a non-pathogenic strain of *F. oxysporum* (Ma et al., 2010). The non-pathogenic recipient aquired virulence on tomato plants, indicating that the genes located on chromosome 14 are involved in *Fol* pathogenicity. Moreover, the gain or loss of LS chromosomes or chromosome regions appears to be related to the genetic diversity of the species in the absence of a sexual cycle, allowing the rapid emergence of new pathogenic lineages (Ma et al., 2014; Vlaardingerbroek et al., 2016).

Fol LS chromosome 14 is small in size (around 2 Mb) and has a low gene content but a high content of transposable elements. Despite a low gene content, this chromosome is rich in genes that encode small secreted proteins called SIX (Secreted In Xylem) proteins (Houterman et al., 2007; Ma et al., 2010). The proteins encoded by six of these *SIX* genes have been identified in the xylem sap of tomato plants infected with *Fol* (Houterman et al., 2007; Schmidt et al., 2013). These genes were characterised by the presence of a miniature impala (*mimp*) transposable element located upstream of the coding region (Schmidt et al., 2013). Eight more SIX genes were identified by the presence of an upstream *mimp* element and the presence of proteins encoded by seven of these genes was confirmed in the xylem sap of *Fol*-infected tomato plants (Schmidt et al., 2013). Three of the *SIX* genes correspond to *Avr* genes with corresponding resistance genes in tomato (Rep et al., 2004; Houterman et al., 2008, 2009; see section 1.2.2). However, the role of the *SIX* genes in pathogenicity and host recognition remains unknown for most of the *Fol SIX* genes identified to date (Brown et al., 2012, Ma et al., 2013, Schmidt et al., 2013).

Homologues of *Fol SIX* genes have been identified by PCR and sequencing in other ff. spp. (Table 1.1). Furthermore, the presence of *mimp* elements was used to identify four *Fol* genes encoding secreted enzymes that may be associated with virulence (Schmidt et al., 2013) and 11 novel candidate effectors in the genome sequence *F. oxysporum* f. sp. *melonis* (*Fom*) (Schmidt et al., 2015). *mimp* elements have also been identified upstream of *FoAve1* (a *Fol* homologue of *Ave1* from *Verticillium dahliae*) and in the *SIX1,*

SIX4, SIX8, SIX9, SIX13 and *SIX14* homologues of legume-infecting ff. spp. of *F. oxysporum* (Schmidt *et al*., 2013; Williams et al., 2016; Table 1.1).

Table 1.1 *Fol-SIX* homologues identified and reported in different *formae speciales* (ff. spp.) of *Fusarium oxysporum*

Host plants	f. sp.	*Fol-SIX* homologous genes													References
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Banana	*cubense*	P	S		S			P S	P S	S				P S		Meldrum et al., 2012 Guo et al., 2014 Taylor et al., 2016
Onion	*cepae*			P S		P S		P S		P S	P S		P S		P S	Sasaki et al., 2015 Taylor et al., 2016
Cucurbitaceae	*melonis*	P	S				P S					S		S		Rocha et al., 2015
	niveum						P S			P S		S		S		Niu et al., 2016
	cucumerinum						P S			P S		S		S		Schmidt et al., 2016 vanDam et al., 2016
Cotton	*vasinfectum*						P S									Chakrabarti et al., 2011
Date palm	*canariensis*	P	S					P S			P S					Laurence et al., 2015
Brassicaceae	*conglutinans*	P	S		P S				S	S						Thatcher et al., 2012 Kashiwa et al., 2013
Legumes	*ciceris*					S			S	S		S		S	S	Li et al., 2016
	medicaginis	S							S	S				S		Rocha et al., 2016 Taylor et al., 2016
	pisi	P	S													Williams et al., 2016
	phaseoli	P	S				P S	P S	P							Nino-Sanchez et al., 2015

P= PCR screening based identification
S= sequencing based identification

1.5 *F. oxysporum* effector proteins

Effectors are small proteins secreted by a broad range of plant pathogenic organisms, including bacteria, fungi, oomycetes and nematodes, which interfere with cellular structure and function in their host plants. Effectors can facilitate the lifestyle of the pathogen and determine the level of host specialisation. They can also dictate the outcome of an attempted infection by either suppressing basal immunity i.e. acting as virulence factors, or triggering the defence response of the host i.e. acting as avirulence factors (Kamoun, 2006, 2007; Hogenhout et al., 2009; Lo Presti et al., 2015).

Common criteria used to identify fungal effector proteins are the presence of a signal peptide region, small size (i.e. less than 300 amino acids in length) and the presence of multiple cysteine residues. However, the lack of sequence similarity, extensive sequence diversification, gene duplication, and other genetic rearrangements, such as recombination with novel protein domains, suggest diverse phylogenetic origins (Stergiopoulos and De Wit, 2012). Therefore, it has been difficult to find similar features in effector sequences that allow their more precise classification as a group or protein family (Stergiopoulos and de Wit, 2009; Lo Presti et al., 2015; Guillen et al., 2015).

As a phytopathogenic fungus, *F. oxysporum* uses effector proteins that are secreted during host colonisation to establish a successful infection. These include enzymes that target plant physical or chemical barriers, such as the previously mentioned cell wall degrading enzymes, small cysteine-rich proteins, such as the majority of the previously mentioned SIX proteins, and necrosis-inducing proteins, among others (Di Pietro et al., 2003; van der Does and Rep, 2007; Michielse and Rep, 2009; de Sain and Rep, 2015).

1.5.1 The role of the *F. oxysporum* RALF protein in pathogenicity

Many phytopathogens have the ability to secrete compounds that mimic plant hormones, enabling them to hijack host defences, metabolism, and development (Chanclud and Morel, 2016). In *Fol*, a homologue of the plant rapid alkalinisation factor (*RALF*) gene has been recently characterised for its role in pathogenicity (Masachis et al., 2016; Thynne et al., 2016). Plant RALF peptides are small secreted cysteine-rich proteins known to be involved in growth and development of roots and pollen tubes (Pearce *et al.*, 2010; Murphy and De Smet, 2014). They represent a large protein family in plants and are widely distributed amongst a diverse range of bacteria and fungi, including phytopathogens (Thynne et al., 2016).

In *Fol*, RALF expression has been observed during root host invasion (3 and 6 days post inoculation) (Thynne et al., 2016). Synthesised *Fol* RALF peptide triggered extracellular alkalinisation in tomato cell suspensions, similar to that produced by the plant homologue in *Nicotiana tabacum* (*Nt* RALF) (Masachis et al., 2016). Moreover, when leaf discs of the solanaceous species *Nicotiana benthamiana* and tomato were treated with the synthesised *Fol* RALF and tomato RALF (*Sl* RALF) peptides, both showed a rapid burst of ROS (reactive oxygen species) production, and similar extracellular alkalinisation for both peptide treatments. (Thynne et al., 2016). These results suggest that plant cells can perceive the *Fol* RALF protein.

Functional analysis of the *Fol RALF* gene in pathogenicity revealed that deletion mutant strains (*Fol*-ΔRALF) show a significant reduction in tomato mortality compared to plants inoculated with the *Fol* wild type strain (WT) (Masachis et al., 2016). Furthermore, the virulence of *Fol*-ΔRALF strains was restored when the pH at the site of the infection was increased to seven (Masachis et al., 2016). These results suggest that RALF may be involved in *Fol* pathogenicity by increasing the extracellular pH of the host roots (Masachis et al., 2016). However, Thynne et al. (2016) reported no significant difference in the development of disease symptoms in plants inoculated with *Fol*-ΔRALF strains, compared with plants inoculated with *Fol*-WT. Nevertheless, Masachis et al. (2016) suggests that the alkalinising effect of RALF may be important for the regulation of the *Fmk1* gene which is involved in host colonisation and is activated upon an increase of pH (Di Pietro et al., 2001; Perez-Nadales et al., 2014; Turra et al., 2014, 2015).

1.5.2 The role of *SIX* genes in *F. oxysporum* host specialisation and pathogen diversification

In *Fol*, proteins encoded by the *SIX* genes have been associated with host specificity as virulence determinants (Lievens et al., 2009). SIX3 (Avr2) is recognised intracellularly by I-2, suggesting that Avr2 may be translocated into the plant cell (Houterman et al., 2009), while SIX1 (Avr3) and SIX4 (Avr1) are thought to be recognised at the plasma membrane by the I-3 and I receptor proteins, respectively (Catanzariti et al., 2015, 2017). Interestingly, a *Fol* knockout showing strong loss of virulence on tomato plants compared to the wild type strain (WT) also showed loss of expression of four *SIX* effector genes (*SIX1*, *SIX2*, *SIX3* and *SIX5*) in infected tomato cell cultures and supports their role in pathogen virulence (Michielse et al., 2009). This gene designated *Sge1* (*SIX*-gene expression 1) appears to encode a transcription factor that regulates expression of the *SIX* genes in *Fol* (Michielse et al., 2009).

In *Fol*, *SIX4* is present in race 1 but generally absent in races 2 and 3. However, some isolates of *Fol* races 2 and 3 contain a non-functional *SIX4* truncated by a transposon insertion (Inami et al., 2012; Kashiwa et al., 2016a). This finding suggests an additional mechanism for the emergence of a new race of *Fol* besides gene deletion. Furthermore, a point mutation in the *SIX3* gene of race 2 resulted in the emergence of *Fol* race 3 (Kashiwa et al., 2016a).

Although *Fol SIX4* is not required for virulence on susceptible tomato plants (Houterman et al., 2008), a gene deletion mutant of the *SIX4* homologue in the *Arabidopsis* infecting strain *Fo5176* showed reduced virulence on susceptible Arabidopsis plants, suggesting a role in pathogenicity (Thatcher et al., 2012). Interestingly, however, *Fol SIX4* is able to suppress the resistance response conferred by the *I-2* and *I-3* resistance genes, so does contribute to virulence on some otherwise resistant tomato plants (Houterman et al., 2008).

SIX1 (Avr3) was the first SIX protein identified in the xylem sap of tomato plants infected with *Fol* (Rep et al., 2002, 2004). Subsequent characterisation showed that SIX1 was involved in pathogenicity, since strains with the *SIX1* gene knocked out showed reduced disease symptoms on a susceptible tomato line, but gained virulence on a tomato line containing the *I-3* gene. These findings suggest that the resistance conferred by the *I-3* tomato line is based on the recognition of the SIX1 effector (Rep et al., 2004, 2005). *Fol SIX1* is strongly upregulated in roots during the early stage of host colonisation (before 4 days post inoculation) and only in the presence of living plant cells (van der Does et al., 2008).

Homologues of *SIX1* have been found in *F. oxysporum* ff. spp. *conglutinans* (*Foc*) *cubense*, *melonis* and *pisi*, but not *cucumerinum, niveum* or *radicis-cucumerinum* (van Dam et al., 2016; Table 1.1). Functional analysis of the *SIX1* homologue in *Foc* showed a significant increase in gene expression during cabbage infection compared to mycelia growth *in vitro* (Li et al., 2016). Furthermore, *Foc SIX1* deletion mutants showed a significant reduction in virulence compared to the wild type strain. Virulence was restored when the deletion mutant was complemented with the *Foc SIX1* gene, as previously demonstrated for *Fol SIX1* in tomato (Rep et al., 2004). However, *Fol* SIX1 did not complement the *Foc SIX1* deletion, suggesting that *SIX1* variants may be host specific (Li et al., 2016). This finding suggests that adaptive evolution of *SIX1* may be required to allow *F. oxysporum* to infect a different host species.

The *SIX5* and *SIX6* effectors also contribute to *Fol* pathogenicity. *SIX5* and *SIX3* (*Avr2*) are clustered together in divergent orientation and share a common bidirectional promoter (Schmidt et al., 2013; Ma et al., 2015). Functional analysis of *Fol SIX5* revealed that gene deletion mutant strains display reduced pathogenicity on a susceptible tomato cultivar and restored *Fol* pathogenicity on tomato *I-2* plants, revealing a functional link between the SIX3 and SIX5 proteins through a dual requirement for activation of I-2-mediated resistance (Ma et al., 2015). Furthermore, *in vitro* protein-protein interaction and *in planta* bimolecular fluorescence complementation assays showed that SIX3 and SIX5 proteins physically interact (Ma et al., 2015). The role of SIX5 in the recognition of Avr2 by I-2 remains to be determined.

The *F. oxysporum* SIX6 effector is conserved among several ff. spp. (Table 1.1). In the cotton-infecting f. sp*. vasinfectum* (*Fov*), *SIX6* was detected only in Australian strains and not in strains with a different origin (Chakrabarti et al., 2011). In f. sp. *phaseoli* the presence of *SIX6* is associated with aggressiveness on soybean (Lanubile et al., 2016), suggesting a role in virulence. In *Fol*, qPCR analysis showed expression of *SIX6* during host infection but no expression was observed in mycelia grown *in vitro* (Gawehns et al., 2014). Plants inoculated with *Fol SIX6* knockout strains showed a significant increase in plant weight with some also showing less severe disease symptoms compared with those inoculated with *Fol* wild type or ectopic transformants, indicating a reduction in virulence (Gawehns et al., 2014).

Interestingly, heterologous expression of SIX6 suppressed cell-death and ion leakage induced by co-expression of *Avr2* and *I-2* in *Nicotiana benthamiana*, but did not suppress resistance to race 2 (carrying Avr2) in *I-2* tomato, suggesting that the resistance mediated by I-2 might not require host cell death (Gawehns et al., 2014). Functional analysis of a *SIX6* homologue in *F. oxysporum* f. sp. *niveum* (*Fon*) suggests that *SIX6* can also act as an avirulence factor, since *Fon SIX6* deletion mutants showed significantly enhanced virulence on a resistant watermelon cultivar (Niu et al., 2016). Furthermore, complementation of race 2 (which lacks the *SIX6* gene) with *Fon SIX6*, resulted in reduced virulence on a race 2-susceptible watermelon cultivar, supporting an avirulence role for *SIX6* in the watermelon-*Fon* pathosystem.

The remaining SIX proteins also have homologues in other ff. spp. (Table 1.1). Expression of *SIX8*, *SIX9* and *SIX13* homologues has been reported in the legume infecting ff. spp. *ciceris*, *medicaginis*, and *pisi* (Williams et al., 2016), while *SIX7, SIX9, SIX10* and *SIX12* homologues have been found in the onion-infecting f. sp. *cepae* and *SIX7* and *SIX10* homologues have been found in the date-palm-infecting f. sp.

canariensis (Laurence et al., 2015; Taylor et al., 2016). However, to date, the role of the above-mentioned *SIX* genes in pathogenicity has not been established in any *F. oxysporum* ff. spp.

1.6 Research project

The research presented in this thesis focuses on the recently described *Foph* pathosystem, due to its impact on cape gooseberry production in Colombia (Barrero et al., 2012). Cape gooseberry has been explored recently for the identification of plant immunity-related genes and SNPs (single nucleotide polymorphisms) that could be associated with resistance against *Foph* (Enciso-Rodriguez et al., 2013; Osorio-Guarin et al., 2016). However, little is known about the interaction between *Foph* and cape gooseberry. Corpoica, Colombia, has conducted an RNAseq analysis from stem and roots of resistant and susceptible cape gooseberry plants inoculated with a highly virulent strain of *Foph,* in order to identify plant genes differentially expressed during infection (Garzon-Martinez et al., unpublished). In this project, the same RNAseq data has been used to identify pathogen genes expressed during infection.

The first aim of this project was to identify putative *Foph* effector genes that may play a role in pathogenicity on cape gooseberry and could potentially serve as avirulence factors corresponding to resistance genes that might already be present in cape gooseberry germplasm or be introduced from other solanaceous plants such as tomato. Two approaches were taken. The first and ultimately unsuccessful approach, was to attempt a de novo assembly of *Foph* transcripts from the RNAseq data to enable identification of transcripts encoding small secreted cysteine-rich proteins that could potentially function as effectors. The second and ultimately successful approach, was to identify RNAseq reads homologous to known or putative effector genes identified in other ff. spp. of *F. oxysporum* and generate full-length gene sequences for further analysis. The second aim of this project was to carry out a functional characterisation of the putative effector genes identified by these two approaches. Unfortunately, this objective was limited by the inability to work directly with *Foph* owing to an export ban imposed by Colombian authorities. Therefore, the tomato/*Fol* interaction was used as a proxy pathosystem to undertake further characterisation of the putative effector genes shared between *Foph* and *Fol.* Again, two approaches were taken. Where a knockout of a shared gene already existed in *Fol* and had an effect on virulence/avirulence, the corresponding *Foph* homologues was tested for ability to complement the virulence/avirulence defect. Where no knockout of a shared gene existed in *Fol* and its role in virulence was unknown, an attempt was made to generate knockouts in *Fol* and

test their effect on virulence in tomato pathogenicity assays, with a view to subsequent complementation tests using their *Foph* homologues.

Chapter 2.

Identification of effector candidates in *Fusarium oxysporum* f. sp. *physali* (*Foph*)

2.1 Introduction

Small proteins secreted by a broad range of plant pathogens, including bacteria, fungi, oomycetes and nematodes, that interfere with the cellular structure and function of their hosts are known as effector proteins (Kamoun, 2006, 2007; Hogenhout et al., 2009). The low level of homology among fungal effectors makes it difficult to identify common features that allow their classification as a group or protein family (Stergiopoulos and de Wit, 2009; Lo Presti et al., 2015; Guillen et al., 2015). Nevertheless, many fungal effectors have been identified based on the presence of a signal peptide sequence for secretion, small size of around 300 amino acids or less, and the fact that they are often cysteine-rich.

Comparative genomics of phytopathogens in the genus *Fusarium* (i.e. *F. graminearum, F. verticillioides* and *F. oxysporum* f. sp. *lycopersici* [*Fol*]), revealed the presence of lineage specific (LS) chromosomes and chromosomal regions in *Fol* that were rich in repetitive elements and contained genes encoding known or putative effector proteins (Ma et al., 2010). Among them, 14 genes were identified that encode small proteins secreted into the xylem sap of tomato plants infected with *Fol* (called SIX proteins) (Houterman et al., 2007; Schmidt et al., 2013). Three of these *SIX* genes are avirulence genes (*Avr*), with resistance (*R*) gene counterparts identified in tomato (Simons et al., 1998; Rep et al., 2004; Houterman et al., 2008, 2009; Catanzariti et al., 2015, 2017).

A large scale search for putative effector genes in 59 strains of various ff. spp., resulted in a set of 104 candidate effectors including the 14 *Fol* SIX genes (van Dam et al., 2016). From this candidate effector repertoire, strains were classified according to the putative effector sequences they shared. Interestingly, all the cucurbit-infecting ff. spp. (i.e. *melonis*, *niveum*, *cucumerinum* and *radicis-cucumerinum*) were grouped together in a separate supercluster, sharing an overlapping set of putative effectors, possibly associated with the ability of those ff. spp. to infect cucurbit host species (van Dam et al., 2016). This supercluster largely excuded *SIX2, SIX3, SIX4, SIX5, SIX7, SIX10, SI12* and *SIX14,* but included substantial overlap with *SIX1, SIX6, SIX8, SIX9, SIX11 and SIX13.* In legume-infecting ff. spp. of *F. oxysporum*, homologues of *Fol SIX1*, *SIX4*, *SIX8*, *SIX9*, *SIX13* and *SIX14* were identfied (Williams et al., 2016). In this case, the effector identification strategy was based on prediction of small-secreted proteins from RNAseq

assemblies of host-infected tissue. The aim of the work decribed in this chapter was to identify any *Foph* homologues of the *Fol SIX* genes or putative effector genes from other ff. spp. of *F. oxysporum.*

An RNAseq analysis was carried out by the Colombian Corporation for Agricultural Research (CORPOICA), Colombia, comparing gene expression in cape gooseberry plants infected by *F. oxysporum* f. sp. *physali* (*Foph*) with non-infected plants (Carolina Gonzalez, Corpoica, 2010, pers. comm.). However, this analysis did not include a study of the *Foph* transcriptome expressed during host infection. In this chapter, unpaired RNAseq reads from cape gooseberry plants infected with *Foph* were obtained from Corpoica and used to analyse the *Foph in planta* transcriptome. The *Foph* RNAseq were mapped against the *Fol* 4287 LS transcriptome, enabling the identification of *Foph* homologues of *Fol SIX1*, *SIX7*, *SIX10*, *SIX12*, *SIX15* and Fo*Ave1*.

2.2 Materials and Methods

2.2.1 *Foph* RNAseq data source

Two biological replicates of susceptible cape gooseberry seedlings (10 cm in height) (accession number 09U274-1 from the *in vitro* germplasm bank at CORPOICA), were inoculated with a suspension of *Foph*, adjusted to $1x10^4$ cfu/ml. Inoculations were performed by the root-dip method (Wellman, 1939; Mes et al., 1999) with a few modifications. Cape gooseberry roots were dipped for 3 minutes before replanting into a sterilized 3:1 ratio of soil:rice husk substrate in plastic pots. Mock-inoculated seedlings were dipped into sterile water. Plant disease progression was observed by two weeks after inoculation.

Root and stem tissues from two plants were collected at 4 days post-inoculation (dpi), and flash frozen in liquid nitrogen. The samples were immediately pooled for RNA extraction and cDNA synthesis (Bio S&T Inc. Montreal, QC, Canada). Total RNA was extracted using a modified TRIzol method (Invitrogen, Carlsbad, California, USA). About 10 µg of purified total RNA was used to carry out a modified SMART^{TM} cDNA synthesis (Clontech, California, USA). RNAseq libraries were generated from purified mRNA and sequenced (100 bp single-end reads) on an illumina HiSeq 2000 platform. A total of 38,874,746 RNAseq reads were obtained from infected cape gooseberry plants. The RNAseq data were generated by Carolina Gonzalez from CORPOICA, Colombia in 2010 and shared as part of a collaboration established between Dr Jones at ANU and Dr Gonzalez at CORPOICA.

2.2.2 *F. oxysporum* transcriptome databases

Transcript sequences for genes in the LS regions (i.e. chromosomes 3, 6, 14, 15 and segments of chromosomes 1 and 2) were originally retrieved from the *Fol* strain 4287 coding sequence (CDS) database at the Broad Institute. However, this database was recently transferred to the EnsemblFungi web site (Kersey et al., 2016). An additional database of 89 candidate secreted *F. oxysporum* effectors was obtained from van Dam et al. (2016). Two additional candidate effectors, CRX1 and CRX2 (GenBank accessions KP965011.1 and KP965012.1), found in f. sp. *cepae* (Taylor et al., 2016) and three, FOXM_15788, FOXM_109214 and FOXM_16306, found in the legume-infecting ff. spp. *medicaginis*, *ciceris* and *pisi* (Williams et al., 2016), were included along with the van Dam et al. (2016) database of putative effectors for further analysis.

2.2.3 RNAseq mapping analysis for identification of homologous effectors

The core genome of *F. oxysporum* is highly conserved among several ff. spp. Therefore, only the *Fol* CDS database from the LS regions (i.e. specific to *Fol* and containing genes involved in host infection), was compared against the RNAseq reads from cape gooseberry plants inoculated with *Foph*, in order to identify homologues of effectors or effector candidates present in the *Fol* LS transcriptome. An additional mapping analysis was performed comparing the RNAseq data with the database of 94 putative effectors described in Section 2.2.2 to identify putative effectors not represented in the *Fol* LS transcriptome. The mapping analysis was performed using CLC Genomics Workbench v7.0 with default parameters except for the similarity and length fraction which were decreased to 70% and 80% respectively, to allow the mapping of homologous sequences from the genomes of various ff.spp. Homologous transcripts less than 3 kb in length with at least seven unique *Foph* reads mapped were chosen. Their length coverage by *Foph* RNAseq reads was then examined manually to select transcripts with complete or nearly complete mapping coverage for further analysis.

2.2.4 PCR testing for presence of *Fol* effector homologues in *Foph*

In silico identifications of *Fol* effector homologues in *Foph* were tested by PCR amplification from *Foph* genomic DNA using primers SIX1F/SIX1R, SIX7F/SIX7R, SIX10F/SIX10R, SIX_inter1F/SIX_inter1R, SIX_inter2F/SIX_inter2R, and Ave1F/Ave1R (Table 2.1), which were designed to amplfy the genomic regions of their counterparts in *Fol*. PCR and sequencing were performed at CORPOICA due to the restricted access to *Foph* and its DNA imposed by Colombian authorities. Despite requests for access made

by the university, permission to import and use *Foph* was not approved during the course of this project. However, the sequences from the PCR products were made available for analysis.

Table 2.1 Primers used to confirm the presence of homologue effectors in *Foph*

Primer	Sequence	homologue region	Predicted size
SIX1F	AAAATGGCGCCCTATAGCAT	*SIX1*[a]	847 bp
SIX1R	GGCTTTGGATCGCAACTTAG		
SIX1.1F	AGAAAGCGTGAGCCTGTCG	*SIX1b*[b]	480 bp
SIX1.1R	CGCATGCCCATGAGTGTC		
SIX1.2F	GGAAAGCGTGAGATTGTCC	*SIX1a*[b]	504 bp
SIX1.2R	AAGGCATGACCATGAGACTG		
SIX7F	CTTTTCCATTTCGCCCTGT	*SIX7*[c]	640 bp
SIX7R	GAAAACGAAAGTCAGCAAGG		
SIX10F	TGAAGCTCTTGTGGTTGATTCC	*SIX10*[c]	409 bp
SIX10R	GGACCATGAGGTGAATAGTCG		
SIX_inter1F	GACCAACTCGAAACGTGACA	Intergenic region 1[d]	1.4 kb
SIX_inter1R	CCAAGCTATGCCACTCCTGA		
SIX_inter2F	GGTCCAACGCTGAGACAACT	Intergenic region 2[e]	2.3 kb
SIX_inter2R	CGTGTTGACATTTTGGGTGA		
Ave1F	TCTCGCGTCACCATTAGTCA	*Ave1*[f]	299 bp
Ave1R	TGTATGCTGCAGCGTGTAGT		

[a] Reported by Rep et al. (2004)

[b] *SIX1* homologues identified in *Foph*

[c] Reported by Schmidt et al. (2013)

[d] Intergenic region between *SIX10* and *SIX12* in *Fol* genome

[e] Intergenic region between *SIX12* and *SIX7* in *Fol* genome

[f] *Fol* homologue of *Verticillium dahliae Ave1* effector gene (Schmidt et al., 2013)

2.2.5 Phylogenetic analysis

Using Blastp searches, the predicted protein sequences of the *Fol* effector homologues identified in *Foph* were compared with homologues reported for other ff. spp. in the UniProt database (The UniProt Consortium, 2015), Protein sequences that showed more than 65% identity with the corresponding *Foph* homologue were retrieved for subsequent analysis. Multiple sequence alignments of the protein sequences retrieved from the Blastp searches were generated using the ClustalW algorithm in MEGA software v.7.0 (Kumar et al., 2015). Phylogenetic trees were constructed using the Maximum Likelihood *and Neighbor-Joining Methods in MEGA.* The statistical significance of each node was assessed using 1000 bootstrap iterations.

2.3 Results

2.3.1 *Fol* effector gene homologues identified in *Foph*

Single-end RNAseq reads were obtained from susceptible cape gooseberry plants inoculated with *Foph* and mapped against the *Fol* LS transcriptome. A total of 104 *Fol* LS sequences were identified and 53 were selected as effector candidates based on the criteria described in section 2.2.3. Of these, 29 corresponded to unpositioned genes in the *Fol* genome and the remainder corresponded to genes located in LS regions and chromosomes including 11 genes located on the pathogenicity chromosome 14 (Table 2.2).

Table 2.2 Putative candidate effector homologues identified by mapping *Foph* RNAseq reads against the *Fol* LS transcriptome

	***Fol* transcripts**	
***Fol* LS chromosome or sub region**	Number of genes to which *Foph* reads mapped	Number of genes selected for further analysis based on length of coverage by *Foph* reads
1-LS sub region	3	1
2-LS sub region	3	-
3	11	3
6	14	8
14	19	11
15	1	1
Unpositioned	53	29
Total	**104**	**53**

The majority of the homologous transcripts encoded proteins with enzymatic activity (23) or proteins with unknown function (15). Six homologues encoded known or putative effector proteins. The remaining nine homologues, encoding proteins predicted to be involved in transcription, intracellular functions, secondary metabolism and transport, were not analysed further (Figure 2.1, Appendix 2.1).

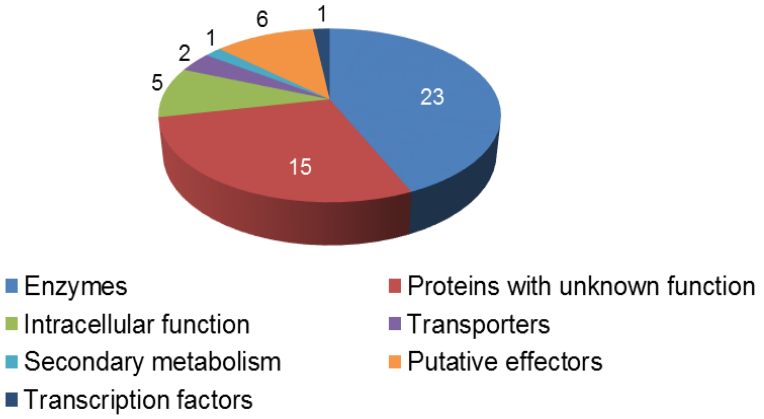


Figure 2.1 Functional categories of the *Fol* homologues expressed in *Foph*-infected cape gooseberry at 4 dpi. Most of the homologues are involved in enzymatic activity or encode proteins of unknown function.

From the homologues identified in *Foph* that encode known or putative effector proteins in *Fol* (Figure 2.1), five corresponded to genes that encode proteins secreted in the xylem i.e. *SIX1*, *SIX7*, *SIX10* and *SIX12*, or predicted to be secreted in the xylem i.e. *SIX15* (Genbank accession KY073750; Gonzalez-Cendales et al., unpublished). A homologue of the *Verticillium dahliae* effector *Ave1*, which is conserved in *F. oxysporum,* was also identified (Table 2.3). Interestingly, four homologues shared between *Foph* and *Fol* (FOXG_16255, FOXG_17645, FOXG_17359), which encoded proteins of unknown function, had characteristics of effector proteins as they are predicted to contain a signal peptide region (except for FOXG_16255), cysteine residues and to be small in size (except for FOXG_17359). Therefore, they were also included in this study as putative effector homologues (Table 2.3). The remaining ten homologues encoding proteins of unknown function were not analysed further.

Table 2.3 *Fol* effector genes or candidate effector genes with homologues expressed at 4 dpi in *Foph*-infected cape gooseberry

Transcript ID	Protein product	*Fol-4287* chromosome location	Predicted protein size (amino acids)	SP[1]	Cysteine residues[2]
FOXG_16418[3]	SIX1	14 (1282269-1283123)[4]	284	Yes	8
FJ755836.1[5]	SIX7	14 (1096828-1097490)[4]	220	Yes	3
FOXG_17457[3]	SIX10	14 (1092281-1092800)[4]	149	Yes	2
KU710369[5]	SIX12	14 (1094024-1094455)[4]	127	Yes	10
KY073750[5]	SIX15	14 (1160235-1160458)[4]	112	Yes	8
JQ283440[5]	Ave1	14 (1367590-1367964)[4]	124	Yes	4
FOXG_16255[3]	Hypothetical proteins	6 (2736879-2737591)[6]	126	No	3
FOXG_17359[3]		Unpositioned genes	371	Yes	2
FOXG_17645[3]			106	Yes	8

[1]Predicted signal peptide sequence, [2]Number of cysteine residues after signal peptide cleavage, [3]Ensembl Fungi ID, [4]Nucleotide position on chromosome 14, [5]Genbank ID, [6]Nucleotide position on chromosome 6.

Of the 23 homologous transcripts encoding proteins with enzymatic activity, 22 were analysed further as they might be potentially involved in pathogenicity (e.g. have a functional annotation associated with polysaccharide degradation or encode enzymes predicted to be secreted in the xylem) (Table 2.4). Transcripts encoding enzymes that lack a predicted signal peptide were also included because they could potentially play a role in pathogenicity (Table 2.4). To examine whether these predicted *Fol* transcripts were incorrect and whether they might contain a signal peptide sequence, a manual analysis of the reads mapping to these transcripts was performed, focusing on any possible extension of the open reading frame (ORF) in the consensus sequences. However, in all cases the predicted ORFs covered the mapped reads with no indication of an extended 5′ end that might encode a signal peptide domain. Nevertheless, these enzymes have putative functions involved in polysaccharide and cell-wall degradation and could potentially be delivered into the xylem through a non-canonical secretion signal, in which case they might also contribute to the pathogenicity (Table 2.4).

Table 2.4 *Fol* genes encoding enzymes known to be involved or potentially involved in *F. oxysporum* pathogenicity with homologues expressed at 4 dpi in *Foph*-infected cape gooseberry

***Fol* homologue**		**Enzyme encoded[1]**	**SP[2]**
Transcript ID	**Chromosome**		
FOXG_17404	Unpositioned	Glycosyl-hydrolase	Yes
FOXG_17421			No
FOXG_17496			No
FOXG_17514			No
FOXG_17608			Yes
FOXG_17498			Yes
FOXG_17013			No
FOXG_16943			Yes
FOXG_16941		Esterase-hydrolase	Yes
FOXG_17106	6	Oxidoreductase	No
FOXG_14234	14	Catalase-peroxidase	Yes
FOXG_17180	Unpositioned		Yes
FOXG_14258	14		Yes
FOXG_17382			No
FOXG_17483	Unpositioned	Amine-oxidase	Yes
FOXG_17523		Metallopeptidase	No
FOXG_17403		Phospholipase	Yes
FOXG_17619	Unpositioned	Oligopeptidase	No
FOXG_17187		Peptidase	No
FOXG_16942		Lyase	No
FOXG_17723		Cyanovirin/LysM	No
FOXG_16979	Unpositioned	Polyketide synthase	No

[1]Putative functions based on annotations at the Ensembl Fungi web site (Kersey et al., 2016).
[2]Signal peptide region predicted as indicated in the protein annotations available at the Ensembl Fungi web site (Kersey et al., 2016)

2.3.2 Putative effectors shared between other *formae speciales* of *F. oxysporum* and *Foph*

An additional mapping analysis was performed comparing the *Foph* RNAseq reads against a curated database of 95 putative effector transcripts (excluding the *Fol* SIX effectors) obtained from several different *ff. spp.* of *F. oxysporum* (van Dam et al., 2016; Williams et al., 2016). This analysis was done in order to identify homologues not retrieved in the previous analysis due to missing annotations, absence from *Fol,* or lack of conservation between *Foph* and *Fol.* In contrast to the analysis described above, this analysis was not limited to transcripts encoded by genes on lineage-specific chromosomes or regions. Ten homologues were retrieved, but three were discarded due to the low transcript coverage by the RNAseq reads (i.e. reads were concentrated in only a short region comprising ≤ 100 bp of the reference transcript).

One of the transcripts identified in this search (FOWG_18016) is identical to two transcripts (FOXG_17088 and FOXG_14004) encoded by genes located in the LS region of *Fol* chromosome 6 and two transcripts (FOXG_12535 and FOXG_12585) encoded by genes located in the LS region of *Fol* chromosome 3 (Table 2.5), which were not retrieved in the previous mapping analysis of the *Fol* LS transcriptome. In this analysis, the sequence FOWG_18016 was partially covered by *Foph* RNAseq reads and was included as a candidate homologue, unlike the four *Fol* homologues, which were not detected in the previous analysis. This was most likely due to reads mapping to more than one location and being assigned across each of the transcripts, and subsequently not meeting the total read number and coverage criteria for any one of the four FOXG homologues. Moreover, six homologues not present in the LS regions of the *Fol* reference transcriptome were also identified as candidate-effector homologues in this analysis. Interestingly, the putative effector FOXM_16306 from legume-infecting *ff. spp*. (Williams et al., 2016), was the only transcript mapped by *Foph* RNAseq reads that did not have a homologous counterpart in *Fol,* but instead this candidate effector has a homologue in the banana infecting f. sp. *cubense* race 1. Moreover, the *Foph* homologue showed 95% protein identity to FOXM_16306 (Appendix 2.3).

Table 2.5 Candidate *F. oxysporum* effectors with homologues identified in *Foph*

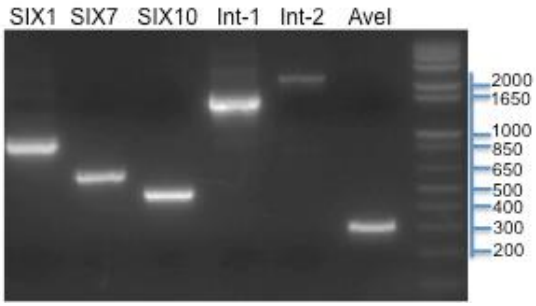
Transcript ID	f. sp.	Enzyme or protein encoded	Genomic location in *Fol*	
			Gene ID	Chromosome
FOXM_16306	*medicaginis*	Candidate effector[2]	-	-
FOMG_18850	*melonis*	Extracellular protease[3]	FOXG_14564	12
FOYG_00459	*FOSC-3a* clinical strain	Tyrosinase[3]	FOXG_04315	4
FOPG_13541	*conglutinans*	Pectinesterase precursor[3]	FOXG_12330	13
FOWG_18016	*lycopersici* MN25	Polygalacturonase[3]	FOXG_17088	6
FOCG_01737	*radicis-lycopersici*	Metallo-β-lactamase[3]	104737-105311[1]	5
FOQG_14751	*raphani*	Hypothetical protein[3]	FOXG_04853	7

[1]Nucleotide position on chromosome 5, [2]Prediction reported by Williams et al. (2016), [3]Prediction reported by van Dam et al. (2016)

2.3.3 Confirming the presence of *Fol* effector homologues in *Foph*

The presence of *SIX1*, *SIX7*, *SIX10*, and *Ave1* homologues in *Foph* was confirmed by PCR amplification and sequencing using the primers SIX1F/R, SIX7F/R, SIX10F/R and Ave1F/R (Table 2.1, Figure 2.2a, Appendix 2.2). Presence of the *SIX12* gene was also confirmed by PCR and sequencing of its cloned product. However, no gel image was obtained for the *SIX12* amplification product. The PCR and sequencing reactions were performed at Corpoica using primers supplied by ANU.

A.



B.

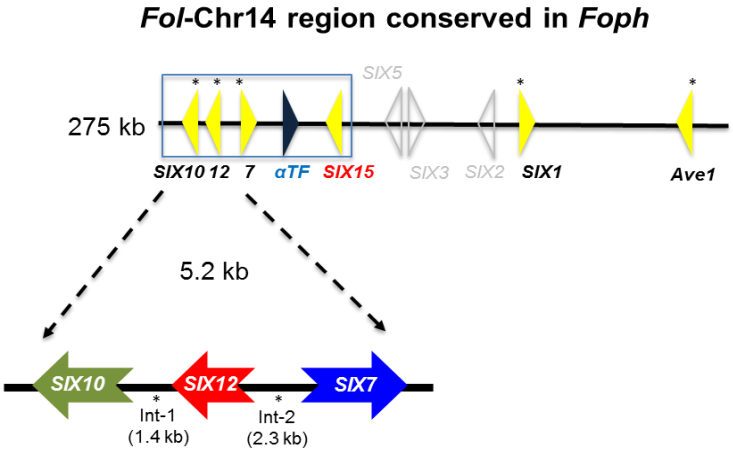


Figure 2.2. A. PCR amplification confirming the presence of *Fol SIX1*, *SIX7*, *SIX10*, and *Ave1* homologues in *Foph*. Homologues of the *Fol* intergenic regions between *SIX10* and *SIX12* (Int-1) and *SIX12* and *SIX7* (Int-2) were also identified in *Foph.* PCR product visualization was carried out following electrophoresis in a 1% agarose gel (gel photograph supplied by Corpoica). **B.** Schematic representation of a conserved region of 274 kb on *Fol* chromosome 14 containing the homologues shared between *Fol* and *Foph.* Homologues denoted by asterisks were amplified from *Foph*, confirming their presence (as shown in A, except for *SIX12*). αTF1= FTF1 transcription factor encoded by FOX_17458 and *SIX15* = a putative SIX effector with a homologue identified in *Foph*. The blue box indicates a segment of Chr14 highly conserved between *Fol* and *Foph*. Effector genes *SIX2*, *SIX3* and *SIX5* shown in grey and located between *SIX15* and *SIX1* were not identified in *Foph.* An expansion of *SIX10*, *SIX12* and *SIX7* gene cluster is shown by the dashed arrows.

2.3.4 Evidence of a conserved segment of *Fol* chromosome 14 in *Foph*

The effector protein sequences encoded by genes located on *Fol* chromosome 14 were aligned with their *Foph* homologues using Clustal-Omega (EMBL-EBI) (Appendix 2.3). *Fol* SIX7, SIX10 and SIX12 showed 98 to 100% protein identity with their *Foph* homologues (Table 2.6). SIX15, recently identified as a putative *Fol* effector

(FOXG_22828, van Dam et al., 2016; KY073750, Gonzalez-Cendales et al., unpublished), was 96% identical to its *Foph* homologue (Table 2.6), but did not have homologues in any other f. sp. of *F. oxysporum*. *Fol* Ave1 showed 88.7% identity to its *Foph* homologue, but like SIX15 had no homologues in any other f. sp. of *F. oxysporum.*

Table 2.6 Protein identity between *Foph* homologues of *Fol* effectors or putative effectors encoded by genes located on *Fol* chromosome 14

Homologue	Size (amino acids)		% Identity
	Fol	***Foph***	
SIX7	220	224	93.6
SIX10	149	149	98.6
SIX12	127	127	100
SIX15	79	79	96
Ave1	125	124	88

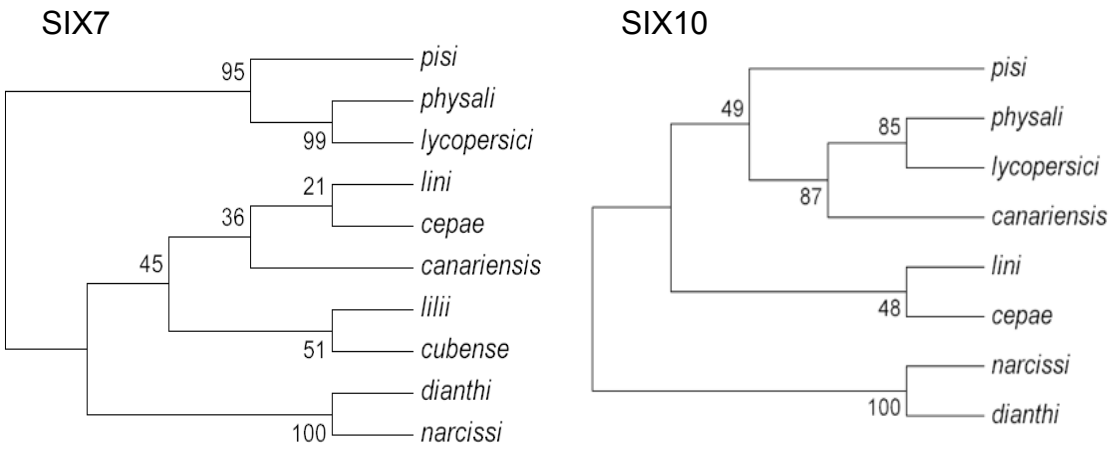
The *SIX7*, *SIX10* and *SIX12* effector genes are located close to each other in the *Fol* genome, forming a gene cluster in a 5.2 kb region on supercontig 51 of chromosome 14 (Schmidt et al., 2013). Therefore, to confirm whether this entire region including the clustered genes was also present in *Foph*, two pairs of primers were designed flanking the intergenic regions between *SIX10* and *SIX12* (Inter1F/R, 1.4 Kb) and between *SIX12* and *SIX7* (Inter2F/R, 2.3 Kb) of *Fol* (Table 2.1, Figure 2.2b). The Inter1F/R and Inter2F/R primer pairs were used by Corpoica to amplify the corresponding regions from *Foph* genomic DNA and the resulting Int-1 and Int-2 PCR products were found to be similar in size to the corresponding *Fol* intergenic regions (Figure 2.2a). The PCR products were cloned and sequenced, confirming their sequence similarity to the corresponding *Fol* intergenic regions (Appendix 2.4). Together these data suggest the presence of an entire SIX-gene cluster in *Foph* homologous to that in *Fol* (Figure 2.2b).

The *SIX15* gene is located close to this cluster, and like *SIX7, SIX10* and *SIX12* is almost identical to its *Fol* counterpart (Table 2.6, Figure 2.2b), raising the possibility that this region of high conservation with *Fol* chromosome 14 extends to and includes *SIX15*. FOXG_17458 is located between *SIX7* and *SIX15* (10.8 kb away from the *SIX7* stop codon) and *Foph* RNAseq reads mapping to FOXG_17458 were identified covering part of the open reading frame, but were not analysed further because FOXG_17458 encodes the FTF1 transcription factor (αTF1), which did not meet the objective of this

analysis. Nevertheless, this gene represented an intervening sequence that could be used to test whether this region is highly conserved between *Fol* and *Foph*. The *Foph* consensus sequence retrieved from the mapping analysis showed 94% identity to *Fol* αTF1 at the nucleotide level (Appendix 2.4), indicating that there is sequence conservation in the interval between *SIX7* and *SIX15*, and suggesting the presence of a larger segment of homology to *Fol* chromosome 14 than the *SIX7, SIX10, SIX12* cluster alone (Figure 2.2b).

2.3.5 Phylogenetic analysis of the SIX proteins encoded by genes in the SIX gene cluster conserved between *Fol* and *Foph*

In order to determine if the *Foph* SIX7, SIX10 and SIX12 proteins are more closely related to their corresponding *Fol* homologues than homologues from other ff. spp., a phylogenetic analysis was performed using sequences in the UniProt database (The UniProt Consortium, 2015) giving blastp hits with >80% identity. The trees based on *SIX7, SIX10* and *SIX12 protein sequences,* showed that the *Foph* sequences were more closely related with *Fol* sequences than they were to homologous sequences from other ff. spp. (Figure 2.3).



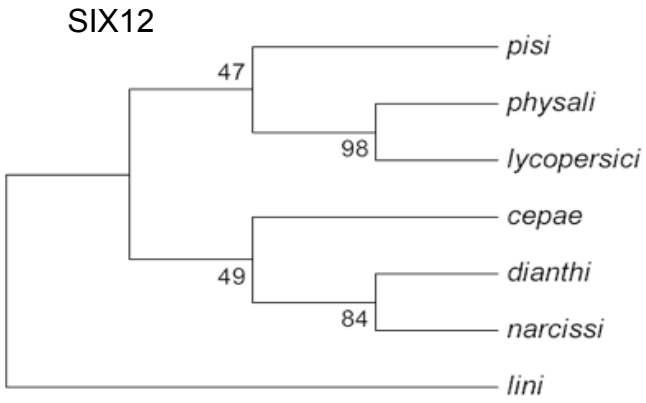


Figure 2.3 Phylogenetic analysis of predicted SIX7, SIX10 and SIX12 proteins from the various ff. spp. of *Fusarium oxysporum*. Phylogenetic analysis was performed using the Maximum Likelihood Method in MEGA. Numbers on the branches represent the percentage of 1000 bootstrap replicates grouping the associated ff. spp. together. The protein sequences were retrieved from the Uniprot database (The UniProt Consortium, 2015), except for Fol SIX12, which was retrieved from GenBank (protein ID ANF89367.1) and the *Foph* sequences.

2.3.6 Two *SIX1* homologues are present in *Foph*

Two *Foph* homologues of *Fol SIX1* were detected during the RNAseq mapping and confirmed by the presence of mixed peaks in the sequence chromatograms of the PCR product (Figure 2.4). To determine the correct sequence of each SIX1 gene in *Foph*, two primer pairs SIX1.1F/R and SIX1.2F/R (Table 2.1) were designed, based on the predicted sequence for each homologue and sent to CORPOICA in order to amplify, clone and sequence the individual homologues from *Foph.*

The sequenced PCR products of *Foph SIX1* were aligned with their counterpart in *Fol* using Clustal-Omega (EMBL-EBI; Figure 2.5), confirming the presence of two homologues of the *SIX1* effector in *Foph.* The two predicted proteins, named SIX1a and SIX1b, were found to have 72% and 80% identity with *Fol* SIX1, respectively, and 74% identity with each other.

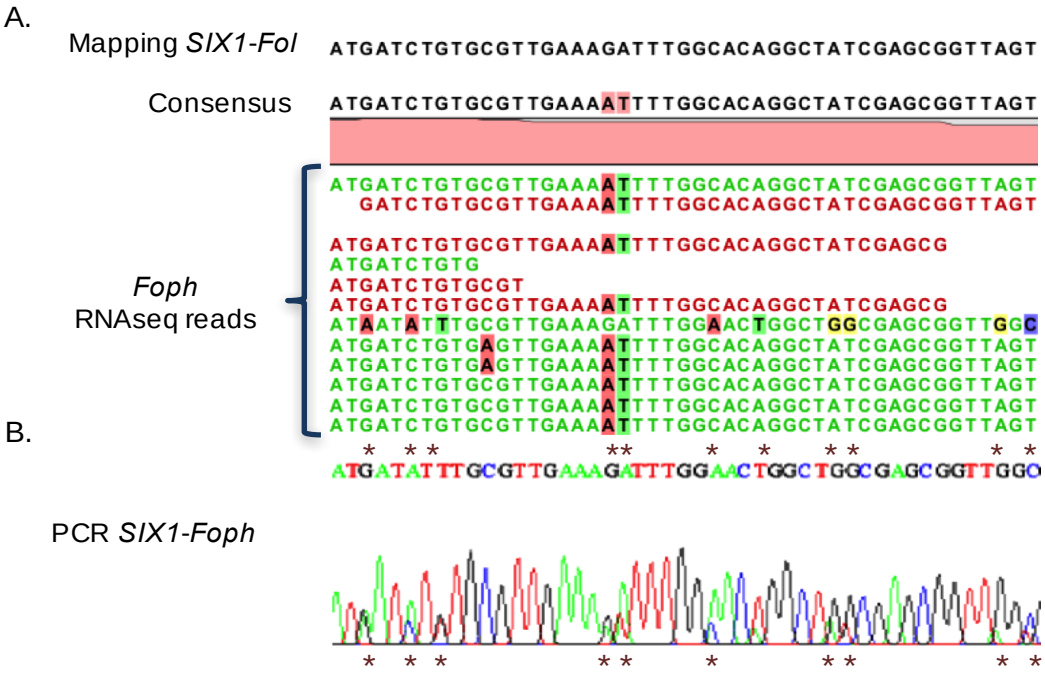


Figure 2.4 Identification of homologues of *Fol SIX1* in *Foph*. **A**. Fragment of the alignment obtained from mapping *Foph* RNAseq reads against *Fol SIX1*. **B.** Part of the sequence chromatogram and corresponding sequence of the *Foph SIX1* amplicon obtained using the primers SIX1F/SIX1R (Table 2.1). SNPs in the mapped reads are marked with asterisks.

```
                ---signal peptide----------------------------------------
Foph-SIX1a      MAPYSMVLLGALSILGFGAYAQEAAVREPQIFFNLTFTEYLDKVAESHGSPPDKSDLPWN
Foph-SIX1b      MAPYSMVLLGALSILGFGAYAQEAAVREPQIFFNLTYTEYLDKVAASHGSPSDKSDLPRN
Fol-SIX1        MAPYSMVLLGALSILGFGAYAQEAAVREPQIFFNLTYTEYLDKVAASHGSPPDKSDLPWN
                ************************************:******** *****.****** *
                ------prodomain----------------------
Foph-SIX1a      DTMSSFPGNETDDGVQTETGSSLSRRGHIVNLGKREIVRGESRNDAVTNDMLQVLHNICV
Foph-SIX1b      DTMSSFPGNETDDGVQTETGSSLSRRGHIVNLRKREPVGEESRNDRVTQDMLQALHDLCV
Fol-SIX1        DTMGSFPGNETDDGVQTETGSSLSRRGHIVNLRKREPFGEESRNDRVTQDMLQALHDLCV
                ***.**************************** *** .  ***** **:****.**::**

Foph-SIX1a      ERFGTGWRAVGGYC--DRR-SRRVKCRRPDDTGLERRANKACPPREECSTFQAYNFITTG
Foph-SIX1b      ENFGTGYRAVSGMCVPGRRATRSFECGTPGVRGGDRAMTRACPKDQECTTFQAYNY----
Fol-SIX1        ERFGTGYRAVSGLCYTDRRATRKIECNKPSVRERDRSVTRACPKGQECTTFNAYNF----
                *.****:***.* *  .** :* .:*  *.    :*  .:***  :**:**:***:

Foph-SIX1a      QYGRTPHNFPVCGHKIEVNDRQDQGSHTEWDGTWYPEEPKSPGTYDSFVQMAGSLNAYFN
Foph-SIX1b      RNGKRQVTFPVCGHKIEVKERHDIGIDTEWDGSWFPEKPESPGTYDSFVQMAGSLGAHFD
Fol-SIX1        RNRHHQVTFPVCGPRIEVKDRHDIGIHTEWQGTWYPESPKSPGTYDYFAQMAGTLNGYFG
                :  :   .***** :***::*:* * .***:*:*:**.*:****** *.****:*..:*.

Foph-SIX1a      FDGVYSDGETMNSRGHAVSWSCLNCPGGKLTITNTYRPTWAVGYSHTV
Foph-SIX1b      FHGVYSDGYEMNSRGNGHSWACVNCPGGELTITSTYRPTWAIGYTTPN
Fol-SIX1        YDGVYSDGYKTSSHGYGHSWSCINCPRGKVTITNTYRATWAFGYTSPH
                :.******   .*:* . **:*:*** *::***.***.***.**: .
```

Figure 2.5 Protein sequence alignment of *Foph* SIX1a and SIX1b, and *Fol* SIX1, generated using Clustal-Omega (Goujon et al., 2010). Signal peptide indicates the signal sequence required for secretion, prodomain indicates the region cleaved off to generate the mature protein (Rep et al., 2004; Houterman et al., 2007). Amino acid residues highlighted in green indicate polymorphisms in the *Foph* SIX1b and *Fol* SIX1 sequences compared to *Foph* SIX1a. Conserved cysteine residues are highlighted in blue.

2.3.7 Phylogenetic analysis of *Foph* SIX1a and SIX1b

The protein sequences of *Foph* SIX1a and SIX1b were compared using a Blastp search with homologues from other ff. spp. present in the UniProt database (The UniProt Consortium, 2015). A phylogenetic analysis based on SIX1 homologues with more than 67% identity to *Foph* SIX1a or SIX1b showed that *Foph* SIX1a and SIX1b are more closely related phylogenetically to *Fol* SIX1 than the SIX1 homologues present in other ff. spp. of *F. oxysporum* (Figure 2.6).

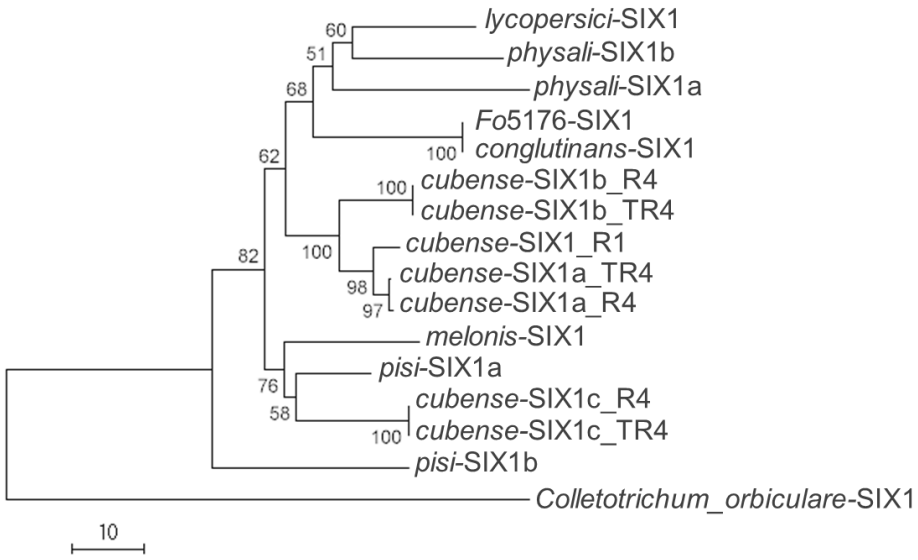


Figure 2.6 Phylogenetic analysis of SIX1 homologues in *Foph* and other ff. spp. of *F. oxysporum*. Phylogenetic analysis was performed using the Neighbour-Joining Method in MEGA using the *Colletotrichum orbiculare* homologue of SIX1 as an outlier. Numbers on the branches represent the percentage of 1000 bootstrap replicates grouping the associated ff. spp. together. The protein sequences were retrieved from the UniProt database (The UniProt Consortium, 2015) except for *Foph* Six1a and Six1b. R = races distinguished within f. sp. *cubense.* TR4 = tropical race 4. The scale bar indicates the number of amino acid differences per sequence.

2.4 Discussion

2.4.1 *Foph* homologues of *Fol* transcripts that encode enzymes and hypothetical proteins

An RNAseq database generated from *Foph*-infected cape gooseberry was used to search for *Foph* transcripts homologous to transcripts from genes encoding effectors or putative effectors in the *Fol* lineage-specific transcriptome (FO2-4287; Kersey et al., 2016) and in other *F. oxysporum* putative effector databases (Taylor et al., 2016; van Dam et al., 2016, Williams et al., 2016). A total of 27 homologous transcripts identified

in *Foph* were found to encode enzymes, which might potentially be involved in pathogenicity (Tables 2.4 and 2.5). Several homologues encode proteins predicted to be secreted in the xylem with functions such as inhibition of host chitinase activity (metallopeptidase) or host cell-wall lysis (polygalacturonase). In *Fol*, knockouts of different metalloprotease and polygalacturonase genes have shown reduced virulence in the host (Karimi Jashni et al., 2015; Bravo Ruiz et al., 2016).

The remaining ten homologues identified in *Foph*, encode putative effectors or hypothetical proteins as annotated in the *Fol* genome available at http://fungi.ensembl.org/Fusarium_oxysporum/Info/Index (Kersey et al., 2015; Table 2.3). Several of the hypothetical proteins have features typical of a fungal effector protein (e.g. signal peptide for protein secretion, multiple cysteine residues and small size). Moreover, one of them (FOXG_16255) is located on LS chromosomes 6 (Table 2.3). The transcript FOXG_04853 is positioned on *Fol* core chromosome 7 and has a homologue in f. sp. *raphani* (FOQG_14751), which was described as a candidate effector by van Dam et al., 2016 (Table 2.5).

A homologue of *FoAve1* (de Jonge et al., 2012; Schmidt et al., 2013) was identified in *Foph*. In the tomato pathogen *Verticillium dahliae, Ave1* knock-out mutants showed significantly reduced pathogenicity on tomato plants, demonstrating that Ave1 is involved in fungal pathogenicity (de Jonge et al., 2012). Furthermore, coexpression of Ave1 with the tomato receptor Ve1 in *Nicotiana tabacum* leaves triggered necrosis, indicating that *Ave1* is an avirulence factor that activates Ve1-mediated resistance (de Jonge et al., 2012). The *Fo*Ave1 homologue of Ave1 is also recognised by the Ve1 receptor, suggesting that Ave1 has the potential to act as an avirulence protein in *Fol* (de Jonge et al., 2012), despite the fact that *Ve1* is not known to confer *Fol* resistance in tomato. Although, no *Ave1* homologues were identified in other *F. oxysporum* ff. spp., the *Foph Ave1* homologue identified in this study is highly similar (88%) at the protein level to its counterpart in *Fol* and less similar (51%) to *V. dahliae* Ave1. The presence of a very similar homologue in *Foph* suggests that the avirulence function of *FoAve1* might be conserved. This hypothesis needs further investigation e.g. by testing for recognition of *Foph* Ave1 by tomato Ve1 or identification of a *Ve1* homologue in cape gooseberry. Unlike *Fol,* where no expression during tomato infection has been detected for *FoAve1* (Catanzariti, personal communication), its counterpart in *Foph* was found to be expressed during cape gooseberry infection, suggesting that *FophAve1* might have a role pathogenicity. Therefore, functional analysis also needs to be performed by generating an *Ave1* gene knockout in *Foph* to test its role in pathogenicity.

2.4.2 Identification of *Foph SIX1a* and *SIX1b* homologues

In the present study, two homologues of *Fol SIX1* were found in *Foph* (named *SIX1a* and *SIX1b*), which were confirmed by PCR amplification and sequencing. At the protein level, *Foph* SIX1b is more similar (80%) to its *Fol* counterpart than *Foph* SIX1a (72%) (Figure 2.5). Phylogenetic analysis based on SIX1 homologues from different ff. spp., including two SIX1 homologues from each of the ff. spp. *cubense* and *pisi* (Meldrum et al., 2012; Williams et al., 2016) and three homologues from f. sp. *cubense* (Guo et al., 2014), suggests that *Foph* SIX1a and SIX1b are more similar to *Fol* SIX1 than they are to SIX1 homologues from other *ff. spp.* (Figure 2.6). These results therefore suggest that *Fol SIX1* and *Foph SIX1b* may be derived from an ancestral *SIX1* gene that had already diverged from the *Fo*5176 and *conglutinans SIX1* genes (Figure 2.6).

In *Fol*, *SIX1* is involved in pathogenicity (Rep et al., 2002, 2004). Furthermore, it has been characterised as an avirulence gene (*Avr3*), since the Avr3 protein is recognised by the I-3 resistance protein in tomato (Rep et al., 2004, 2005; Catanzariti et al., 2015). Although, the *SIX1* homologue present in f. sp. *conglutinans* is also involved in cabbage pathogenicity, virulence was not restored when *Fol SIX1* was used in an attempt to complement a *conglutinans* (*Foc*) *SIX1* deletion mutant, suggesting that *SIX1* variants in *F. oxysporum* may be host specific (Li et al., 2016). The phylogenetic analysis of *SIX1*, performed in this study (Figure 2.3), showed that *Foc*, *Foph* and *Fol SIX1* proteins were grouped together forming a distinct clade compared with the other ff. spp., suggesting that their function in pathogenicity might be acquired from the same ancestor and possibly diverged according to the host they infect. However, this hypothesis needs further functional analysis of the *Foph SIX1* homologues. Chapter 3 reports an investigation of the ability of *Foph SIX1a* and *SIX1b* to complement the loss of virulence that occurs in a *Fol SIX1* deletion strain as a preliminary functional analysis.

2.4.3 A region of *Fol* Chromosome 14 conserved in *Foph*

The homologous (putative) effectors identified in *Foph* correspond to *Fol* genes located in a 274 kb region on *Fol* chromosome 14 (Figure 2.2). This region can be divided into two segments according to the conservation of homologues between *Fol* and *Foph.* The first segment (67 kb in length, Figure 2.2, dashed box) includes the highly conserved homologues of *SIX7*, *SIX10*, *SIX12* and *SIX15* (Table 2.6) with protein identity ranging from 93 to 99% and the homologue of the αTF1 transcription factor showing 94% nucleotide identity. Subsequent analysis of the sequenced PCR products of *SIX7*, *SIX10*, *SIX12* and homologous intergenic regions between *SIX7* and *SIX12* and between *SIX10*

and *SIX12*, confirmed that the entire 5.2 kb *SIX7*, *SIX10*, *SIX12* gene cluster is conserved between *Fol* and *Foph* (Figure 2.2b). This result suggests that the *SIX* gene cluster might have been acquired by horizontal gene transfer as proposed for the presence of homologues of *Fol SIX* genes in several ff. spp. of *F. oxysporum* (Ma et al., 2010; Fraser-Smith et al., 2014; Laurence et al., 2015; Rocha et al., 2015; Taylor et al., 2016; van Dam et al., 2016; Williams et al., 2016). Interestingly, the conservation of *SIX15* and the transcription factor αTF1 between *Fol* and *Foph*, might extend such a horizontally transferred segment from 5.2 kb to 67 kb. Sequencing and assembly of the *Foph* genome will help define the full extent of this segment. Based on the findings of this study, the homologues SIX7, SIX10, SIX12 and SIX15 shared between *Foph* and *Fol*, may represent a SIX-effector repertoire associated with the infection of solanaceous hosts species. However, additional *Foph* strains and other solanaceous-infecting ff. spp. need to be evaluated for the presence of this *SIX* gene cluster to test this hypothesis further.

Homologues of *SIX7, SIX10* and *SIX12* have been identified in pathogenic isolates of the onion-infecting f. sp. *cepae* and date-palm-infecting f. sp. *canariensis* (Laurence et al., 2015; Taylor et al., 2016). Homologues of *SIX3* and *SIX5* were also identified in pathogenic strains of f. sp. *cepae* (Taylor, 2016). However, no evidence for gene clustering or a role in pathogenicity has yet been reported for *SIX7*, *SIX10* and *SIX12* or SIX3-SIX5 in either of these two *ff. spp.* Nevertheless, sequencing of f. sp. *cepae* is underway (Taylor, 2016, personal communication). In *Fol*, the clustered effectors *SIX3* and *SIX5*, located on chromosome 14 (Figure 2.2b), are functionally related as their interaction is required for I-2-mediated resistance on tomato. Future functional analysis of the SIX7, SIX10 and SIX12 could potentially reveal a similar functional relationship during host infection.

Homologues of *SIX1* and *Ave1,* also located on *Fol* LS chromosome 14, are conserved in *Foph*, indicating another possible conserved segment (Figure 2.2). These homologues are less identical (80 to 88% at the protein level), compared with the highly conserved segment of the *Fol* chromosome 14 (Figure 2.2 dashed box). The *Fol* genomic region between *SIX15* and *SIX1* also includes the *SIX5*, *SIX3* and *SIX2* effector genes, (Figure 2.2b). Nevertheless, in this study no homologous transcripts of *Fol SIX5*, *SIX3* and *SIX2* were identified at 4 dpi in *Foph*-infected cape gooseberry plants, suggesting that the chromosomal segment between *SIX15* and *SIX1* is not conserved in *Foph*. The phylogenetic analysis based on the two copies of *Foph SIX1* showed a relationship with the *Brassicaceae* infecting f. sp. *conglutinans* (Figure 2.6), and suggests a different ancestry compared to the phylogeny of *Foph SIX7*, *SIX10*, and *SIX12*, which share a

common ancestor related to f. sp. *pisi* (Figure 2.3). These results might indicate that *SIX7*, *SIX10*, and *SIX12* were more recently acquired in *Fol* or *Foph* by horizontal transfer of a chromosomal segment, while perhaps *SIX1* and *Ave1* are part of an ancestral segment involved in pathogenicity that was acquired earlier. An analysis of the *Foph* genome sequence will allow this hypothesis to be examined further.

The identification of a region of *Fol* chromosome 14 conserved in *Foph*, suggests a horizontal acquisition of a chromosomal segment that contains the candidate effectors *SIX7*, *SIX10*, *SIX12* and *SIX15* by either *Fol* or *Foph*. In the absence of functional evidence to the contrary, it is possible that the acquired chromosomal segment could be associated with a recent host adaptation or could simply be a remnant of a horizontal chromosome transfer event with no role in pathogenicity.

Although no functional evidence indicating a role for *SIX7*, *SIX10, SIX12* and *SIX15* in *Fol* pathogenicity has been reported to date, their expression is induced *in planta* (van Dam et al. 2016; Gonzalez-Cendales et al., unpublished) and SIX7, SIX10 and SIX12 proteins are found in the xylem sap of infected tomato plants (Schmidt et al., 2013), suggesting a role in pathogenicity. Moreover, homologues present in f. sp. *cepae* were identified only on highly pathogenic isolates of onion plants, compared with moderate pathogenic and non-pathogenic isolates, suggesting that the presence of *SIX7*, *SIX10* and *SIX12* might be associated with onion pathogenicity (Sasaki et al., 2015; Taylor et al., 2016). In this study, closely-related homologues of *Fol SIX7*, *SIX10* and *SIX12* were identified at 4 dpi in *Foph*-infected cape gooseberry plants suggesting they might be functionally conserved. Based on this hypothesis, a gene deletion strategy to test the role of this SIX gene cluster in *Fol* pathogenicity was developed as described in Chapter 4.

Chapter 3.

Functional analysis of *Foph SIX1a* and *SIX1b*

3.1 Introduction

The secreted in xylem (SIX) proteins in *Fol*, have been associated with host specificity as virulence determinants (Ma et al., 2010; Lievens et al., 2008; Rep et al., 2004; Rep, 2005; van der Does and Rep, 2007). SIX1 was the first SIX protein identified in the xylem sap of tomato plants infected with *Fol* (Rep et al., 2002, 2004). *Fol*-susceptible tomato plants inoculated with *Fol SIX1* knockout strains, showed reduced disease symptoms compared to plants inoculated with wild type *Fol*. These results demonstrated that *SIX1* was involved in pathogenicity. Moreover, *I-3* tomato plants resistant to *Fol* race 3 showed loss of resistance upon infection with *Fol SIX1* knockout strains (Rep et al., 2004, 2005) indicating that SIX1 is the avirulence factor recognised by I-3. The *I-3* resistance gene encodes a membrane-anchored S-receptor-like kinase suggesting that the SIX1 protein is recognised at the plasma membrane (Catanzariti et al., 2015).

Homologues of the *Fol SIX1* gene have been identified in several other *ff. spp*. of *F. oxysporum* such as *cubense*, *conglutinans*, *canariensis* and cucurbit -and legume-infecting strains, among others *(*Meldrum et al., 2012; Thatcher et al., 2012; Laurence et al., 2015 Nino-Sanchez et al., 2015; Rocha et al., 2015; Li et al., 2016; Schmidt et al., 2016; Williams et al., 2016). In the cape-gooseberry-infecting f. sp. *physali* (*Foph*), two homologues of *Fol SIX1* (*SIX1a* and *SIX1b*) have been identified (Chapter 2). These two homologues are more closely related to *Fol SIX1* than *SIX1* from any other *ff. spp.* and form a distinct phylogenetic clade with the *SIX1* homologue from the *Brassica*-infecting f. sp. *conglutinans* (identical to Fo5176 SIX1 shown in Figure 2.6), suggesting that their function in pathogenicity might be acquired from a common ancestor and may have diverged according to the host they infect.

Functional analysis of the closely related homologue of *Fol SIX1* in the *Brassica*-infecting f. sp. *conglutinans* demonstrated that it is involved in cabbage pathogenicity, as gene knockout strains showed a significant reduction in virulence, compared to the wild-type strain (Li et al., 2016). Attempts were made to complement the knockout strains with the *conglutinans* and *Fol SIX1* genes but restoration of symptoms was only observed for *conglutinans SIX1*. This suggested that SIX1 variants are host specific and might be involved in the adaptive evolution that allowed *F. oxysporum* to expand its range of host species.

The aim of the work described in this chapter was to test whether the *SIX1* homologues identified in *Foph* (*SIX1a* and *SIX1b*) could perform the role of *SIX1* in *Fol*. A *Fol SIX1* knockout strain (*Fol-ΔSIX1*) was therefore transformed with constructs that contain *Foph SIX1a* or *SIX1b*. The resulting transformants were tested for their ability to complement the loss of *SIX1* function by looking for restoration of full pathogenicity on susceptible tomato plants. An additional aim of this work was to test whether *Foph*-SIX1a and/or SIX1b could be recognised by the tomato *I-3* gene which recognises *Fol* SIX1 (Avr3). The SIX1a and SIX1b transformants in the *Fol-ΔSIX1* background were therefore also tested for pathogenicity on tomato plants carrying the *I-3* gene.

3.2 Materials and methods

3.2.1 Generation of the vector for *Fol-Δ*SIX1 complementation with *Foph SIX1a* and *SIX1b*

The coding sequences of *Foph* SIX1a and SIX1b (Chapter 2) were synthesised and cloned into pUC57 by GeneScript, (Piscataway, NJ, USA). The open reading frame of SIX1a and SIX1b were amplified from the pUC57 constructs using the primer SIX1-F (for both SIX1a and SIX1b) and SIX1a-R or SIX1b-R, which introduced *Xba*I sites for binary vector cloning (Table 1). The PCR reactions were performed with proof reading Phusion DNA polymerase (New England Biolabs, Ipswich MA, USA) in a 50 µl reaction volume. The PCR reaction consisted of 1 u/µl Phusion DNA polymerase, 1X Phusion HF buffer (New England Biolabs), 0.3 mM of each primer, 200 µM of dNTPs and 50 ng of DNA template. PCRs were carried out with an initial denaturing step at 98°C for 30 seconds followed by 30 cycles of denaturing at 98°C for 10 seconds, annealing of primers at 54°C for 30 seconds and primer extension at 72°C for 15 seconds, with a final extension step at 72°C for 5 min.

Table 3.1 Primers used for gene complementation vectors construction and transformation screening

Primer	Sequence	Restriction site
SIX1-F	tctagaATGGCGCCCTATAGCATGGT	*Xba*I
SIX1a.1-R	tctagaTTGGATCGCAACTTAGACGG	*Xba*I
SIX1b-R	tctagaGCTTTGGATCGCAACTTAGT	*Xba*I
SIX1p-F	TGAGCTCGGCAGTTGAATA	-
SIX1a.2-R	CCAGGGCAAATCACTCTTGT	-
SIX1b_Ter-F	GCGCACTTCGACTTTCATG	-
SIX1_Ter-R	GCATTAATGAATATACTCAAC	-
RT_SIX1a-F	AGGATCAAGGGAGTCACACG	-
RT_SIX1a-R	TGGGCGATACGTGTTAGTGA	-
RT_SIX1b-F	ATAGACACGGAGTGGGATGG	-
RT_SIX1b-R	AAGTTGGGCGATATGTGCTT	-
RT_Fem1-F	AGCCTTACACCATCCGCTAC	-
RT_Fem1-R	CGCTGTAGTTGACCTCACCA	-
q_Fem1-F	CTGTCACCAAGTCTGCCACC	-
q_Fem1-R	CGAGGATGTCATGCGAGCAG	-

The *F. oxysporum* transformation vector pPZP200-pSIX1GFP (provided by Martijn Rep, University of Amsterdam) was used as a backbone to assemble the *SIX1a* and *SIX1b* gene complementation cassettes. Briefly, pPZP200-pSIX1GFP vector contains a selectable marker cassette (*BLE*) inserted near the left border. The *BLE* selection cassette consists of the zeocin resistance gene (*ble*) driven by the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter and the tryptophan biosynthesis (*trpC*) terminator. The *BLE* cassette is followed by a GFP cassette, which consists of the jellyfish *Aequorea victoria* green fluorescent protein (GFP) coding sequence, driven by the *Fol S*IX1 promoter and terminator (van der Does et al., 2008, Appendix 3.1). *Foph* SIX1a and SIX1b were each cloned via *Xba*I sites, replacing the GFP open reading frame, to generate the vectors pPZP200-pSIX1:SIX1a (Appendix 3.2a) and pPZP200-pSIX1:SIX1b (Appendix 3.2b). The correct insertion of each gene in these construct was confirmed by sequencing.

3.2.2 Transformation of *F. oxysporum* with gene complementation vectors

Fol race 3 isolate #029 (designated *Fol*-WT) and a *ΔSIX1* derivative (designated *Fol-ΔSIX1*) were provided by Martijn Rep (University of Amsterdam). *Fol-ΔSIX1* was transformed using the *Agrobacterium tumefaciens* strain LBA4404 containing the appropriate binary vector. The *A. tumefaciens* mediated transformation (ATMT) protocol was adapted from Mullins et al. (2001). The *Agrobacterium* was prepared by growing at 28°C for 48 hours in Lysogeny broth (LB) supplemented with kanamycin (50 µg/ml). The *A. tumefaciens* suspension was adjusted to an optical density at 600 nm (OD_{600}) of 0.45

in 10 ml of induction medium (IM, Appendix 3.2), supplemented with 200 µM acetosyringone and incubated at 28°C for 6 hours without shaking.

Fol-ΔSIX1 was grown on 50 ml of potato dextrose broth (PDB) at 25°C in a rotary shaker (200 rpm) for 5 days until sporulation. Conidia were harvested by filtering the culture through four layers of miracloth and pelleted by centrifugation at 6,000 rpm for 10 min and washed with sterile deionised water. The conidial suspension was then centrifuged again at 6,000 g for 10 min. The washing step was repeated three times. The concentration of conidia was then estimated using a hemocytometer and adjusted to $1x10^5$ conidia/ml in the above-mentioned 10 ml of IM containing LBA4404. Five to seven pieces per plate of sterile Whatman No.2 filter paper (approximately 1 cm x 1 cm) were placed on co-cultivation medium (CM, Appendix 3.3), supplemented with 200 µM acetosyringone. Twenty-five microliters of the LBA4404-*Fol*3 mixture were then pipetted onto each filter and the plates were then incubated for 2 days at 25°C.

After 2 days incubation, filter papers that contained the mixture of LBA4404 and *Fol-ΔSIX1* were transferred to Czapek Dox agar (CDA) supplemented with 75 µg/ml hygromycin, 0.3 µg/ml augmentin and 100 µg/ml zeocin. The filter papers were incubated for 5-10 days at 25°C until hyphae of putative transformants grew out of the filter onto the media. Putative transformants were transferred to new plates containing the same media and incubated for 5 days at 25°C to allow further growth. Sections of mycelia from the putative transformants were transferred again to new plates containing the same media and grown until the fungus covered the surface of the media. Alternatively, sections of mycelia were transferred to Czapek Dox Broth (CDB) with appropriate antibiotics for further analysis.

3.2.3 Screening of *Fol-ΔSIX1* transformants

Initially, DNA was prepared from mycelia of putative transformants using the method described by Liu et al. (2011). PCR was used to screen the mycelial DNA preps for T-DNA insertion with the primers SIX1p-F/SIX1a.2-R, which amplify a predicted product of 492 bp that corresponds to the *Fol* SIX1p:*Foph* SIX1a junction, and the primers SIX1b_Ter-F/SIX1-Ter-R, which amplify a predicted product of 344 bp that corresponds to the *Foph* SIX1b:*Fol* SIX1 terminator junction (Table 3.1, Figure 3.1) Genomic DNA was extracted and purified from mycelia of positive transformants grown for 5 days on PDB containing hygromycin and zeocin and used as a PCR template to corroborate the presence of either *SIX1a* or *SIX1b*. Subsequently, monoconidial cultures were obtained

from the PDB cultures. The protocol for *Fol* genomic DNA extractions is described in Appendix 3.4.

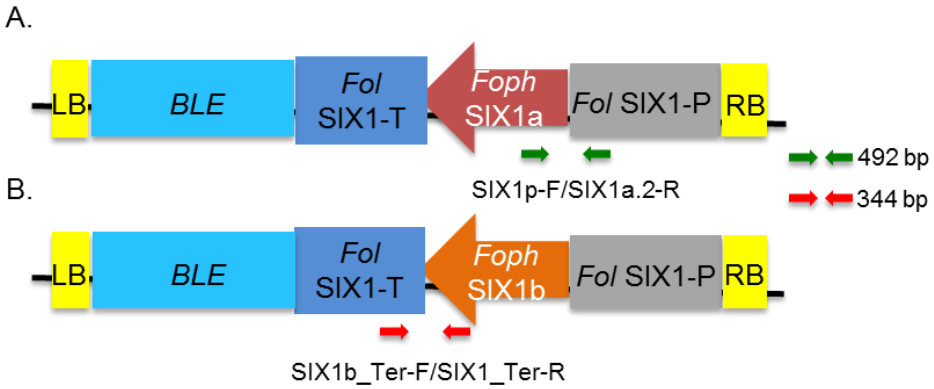


Figure 3.1 Schematic representation of the PCR screening of *Fol-ΔSIX1* transformants for T-DNA insertions carrying *Foph SIX1a* or *Foph SIX1b.* **A**. *FophSIX1a* complementation vector. **B.** *Foph SIX1b* vector. Green arrows indicate the primer binding sites and amplicon sizes for the *SIX1a* and red for the *SIX1b* insertion into the *Fol* genome.

PCRs for screening were performed with MyTaq™ DNA polymerase (BIOLINE, London-UK) in a 15µl reaction volume. The PCR reaction consisted of 0.05 u/µl MyTaq DNA polymerase, 1X MyTaq reaction buffer (BIOLINE, London-UK), 0.3 mM of each primer and 50 ng of DNA template. PCRs were carried out with an initial denaturing step at 95°C for 3 minutes followed by 35 cycles of denaturing at 95°C for 15 seconds, annealing of primers at 56°C for 30 seconds and primer extension at 72°C for 20 seconds, with a final extension step at 72°C for 5 min.

3.2.3 Pathogenicity test of *Fol-ΔSIX1*:*Foph* SIX1a/b complementation transformants on tomato plants

Foph SIX1a/b transformants of *Fol-ΔSIX1* (named *Fol-ΔSIX1:SIX1a* and *Fol-ΔSIX1:SIX1b*) were tested for pathogenicity, compared to the *Fol*-WT and *Fol-ΔSIX1*, on susceptible tomato cultivars M82 and Cf0 and on the *Fol* race 3 resistant tomato line IL7-3.

Fol-WT, *Fol-ΔSIX1* and *Fol-ΔSIX1:SIX1a* and *Fol-ΔSIX1:SIX1b* transformants were grown in PDA for 5 days at 25°C until sporulation. A small portion of each culture was used to inoculate 100 ml of PDB media and was incubated for 7 days at 25°C with a constant shaking at 200 rpm. Conidia were harvested by filtering the cultures over four layers of miracloth, pelleted by centrifugation at 6,000 rpm for 10 min, and washed with deionized water. The conidial suspension was then centrifuged again at 6,000 g for 10

minutes. The washing step was repeated three times. The conidial concentration was determined using a hemocytometer and adjusted to $5x10^6$ conidia/ml in deionized water.

Ten plants of each tomato line were used to test each *Fol* transformant. *Fol*–WT, *Fol*-ΔSIX1 and water (mock inoculation) were used as controls. Inoculations were performed by the root-dip method (Wellman, 1939; Mes et al., 1999) with a few modifications. Tomato seedlings were grown in seed-raising mix and removed when only cotyledons were present (11-day-old plants). Their roots were washed with deionized water, trimmed and then dipped into the above mentioned fungal suspension for 3 minutes before replanting in potting mix (Martins Fertilizers, Yass, Australia). After inoculation, plants were kept in a controlled-environment growth room with a 16 h, 25 °C day/8 h, 20 °C night cycle. After 21 days, wilting symptoms and vascular browning were recorded (vascular browning was assessed by counting the number of brown xylem bundles from a horizontal section taken at the base of each plant), and used to calculate disease scores according to the following criteria described by Rep ***et al***. (2005) and Gonzalez-Cendales et al. (2016): 0 = healthy plant; 1= slightly swollen or bent hypocotyl; 2 = one or two brown vascular bundles in hypocotyl; 3 = at least two brown vascular bundles and growth distortion; 4 = all vascular bundles are brown and plant either dead or very small and wilted.

Differences between treatments, were tested for statistical significance by pairwise comparisons of the distributions of disease symptoms using the non-parametric Mann Whitney test (IBM SPSS statistics for Macintosh, version 22.0, 2013), with p-values ≤ 0.05 indicating statistical significance.

3.2.4 Reverse transcriptase PCR analysis of *Fol-ΔSIX1* transformants carrying *Foph SIX1a* or *Foph SIX1b*

Two-week-old susceptible tomato cv. M82 seedlings were inoculated by dipping their roots in a suspension of $5x10^6$ conidia/ml of *Fol*-WT, *Fol-ΔSIX1*, *Fol*-ΔSIX1:SIX1a or *Fol*-ΔSIX1:SIX1b, or mock inoculated by dipping in water. Plants were then grown in a controlled-environment growth room on a 25°C 16-hour day/20°C 8 hour night cycle until collection of samples. Roots of 3-4 *Fol*-infected or mock-inoculated plants were collected at 3 and 6 days post inoculation (dpi), washed with sterile deionised water, pooled in a microcentrifuge tube and frozen in liquid nitrogen ready for RNA extraction.

Frozen root samples were ground in liquid nitrogen and total RNA was extracted using a Plant RNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Total RNA (2 µg) was treated with 2 µl of RQ1 RNase-Free DNase (Promega, Madison, Wisconsin, USA) in a reaction volume of 20 µl containing 1x RQ1 DNAse reaction buffer, followed by an inactivation step at 65°C for 20 minutes. Treated RNA (1µg) was reverse transcribed into cDNA using Superscript III Reverse Transcriptase and an oligo [dT] 12-18 primer (Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions.

PCRs, containing 1µl (approximately 50 ng) of cDNA template, with the primers RT_SIX1a-F/RT_SIX1a-R, for the *Fol*ΔSIX1:SIX1a transformants and RT_SIX1b-F/RT_SIX1b-R for the *Fol*ΔSIX1:SIX1b (Table 3.1) were carried out as described in section 3.2.1 using MyTaq DNA polymerase instead of Phusion DNA polymerase. The *F. oxysporum* Extracellular Matrix 1 gene (FEM1), amplified using the primers RT-FEM1-F/FEM1-R and q_FEM1-F/q_FEM1-R (Table 3.1), was used as a positive control for fungal gene expression.

3.3 Results

3.3.1 Generation of *Fol-ΔSIX1*:*Foph* SIX1a/b transformants

Fol-ΔSIX1:*Foph* SIX1a/b transformants were obtained by ATMT of *Fol-ΔSIX1* using the vectors pPZP200-pSIX1:SIX1a and pPZP200-pSIX1:SIX1b (Supplementary material 3.1b). Seventy putative transformants for both constructs were observed as filter outgrowths after five days of incubation on selection media (Figure 3.3).

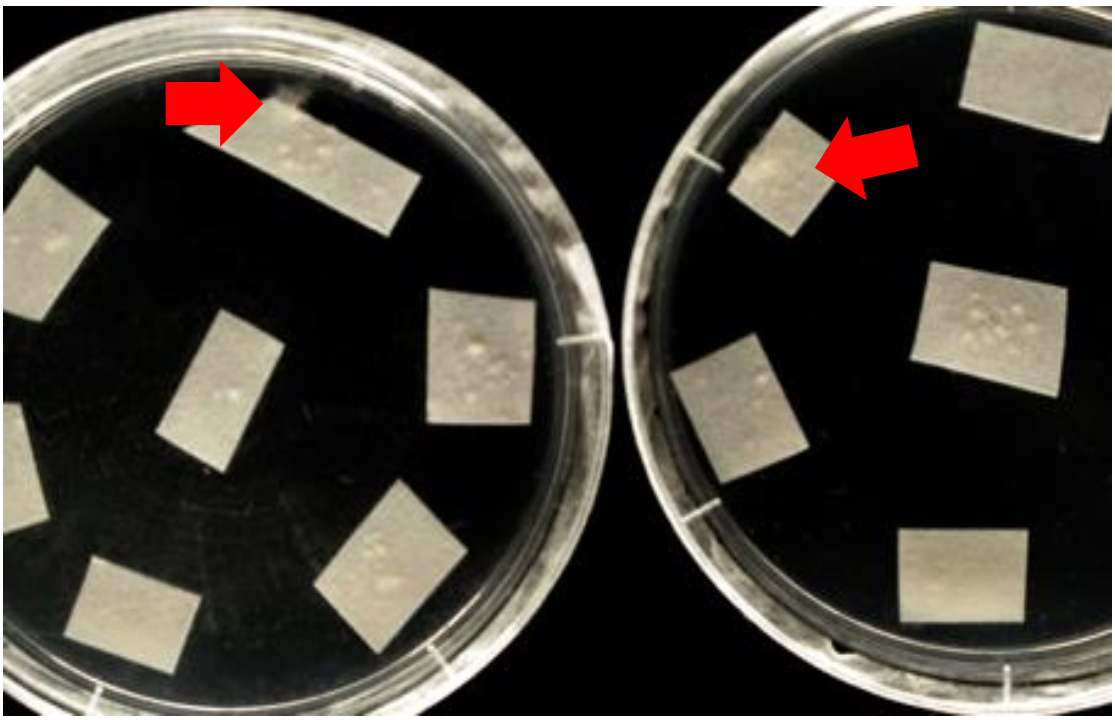


Figure 3.3 Putative *Foph* SIX1a transformants of *Fol-ΔSIX1*, growing on CDA plates amended with 75µg/ml hygromycin, 100 µg/ml zeocin and 0.3 µg/ml augmentin. Red arrows indicate transformants that were selected for further PCR screening.

PCR screening of these 70 putative transformants (38 transformed with *Foph* SIX1a and 32 with *Foph* SIX1b) generated 16 candidates for complementation testing in *Fol*-ΔSIX1. Ten *Foph* SIX1a transformants and six *Foph* SIX1b transformants were confirmed by PCR (Table 3.2, Figures 3.1 and 3.4) Subsequent screening with purified DNA confirmed the insertion of *Foph* SIX1a or *Foph* SIX1b in the selected transformants (Figure 3.4B).

Table 3.2 Generation and recovery of *Fol-ΔSIX1* transformants generated by ATMT in this study with *Foph* SIX1a/b complementation constructs

Construct	Transformation filters placed	zeocin resistant outgrowths	SIX1a/b positives*	Tested for pathogenicity
ΔSIX1:SIX1a	100	38	10	10
ΔSIX1:SIX1b	140	32	6	6

*Confirmed by PCR screening

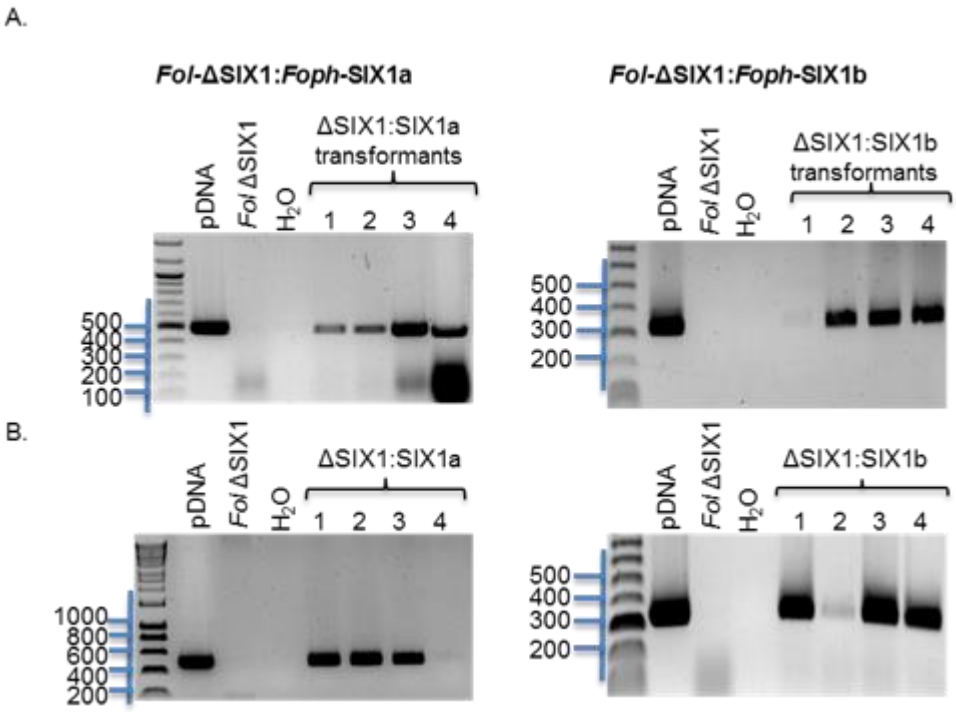


Figure 3.4 PCR screening of eight *Fol-ΔSIX1* transformants for the presence of *Foph SIX1a/b* transgenes. Left gel images show PCR products (492 bp) of four transformants generated with the *Foph SIX1a* construct. Gel images on the right show PCR products (344bp) of four transformants generated with the *Foph SIX1b* construct. **A**. Initial PCR screening with crude DNA preparations of eight candidate transformants with both *Foph SIX1a* and *Foph SIX1b* constructs **B.** PCR screening of purified DNA from monosporic cultures of the transformants tested in A. PCR product visualization was carried out following electrophoresis in 1.5% agarose gels. Controls included *Fol-ΔSIX1* genomic DNA, pPZP200-pSIX1:SIX1a or pPZP200-pSIX1:SIX1b DNA (pDNA) or no template (H_2O).

3.3.2 Expression of *Foph SIX1a* and *Foph SIX1b* transgenes in *Fol* during tomato infection

To test whether SIX1a and/or SIX1b transgenes were expressed during infection, a reverse transcriptase (RT) PCR analysis using the *Foph SIX1a*, *Foph SIX1b* and *FEM1* primers described in section 3.2.4 was performed on roots of the susceptible tomato cultivar M82 infected with three of the ten *Fol-ΔSIX1:SIX1a* transformants and three of the six *Fol-ΔSIX1:SIX1b* transformants. These were compared to mock inoculated plants and plants inoculated with *Fol*-WT and *Fol-ΔSIX1* strains, at 3 and 6 days post inoculation (dpi) and four *Fol-ΔSIX1:SIX1a* transformants at 5 dpi. (Figure 3.5, Appendix 3.5). No *Foph SIX1a* or *Foph SIX1b* expression was detected in root samples from *Fol*-WT, *Fol-ΔSIX1* or mock-inoculated plants. In contrast, expression was detected in the 3 and 6 dpi samples from tomato roots inoculated with *Fol-ΔSIX1:SIX1a* or *Fol-ΔSIX1:SIX1b* transformants.

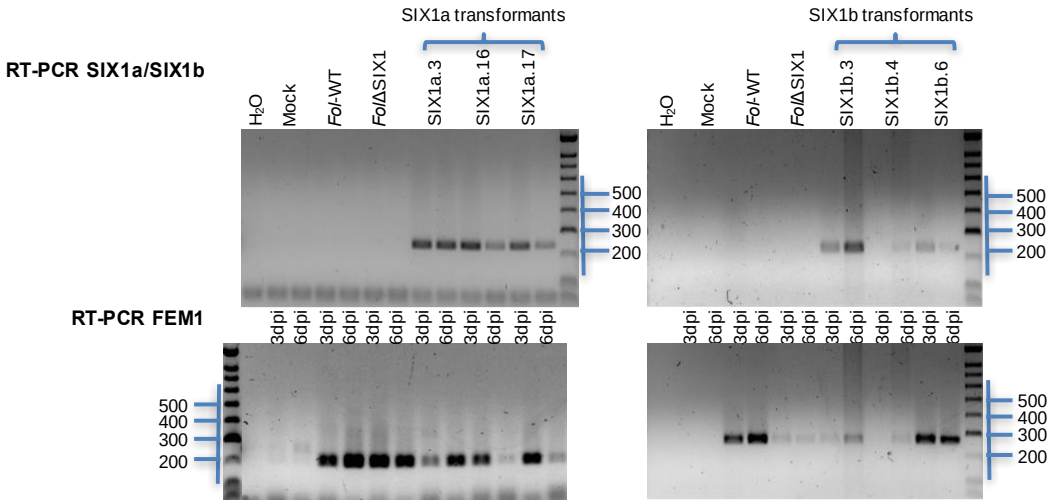


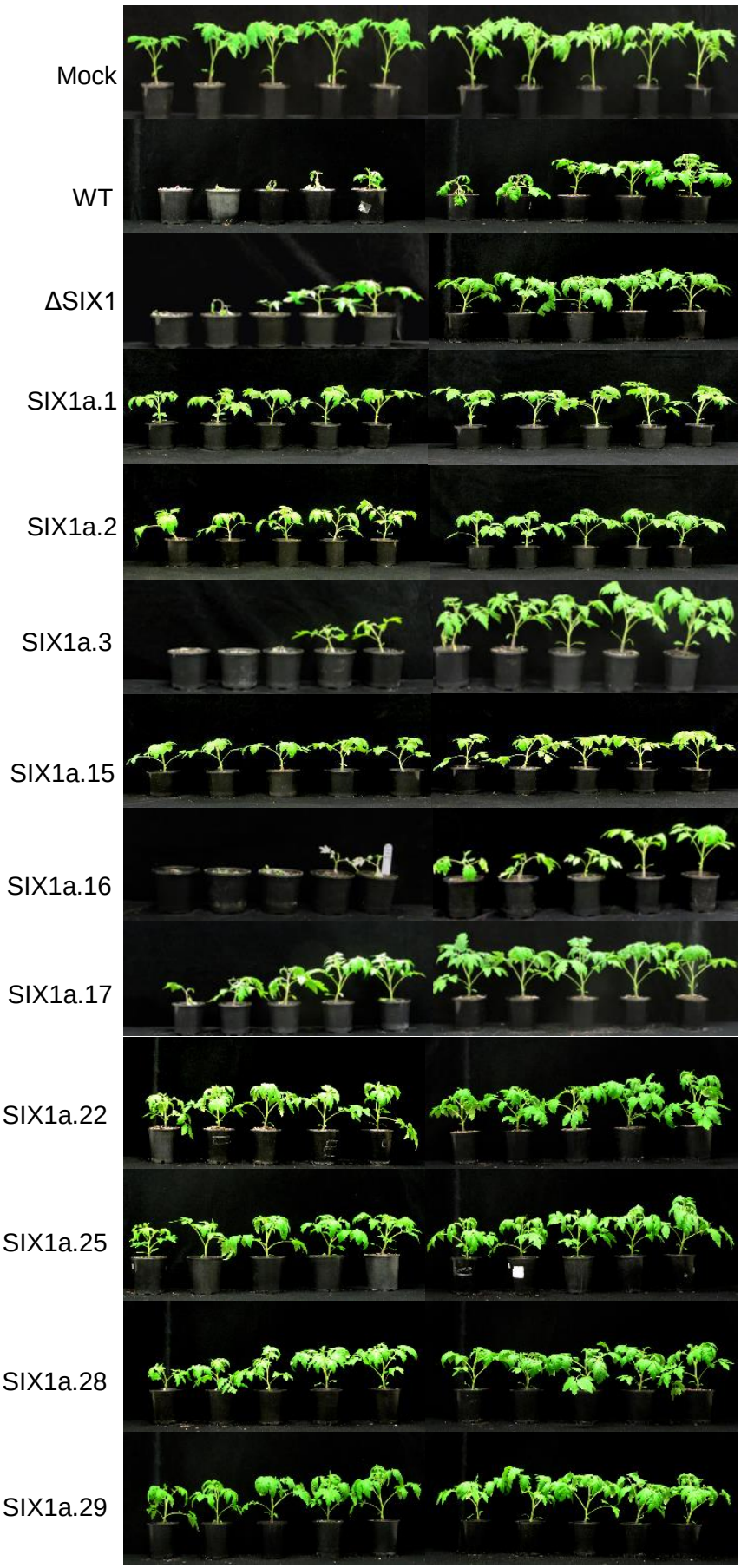
Figure 3.5 Reverse transcriptase PCR analysis showing the expression of *Foph SIX1a* or *Foph SIXb* transgenes in tomato roots infected with *Fol-ΔSIX1:SIX1a/b* transformants at 3 and 6 dpi. Upper gel images show bands (expected size 250 bp) consistent with *SIX1a* and *SIX1b* expression in *Fol-ΔSIX1:SIX1a/b*-infected roots, compared with mock, *Fol*-WT or *Fol-ΔSIX1*-inoculated controls. Lower gel images show bands (expected size 201 bp with RT_Fem1 primers in SIX1a experiment and 250 with q_Fem1 primers in SIX1b experiment) consistent with *FEM1* expression in *Fol*-infected tomato roots.

3.3.3 Functional analysis of *Foph SIX1a* and *Foph SIX1b* in the *Fol* pathosystem

3.3.3.1 *Foph SIX1a* does not appear to complement the loss of virulence in *Fol-ΔSIX1*

Ten *Fol-ΔSIX1:SIX1a* transformants were used to test whether *Foph* SIX1a can restore the virulence of *Fol*-ΔSIX1 in tomato plants. Eleven-day-old tomato plants (M82 cultivar susceptible to *Fol* race 3) were inoculated with *Fol*-WT, *Fol-ΔSIX1* and the *Fol-ΔSIX1:SIX1a* transformants. Disease symptoms were measured according to the disease scoring index described in section 3.2.3. The pathogenicity test was repeated three times and *Foph SIX1a* did not appear to restore full virulence of *Fol-ΔSIX1* on tomato, as no significant differences in disease symptoms or disease scores were observed when tomato plants inoculated with *Fol-ΔSIX1* were compared to those inoculated with nine of the ten *Fol-ΔSIX1:SIX1a* transformants (Figure 3.6). This result indicates that *Foph SIX1a* does not complement the loss of virulence function in *Fol-Δ*SIX1. However, a significant difference in virulence was observed between plants inoculated with *Fol-ΔSIX1* and those inoculated with transformant *Fol-ΔSIX1:SIX1a*.16 (Figure 3.6), suggesting that in this transformant virulence function had been restored.

A.


Mock
WT
ΔSIX1
SIX1a.1
SIX1a.2
SIX1a.3
SIX1a.15
SIX1a.16
SIX1a.17
SIX1a.22
SIX1a.25
SIX1a.28
SIX1a.29

B.

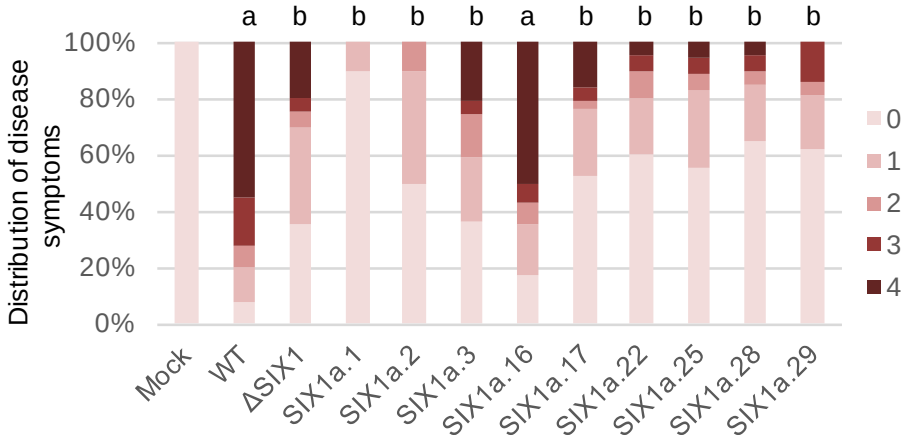
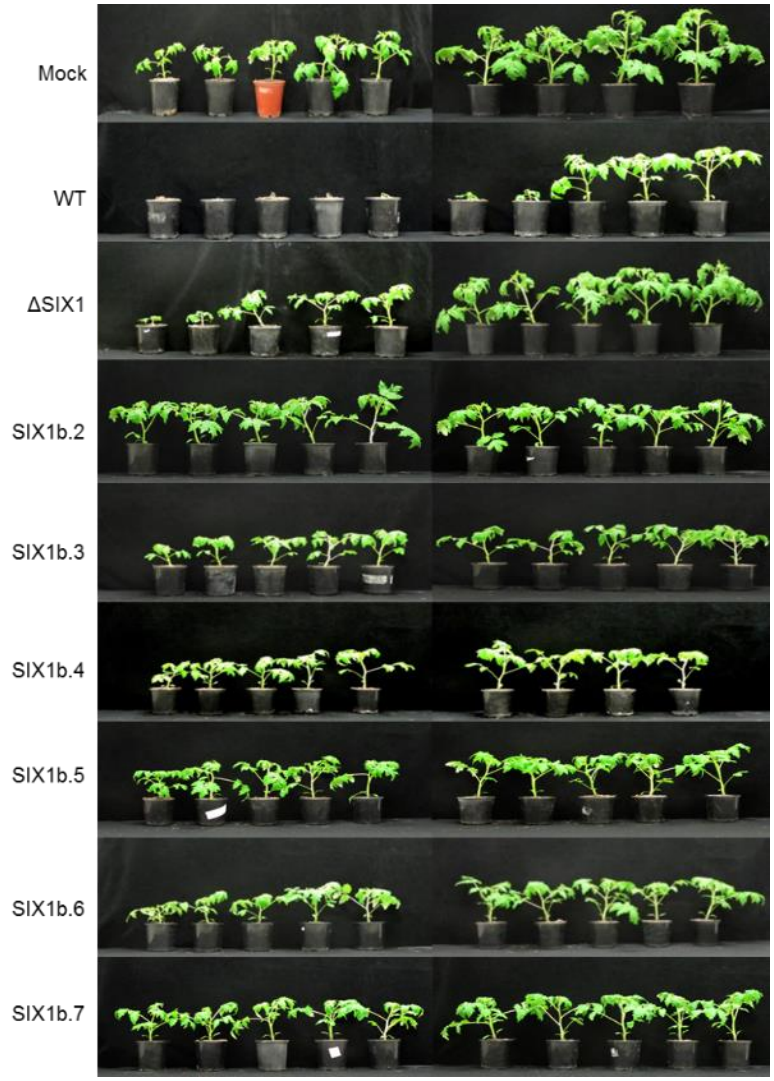


Figure 3.6 Pathogenicity test on M82 plants with either *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*)or ten *Fol-ΔSIX1:SIX1a* (*SIX1a*) transformants **A**. Photographs of infected M82 plants, were taken at 21 dpi, showing ten plants per treatment from one of three replicate experiments. **B**. Disease scoring of the symptoms observed on plants infected with WT, ΔSIX1, or ten *SIX1a* transformants at 21 dpi, from three replicate experiments (n=30 plants per treatment). Treatments with different letters are significantly different at p = 0.05.

3.3.3.2 *Foph* SIX1b does not complement the loss of virulence in *Fol-ΔSIX1*

Six *Fol-ΔSIX1:SIX1b* transformants were used to test whether *Foph SIX1b* can restore the virulence of *Fol-ΔSIX1* in tomato plants, as described above for *Foph SIX1a*. Due to a shortage of M82 seeds, eleven-day-old Cf0 tomato plants (also susceptible to *Fol* race 3), were inoculated with *Fol*-WT, *Fol-ΔSIX1* and the *Fol-ΔSIX1:SIX1b* transformants. The pathogenicity test was repeated three times and *Foph SIX1b* did not restore full virulence of *Fol-ΔSIX1* on tomato, as no significant differences in disease symptoms or disease scores were observed when tomato plants inoculated with *Fol*-ΔSIX1 were compared to those inoculated with *Fol-ΔSIX1:SIX1b* transformants (Figure 3.7). This result indicates that *Foph SIX1b* does not complement the loss of virulence function in *Fol*-ΔSIX1.

A.



B.

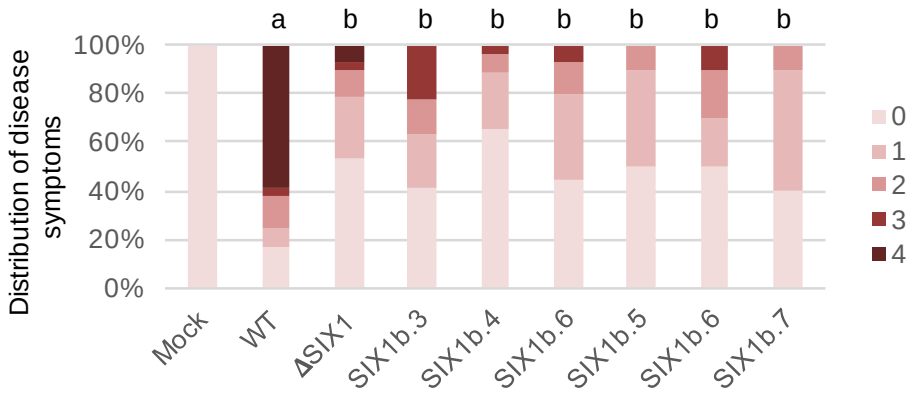


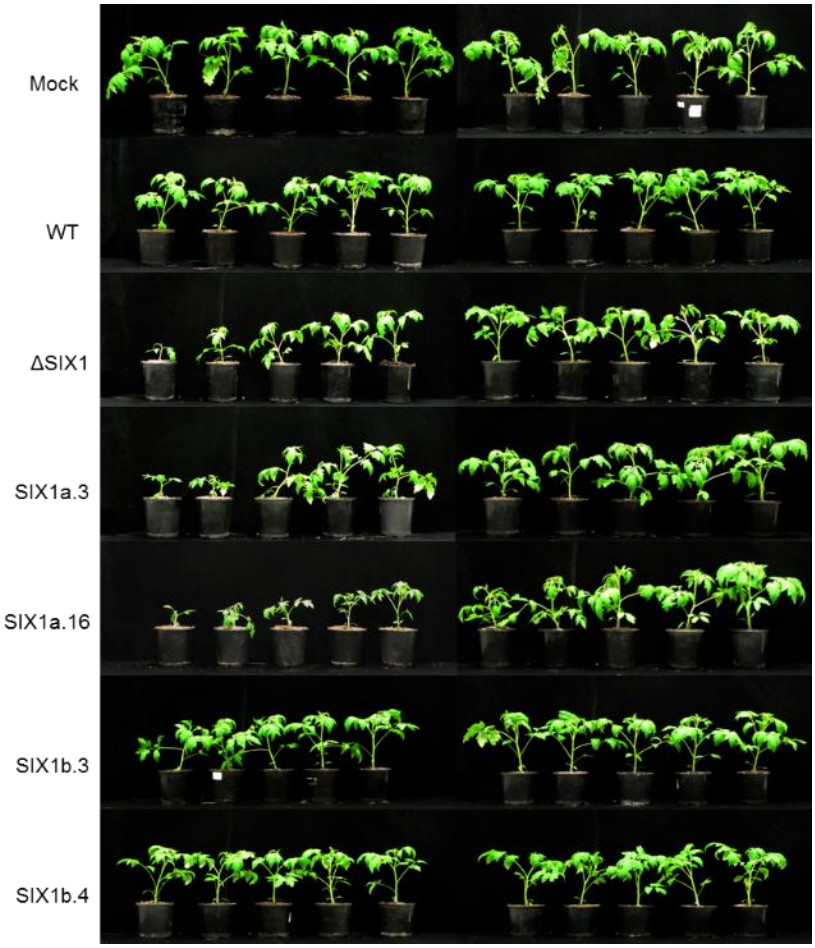
Figure 3.7 Pathogenicity tests on Cf0 plants with either *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) or six *Fol-ΔSIX1:SIX1b* (*SIX1b*) transformants. **A**. Photographs of infected Cf0 plants were taken at 21 dpi, showing nine or ten plants per treatment from one of three replicate experiments. **B**. Disease scoring of the symptoms observed on plants infected with WT, ΔSIX1, or six *SIX1b* transformants at 21 dpi, from three replicate experiments (n=30 plants per treatment). Treatments with different letters are significantly different at p = 0.05.

3.3.3.3 *Foph SIX1b* complements the loss of avirulence of *Fol-ΔSIX1* on tomato plants carrying the *I-3* resistance gene

The tomato *I-3* resistance gene confers resistance to *Fol* race 3 through recognition of the SIX1 effector (Rep et al., 2004; Catanzariti et al., 2015). To test whether *I-3* can also recognise the homologues of *Fol SIX1* presented in *Foph* (*SIX1a* and *SIX1b*), eleven-day-old IL7-3 tomato plants were inoculated with two *Fol-ΔSIX1:SIX1a* transformants and two of *Fol-ΔSIX1:SIX1b* transformants. The IL7-3 introgression line of tomato contains the *I-3* gene and is resistant to *Fol*-WT but susceptible to *Fol-ΔSIX1*.

The pathogenicity test was repeated three times and significant differences in disease symptoms were observed when tomato plants inoculated with *Fol-ΔSIX1* or the *Fol-ΔSIX1:SIX1a* transformants were compared to those inoculated with *Fol*-WT (Figure 3.8). Tomato plants inoculated with the *Fol-ΔSIX1:SIX1a*.16 transformant showed significantly more severe disease symptoms compared with plants inoculated with *Fol-ΔSIX1* or *Fol-ΔSIX1:SIX1a*.3 (Figure 3.8), consistent with previous observations of disease symptoms on the susceptible M82 cultivar of tomato (see section 3.3.3.1). Interestingly, plants inoculated with the *Fol-ΔSIX1:SIX1b* transformants showed significantly less disease symptoms compared with those inoculated with *Fol-ΔSIX1* or the *Fol-ΔSIX1:SIX1a* transformants and no significant differences with plants inoculated with *Fol*-WT (Figure 3.8), suggesting that *Foph-SIX1b* is recognised by the tomato *I-3* resistance gene.

A.



B.

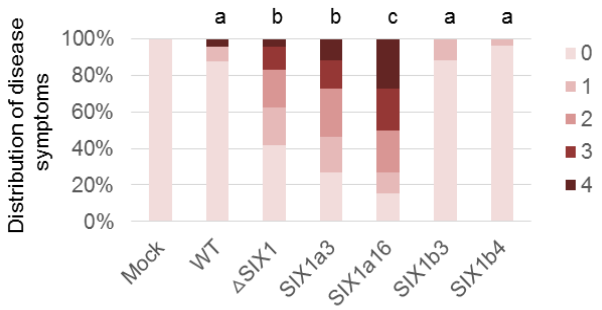
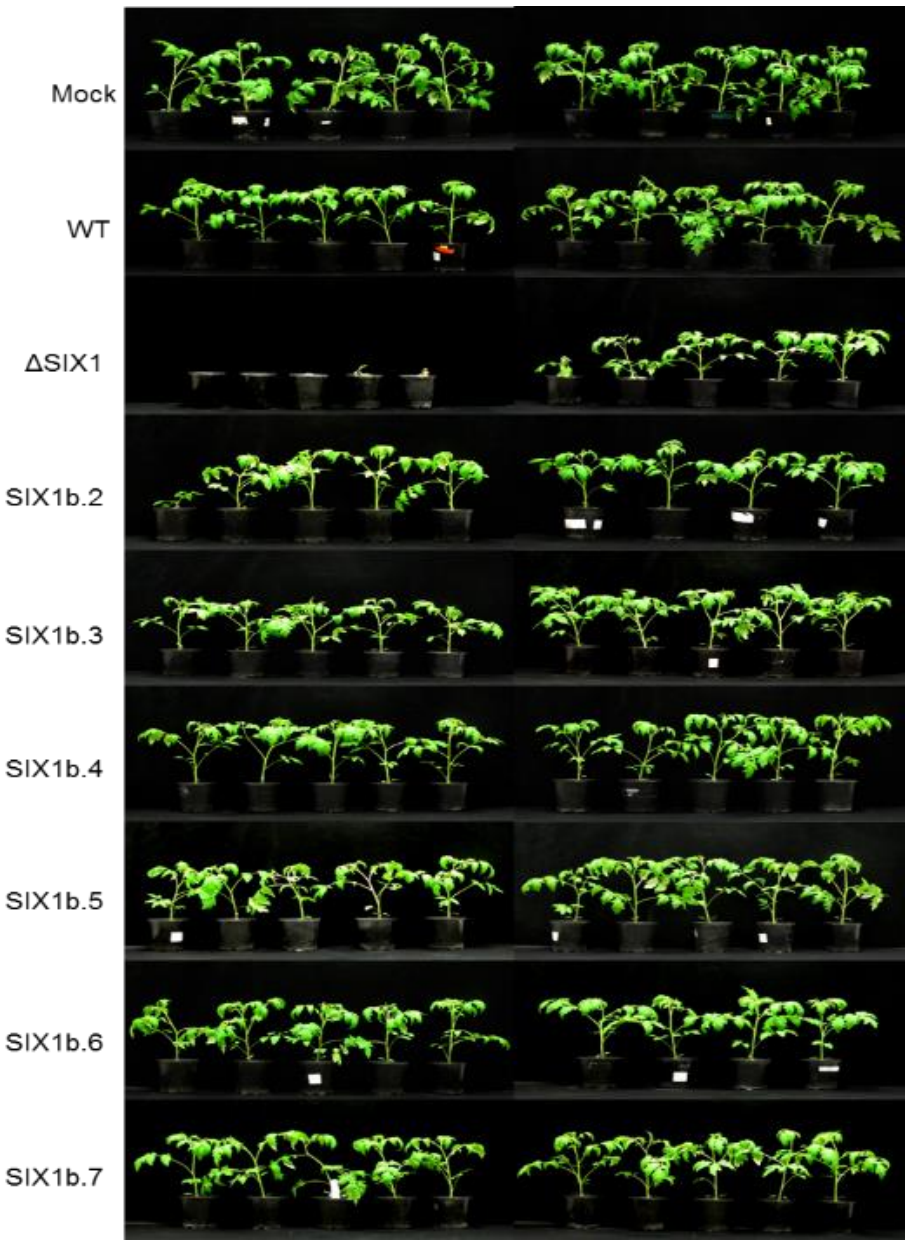


Figure 3.8 Pathogenicity test on IL7-3 tomato plants with either *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) or *Fol-ΔSIX1:SIX1a/b* transformants. **A**. Photographs of infected IL7-3 plants were taken at 21 dpi, showing ten plants per treatment from one of three replicate experiments. **B**. Disease scoring of the symptoms observed on plants shown in A at 21 dpi (n=30 plants per treatment from three replicate experiments). Treatments with different letters are significantly different at p= 0.05.

To confirm the avirulence of the *Fol-ΔSIX1:SIX1b* transformants observed on *Fol* race 3 resistant tomato plants, a second pathogenicity test was conducted by inoculating eleven-day-old IL7-3 tomato plants with all six *Fol-ΔSIX1:SIX1b* transformants generated in this study (Figure 3.9). No significant difference in distributions of disease scores were observed when plants inoculated with the six *Fol-ΔSIX1:SIX1b* transformants were compared to plants inoculated with *Fol*-WT, while plants inoculated with *Fol-ΔSIX1* were significantly different (Figure 3.9). These results consolidate the previous finding suggesting that *Foph*-SIX1b is recognised as an avirulence factor by the I-3 resistance protein.

A.



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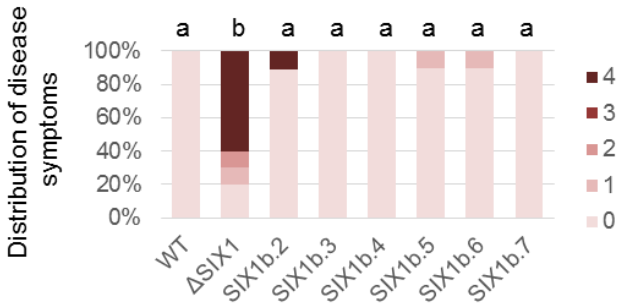


Figure 3.9 Pathogenicity test on IL7-3 tomato plants with either *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) or all six *Fol-ΔSIX1:SIX1b* transformants. **A**. Photographs of infected IL7-3 plants were taken at 21 dpi, showing ten plants per treatment. **B**. Disease scoring of the symptoms observed on plants shown in A i (n=10 plants per treatment). Treatments with different letters are significantly different at *p*= 0.05.

3.4 Discussion

3.4.1 *Foph* SIX1a and *Foph SIX1b* do not complement the loss of virulence of *Fol-ΔSIX1* on tomato

In *Fol*, *SIX1* is required for full pathogen virulence (Rep et al., 2002, 2004). It has also been characterised as an avirulence gene (*Avr3*), since SIX1 is recognised by the I-3 resistance protein, which mediates *Fol*-resistance in tomato (Rep et al., 2004, 2005; Catanzariti et al., 2015). In this study, *Foph SIX1a* and *Foph SIX1b* were tested for their ability to complement the loss of pathogen virulence found for *Fol-ΔSIX1* on tomato. With one possible exception, *Foph SIX1a* and *SIX1b* were unable to complement the loss of *Fol SIX1* function in tomato pathogenicity despite evidence indicating they were expressed in *Fol* (Figure 3.5).

The results of the complementation analysis of *Foph SIX1a* or *SIX1b* in *Fol-ΔSIX1* obtained in this study (Figures 3.6 and 3.7) are similar to those reported for the complementation analysis of *Fol SIX1 in F. oxysporum* f. sp. *conglutinans* (*Foc*) (Li et al., 2016). As observed for *Fol-ΔSIX1* pathogenicity, virulence was reduced when cabbage plants were inoculated with a *SIX1* knock out derivative of *Foc* compared with its wild type parent. Complementation tests showed restoration of full virulence by *Foc SIX1* but not by *Fol SIX1*, suggesting that *SIX1* variants in *F. oxysporum* may be associated with host specificity. The inability of *Foph SIX1a* and *Foph SIX1b* to complement *Fol-ΔSIX1*

is consistent with this hypothesis and suggests that these two genes might therefore be associated with host specificity towards cape gooseberry.

Interestingly, one of the ten *SIX1a* transformants generated in this study (*Fol-ΔSIX1:SIX1a.*16), showed disease symptoms comparable to those of *Fol*-WT in tomato. It is unlikely that this difference is due to a difference in transgene expression as all of the *SIX1a* transformants tested showed expression of the transgene at 3 and 6 dpi (Figure 3.5). If *Fol-ΔSIX1:SIX1a.*16 really is showing complementation, then the question arises as to why nine other transformants did not. Further analysis of the transgene sequence in *Fol-ΔSIX1:SIX1a.*16 may help answer this question. The T-DNA inserted in the transformant *Fol-ΔSIX1:SIX1a.*16 will be sequenced and compared to the *Foph-SIX1a* sequence, to see if any mutation or rearrangement involving the remainder of the *Fol SIX1* gene occurred during transformation. Expression of the transgene in plants inoculated with all *SIX1a* transformants will also be analysed by quantitative RT-PCR to look for differences in expression of *SIX1a* between transformants that might explain the phenotypic difference between *Fol-ΔSIX1:SIX1a.*16 and the other *SIX1a* transformants.

3.4.2 Recognition of *Foph* SIX1b by the tomato I-3 resistance protein

The tomato *I-3* gene confers resistance to *Fol* race 3 through recognition of the SIX1 effector (Rep et al., 2004; Catanzariti et al., 2015). In this study, the tomato introgression line IL7-3, which carries the I-3 resistance gene (Catanzariti et al., 2015), was used to test whether *Foph* SIX1a and/or SIX1b could be recognised by I-3. Two *SIX1a* transformants were tested (*Fol-ΔSIX1:SIX1a.*3 and *Fol-ΔSIX1:SIX1a.*16) and plants inoculated with both transformants showed disease symptoms comparable with or more severe than those inoculated with *Fol-ΔSIX1* (Figure 3.8), suggesting no recognition of *Foph* SIX1a by I-3.

In contrast, the *SIX1b* transformant strains like *Fol*-WT but unlike *Fol-ΔSIX1,* showed no disease symptoms on IL7-3 tomato plants (Figures 3.8 and 3.9), indicating recognition of *Foph* SIX1b by I-3. At the protein level, *Foph* SIX1b was found to have more identity with *Fol S*IX1 (80%) than *Foph* SIX1a (72%) (Chapter 2). The signal peptide and prodomain regions are highly conserved between *Fol S*IX1, *Foph* SIX1a and SIX1b, while the alignment of the mature proteins shows a much higher degree of polymorphism (Figure 3.10). The 34 polymorphic residues in the mature protein sequence shared between *Fol S*IX1 and *Foph* SIX1b but differing from *Foph* SIX1a, might be correlated with recognition by *I-3* in tomato. The role of these residues in recognition by I-3 could be investigated in future work by domains swaps, mutations and/or deletions. There are

also 49 polymorphic residues unique to *Fol* SIX1 or *Foph* SIX1b or both, which may indicate that residues at these positions are not essential for recognition.

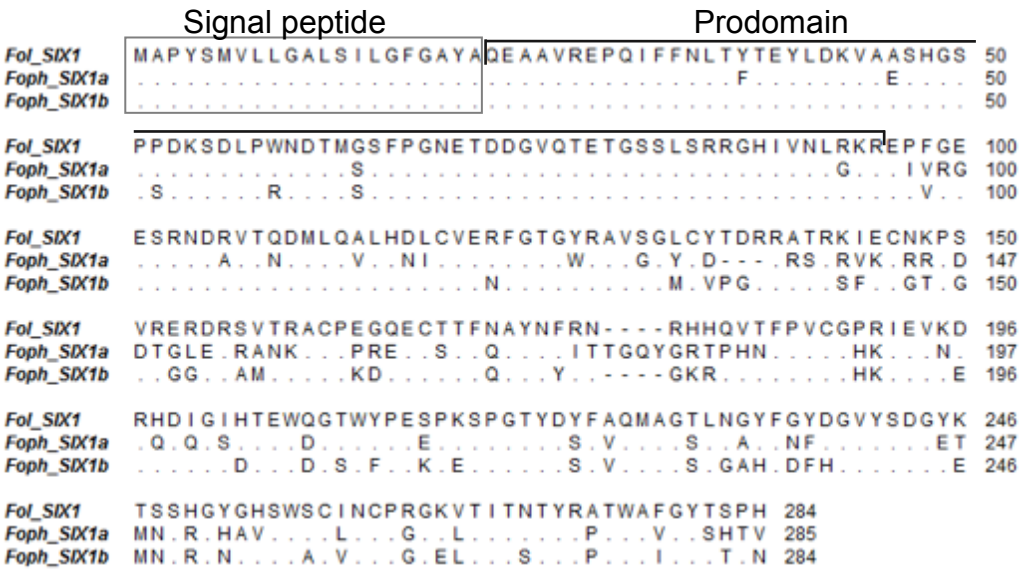


Figure 3.10 Protein sequence alignment of *Foph SIX1a* and *SIX1b* with *Fol SIX1*, generated using Clustal-Omega (Goujon et al., 2010). Signal peptide indicates the signal sequence for protein secretion; Prodomain indicates the region cleaved off the mature protein (Rep et al., 2004; Houterman et al., 2007). Dots represent amino acid residues in *Foph* homologues conserved with *Fol* SIX1.

Despite the absence of an effect on *Fol* virulence by *Foph* SIX1b, it was found to be recognised as an avirulence protein by the I-3 resistance protein. Therefore, it might be possible that the resistance to *Foph* observed in some cape gooseberry cultivars could be mediated by recognition of SIX1b by a homologue of the tomato I-3 protein. Future identification and characterisation of *I-3* homologues in cape gooseberry germplasm might enable this hypothesis to be tested. Alternatively, the possibility of transferring *I-3* mediated resistance to cape gooseberry plants could be explored through the introduction of the *I-3* resistance gene from tomato. Additionally, natural variants of SIX1 in *Foph* or *ff. spp.* affecting other *Physalis* or *Solanum* species could be examined for ability to complement both the loss of virulence of *Fol SIX1* knockouts on susceptible tomato plants as well as their loss of avirulence on *I-3* tomato plants.

Chapter 4.

Generation of a gene deletion construct for *Fusarium oxysporum* transformation

4.1 Introduction

The secreted in xylem (*SIX*) genes *SIX7*, *SIX10* and *SIX12* encode putative effector proteins that were previously identified via xylem sap proteomics and RNAseq analysis of tomato plants infected with *Fol* race 2 and 3 (Houterman et al., 2007; Schmidt et al., 2013). These genes are clustered together on *Fol* chromosome 14 (Ma et al., 2010; Schmidt et al., 2013). Although homologues of these genes have been identified in other *formae speciales* (ff. spp.) such as *cepae* (onion infecting), *pisi* race 1 (pea infecting), *canariensis* (date palm infecting) and *physali* (cape gooseberry infecting) encoding proteins with more than 90% identity (Laurence et al., 2015; Taylor et al., 2015; Chapter 2), their role in pathogenicity has not been established.

The role of candidate effector genes in pathogenicity can be evaluated by gene disruption, deletion or overexpression. Gene knockouts based on targeted deletions of known gene sequences are frequently used to study fungal gene function (Liang et al., 2014). In *Fol*, targeted deletions of *SIX1*, *SIX3*, *SIX4*, *SIX5* and *SIX6* have been used to test their role in pathogenicity (Rep et al., 2004; Houterman et al., 2008, 2009; Gawehns et al., 2014: Ma et al., 2015). Knockouts of the *SIX1* and *SIX4* homologues in *F. oxysporum* f. sp. *conglutinans* and recently the *SIX6* homologue in *F. oxysporum* f. sp. *niveum* have also been generated and tested for their effect on pathogenicity on cabbage, Arabidopsis and watermelon (Thatcher et al., 2012; Li et al., 2016; Niu et al., 2016).

The gene knockout strategy involves three basic steps: generate a homologous gene disruption or knockout cassette, transformation into the target organism, and selection on appropriate media. The gene knockout cassette consists of a vector that carries two flanking DNA fragments from the targeted locus to promote homologous recombination (Hamer et al., 2001; Liang et al., 2014). The flanking DNA fragments can be generated by PCR amplification, then cloned into the knockout vector.

All of the reported *Fol* effector knockouts reported to date have been generated by *Agrobacterium tumefaciens*-mediated transformation (ATMT). The ATMT method is preferred due to the high homologous recombination efficiency between the transfer DNA (T-DNA) and the target locus, compared with transformation by electroporation as

observed in the yeast *Kluyveromyces lactis* of by $CaCl_2$/PEG-mediated transformation as observed in the filamentous fungus *Aspergillus awamori* (Bundock et al., 1999; Michielse et al., 2005). However, several fungal species such as *Aspergillus awamori, F. oxysporum* and *M. oryzae* have a low homologous recombination efficiency with ATMT and require generation and screening of a large number of transformants (Mullins and Khang, 2001). To improve the efficiency of recovering homologous recombination events during ATMT, Khang et al. (2005) developed a targeted gene replacement method involving dual positive and negative selection. This method is based on the generation of a knockout cassette with an adjacent herpes simplex virus thymidine kinase (HSV*tk*) gene as a conditional negative-selection marker against ectopic insertions of the knockout cassette, i.e. T-DNA insertions as opposed to homologous recombination events.

In this chapter, a knock out strategy was proposed for the entire *SIX10*, *SIX12* and *SIX7* gene cluster (named SIXcl) to obtain a *Fol* mutant that, if it had an effect on pathogen virulence, could be complemented with each individual gene in subsequent experiments. Binary vectors with T-DNAs containing a gene knockout cassette and a HSV*tk* negative selection marker were generated to target the SIXcl, the *SIX7* and *SIX12* gene pair or the *SIX10* gene alone in *Fol* race 3, and used in fungal transformation experiments.

4.2 Materials and methods

4.2.1 Gene deletion vector construction

Regions flanking the *SIX* gene cluster (SIXcl), which includes the *SIX7*, *SIX10* and *SIX12* genes, were amplified from *Fol* race 3 genomic DNA using primers SIX10_3′FR1 and SIX10_3′FR2 for the *SIX10* 3′ flanking region, and SIX7_3′FR1 and SIX7_3′FR2 for the *SIX7* 3′ flanking region (Table 4.1, Figure 4.1). All PCRs were performed with MyTaq™ DNA polymerase (BIOLINE, London, UK) in a 15µl reaction volume. The PCR mix consisted of 0.75 units of MyTaq™ Red DNA polymerase, 1X MyTaq Red reaction buffer (BIOLINE, Sydney, Australia), 0.3 mM of each primer and 50 ng of genomic DNA. For all primer combinations (except for intergenic region 2 primer combinations), PCRs were carried out with an initial denaturing step at 95°C for 3 minutes followed by 35 cycles of denaturing at 95°C for 15 seconds, annealing of primers at 56°C for 30 seconds and primer extension at 72°C for 20 seconds. The PCR was completed by a final extension at 72°C for 5 min.

Table 4.1 Primers used for construction of the gene deletion vectors.

Primer	Sequence	Restriction site
SIX10_3′FR1	atcatgataTCCCTTGAATTGTGTTGAACCT	*Eco*RV
SIX10_3′FR2	gactgtacaGCAAAGCCTGCATCGACTAT	*BsrG*I
SIX7_3′FR1	gagactagTGACTACGGTCTGCCTTCAA	*Spe*I
SIX7_3′FR2	accaagcttACCAGCAGATGGGAAGAATGAGG	*Hin*dIII
SIX7_5′FR-F*	GTGCCAATCTTAGCGCACTT	
SIX7-R*	ACGGGCATTCATTAAGCGGT	
SIX10_5′FR1	gagactagGACCAACTCGAAACGTGACA	*Spe*I
SIX10_5′FR2	accaagcttCAAGCTATGCCACTCCTGA	*Hin*dIII
SIX12_3′FR1	cggattaattaaGACCAACTCGAAACGTGACA	*Pac*I
SIX12_3′FR2	attggatatCAAGCTATGCCACTCCTGA	*Eco*RV
SIX12_5′FR-R*	AGTGTGACAGACAAGTGAGT	
SIX12_F*	CAGGAGTGGCATAGCTTGGG	
Inter2-F*	GGTCCAACGCTGAGACAACT	
Inter2-R*	CGTGTTGACATTTTGGGTGA	

* Primers used in the generation of *SIX7* and *SIX12* independent gene deletion constructs

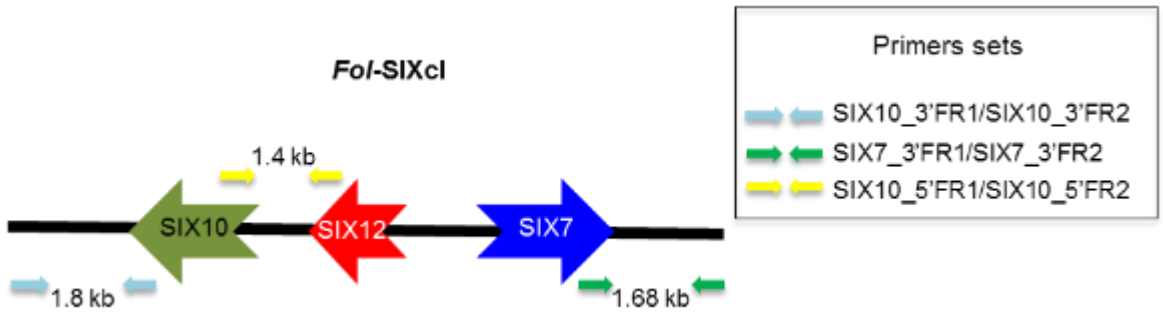


Figure 4.1 Schematic representation of the *SIX7*, *SIX10* and *SIX12 Fol* gene cluster (SIXcl) showing positions of the primers used for PCR amplification of *SIX*-gene flanking regions used in the knockout vector construction.

The *F. oxysporum* binary transformation vector pPK2HPH (provided by Martijn Rep, University of Amsterdam) was used as a backbone to assemble the SIXcl gene deletion cassette. Briefly, pPK2HPH carries the selectable marker cassette (HPH) consisting of the hygromycin resistance gene (*hph*) controlled by the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter and *A. nidulans trpC* terminator (Covert et al., 2001; Michielse et al., 2009; Appendix 4.A).

The 3′ flanking region of the *SIX10* gene was cloned via *Eco*RV and *BsrG*I at the 5′ end of the *hph* cassette and the 3′ flanking region of the *SIX7* gene was cloned via *Spe*I and *Hin*dIII sites at the 3′ end of the *hph* cassette (Table 4.1), to generate pPK2HPH::ΔSIXcl (Appendix 4.B). Two additional deletion constructs were generated, one to delete *SIX10*, and the other to delete both *SIX12* and *SIX7*. For *SIX10*, the 5′ flanking region was amplified using primers SIX10_5′FR1 and SIX10_5′FR2 (Table 4.1) and cloned via *Spe*I and *Hin*dIII sites at the 3′ end of the HPH cassette of the SIX10-3′FR+pPK2HPH intermediate vector. The deletion vector was named pPK2HPH::ΔSIX10 (Appendix 4.E).

For the *SIX12* and *SIX7* genes, the 3′ flanking region of *SIX12* which corresponds to the 5’ flanking region of SIX10, was amplified with the primers SIX12_3′FR1 and SIX12_3′FR2 (the primer sequences for both 5’ flanking region of SIX10 and 3′ flanking region of *SIX12* are identical, except for the cloning sites used) (Table 4.1) and cloned via *Pac*I and *Eco*RV at the 5′ end of the HPH cassette in pPK2HPH. Then, the 3′ flanking region of *SIX7* was amplified with the primers SIX7_3′FR1 and SIX7_3′FR2 (Table 4.1) and cloned via *Spe*I and *Hin*dIII sites at the 3′ end of the HPH cassette, to generate the pPK2HPH::ΔSIX12/7 vector (Appendix 4.F).

4.2.2 Construction of counter selectable marker gene

A modified HSV*tk* gene cassette was generated and used as a second selectable marker as described by Khang et al. (2005). Briefly, the promoter of the *trpC* gene of *A. nidulans* was amplified from the pGpdGFP binary vector (Sexton and Howlett, 2001, provided by Peter Solomon, Australian National University), using the primers trpC_p-F and trpC_p-R (Table 4.2) and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The HSV*tk* gene was obtained from DNA of herpes simplex virus (HSV) isolate UL23 (provided by David Tscharke, Australian National University) using the primers HSV*tk*-F and HSV*tk*-R (Table 4.2) and cloned into the pGEMt:trpC promoter intermediate vector via *Spe*I and *Sal*I sites. The terminator sequence of the *Fol* β-tubulin gene was obtained from *Fol* genomic DNA using the primers βtub-F and βtub-R (Table 4.2) and cloned into the pGEMt:trpC promoter: HSVtk coding sequence intermediate vector via *Sal*I and *Pme*I restriction sites (Appendix 4.C). The entire HSV*tk* cassette was then transferred to all deletion vectors and placed next to the gene deletion cassette, using *Hin*dIII and *Pme*I sites to generate the vectors pPK2HPH::ΔSIXcl::HSV*tk*, pPK2HPH::ΔSIX10::HSV*tk* and pPK2HPH::ΔSIX12/7::HSV*tk* (Appendix 4.D, E and F).

Table 4.2. Primers used to generate the counter selection cassette

Primer	Sequence	Restriction site	Origin
βtub-F	actgtcgacACTAACTAATTTAAACTGGT	*Sal*I	*Fol*
βtub-R	tgtttaaacGGCGGCTTGGGAAGATGCG	*Pme*I	
trpC_p-F	CTTGGGTAGAATAGGTAAGT		*A. nidulans*
trpC_p-R	ACAGAAGATGATATTGAAGG		
HSV*tk*-F	gagactagtATGGCTTCGTACCCCGGCCA	*Spe*I	Herpes simplex virus UL23
HSV*tk*-R	actgtcgacTTAGTTAGCCTCCCCCATCT	*Sal*I	

4.2.3 Transformation of *F. oxysporum* with gene deletion vectors

Fol race 3 isolate #1943, provided by Des McGrath (Agri-Science Queensland, Australia), was transformed using the ATMT method described in Section 3.2.2. After 2 days in co-cultivation, filter papers were transferred to CDA supplemented with 75 µg/mL hygromycin and 0.3 µg/mL augmentin. Transformants that grew out of the filters were transferred to CDA with 75 µg/mL hygromycin. To select against ectopic insertions of the *hph* gene, *Fol* transformation was also carried out using vectors containing the HSV*tk* cassette and transformants were selected on CDA plates with 75 µg/mL hygromycin and 5 mM 5-fluoro-2'-deoxyuridine (F2dU). After 5 to 10 days of selection, putative transformants were transferred to the same media without F2dU and incubated for 5 days at 25°C to allow further growth for subsequent analysis.

4.2.4 Screening of *Fol* race 3 transformants

Initially, DNA was prepared from mycelia of putative transformants using the method described by Liu et al., (2011). Multiplex PCR was used to differentiate between gene deletion transformants and transformants with ectopic insertion of the gene deletion cassette. A combination of three primers was used to amplify a fragment of 403 bp corresponding to the 3′ flanking region and coding sequence of *SIX10* (scSIX10_3′F/ scSIX10_int-R), and a fragment of 297 bp corresponding to the 3′ flanking region of *SIX10* and the *gpdA* promoter of the HPH cassette (scSIX10_3′F/ scGPD-R) (Table 4.3, Figure 4.2). Amplification of the 403 bp product indicates no target gene deletion has occurred. Amplification of the 297 bp product indicates insertion of the gene deletion cassette. The presence of the 297 bp product alone suggests a target gene deletion has occurred whereas the presence of both products indicates ectopic insertion of the gene deletion cassette and no target gene deletion (Figure 4.2). To corroborate deletion of the

SIX gene cluster, genomic DNAs extracted and purified from mycelia of monosporic cultures (see section 3.2.3) of positive transformants grown for 5 days on PDB containing hygromycin were used as PCR templates.

Table 4.3 Primers used to screen *Fol* transformants for gene deletions

Primer	Sequence	Screening for
scSIX10_int-R	TCTGTGTACGTCCAGGATGG	Ectopic T-DNA insertion
scSIX12_int-R	CAAGCGTCCAGTTGTCTCAG	
scSIX10_3′F	GAATAAGCGCGAAAGTGTCA	SIXcl, *SIX10* or *SIX12*/*7* gene deletion
scSIX12_3′F	TCTTCCTTCGCGTACTTGCT	
scGPD-R	AATCATCCACTGCACCTCAG	

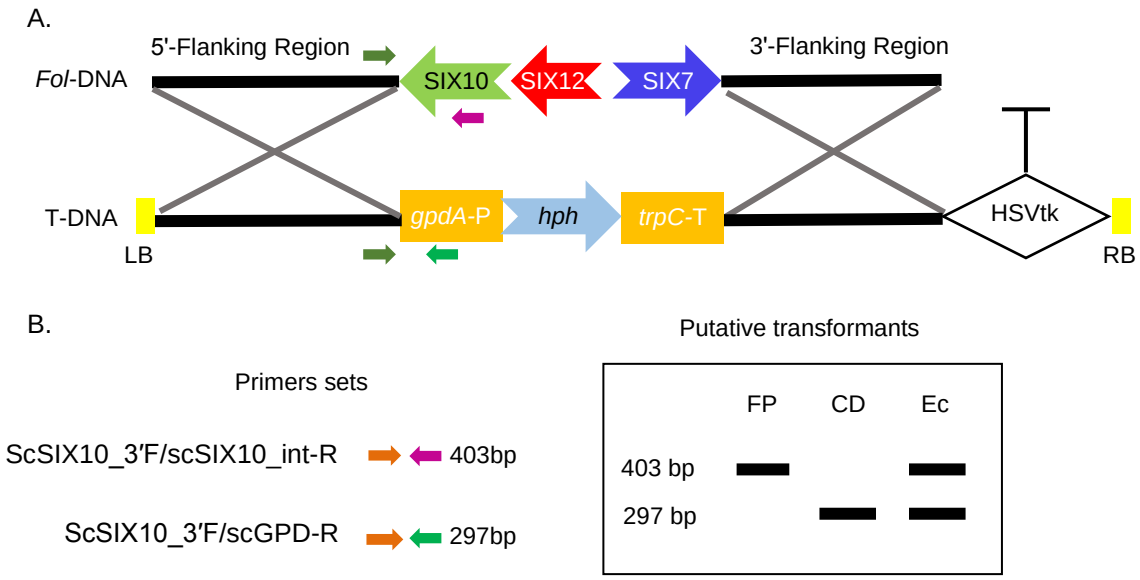


Figure 4.2 A. Schematic representation of SIXcl gene replacement by the *hph* cassette in *Fol* transformants. Arrows indicate primer-binding sites, T-DNA = transfer DNA, LB = left border of the T-DNA, RB = right border of the T-DNA, HSV*tk* = Herpes Simplex Virus thymidine kinase cassette. **B.** Arrows indicate the combination of primers pairs used for the PCR screening of SIXcl or *SIX10* knockouts. The putative transformants are predicted to be false positives (FP) with no insertion of the *hph* cassette, candidate deletion (CD) mutants, or mutants with ectopic (Ec) insertions of the *hph* cassette.

To screen for insertion of the *hph* cassette in transformants generated with the pPK2HPH::ΔSIX12/7::HSV*tk* vector, the primers scSIX12_3′F and scGDP-R amplify a product of 403 bp (corresponding to the gene deletion cassette) while the primers scSIX12_3′F and scSIX12_int-R amplify a product of 297 bp corresponding to an intact *SIX12* coding region (Table 4.3, Figure 4.3), opposite to that of the other two deletion vectors. To corroborate either the double deletion of *SIX7* and *SIX12* or single deletion of *SIX10,* genomic DNAs extracted and purified from mycelia of monosporic cultures of

positive transformants grown for 5 days on PDB containing hygromycin were used as PCR templates.

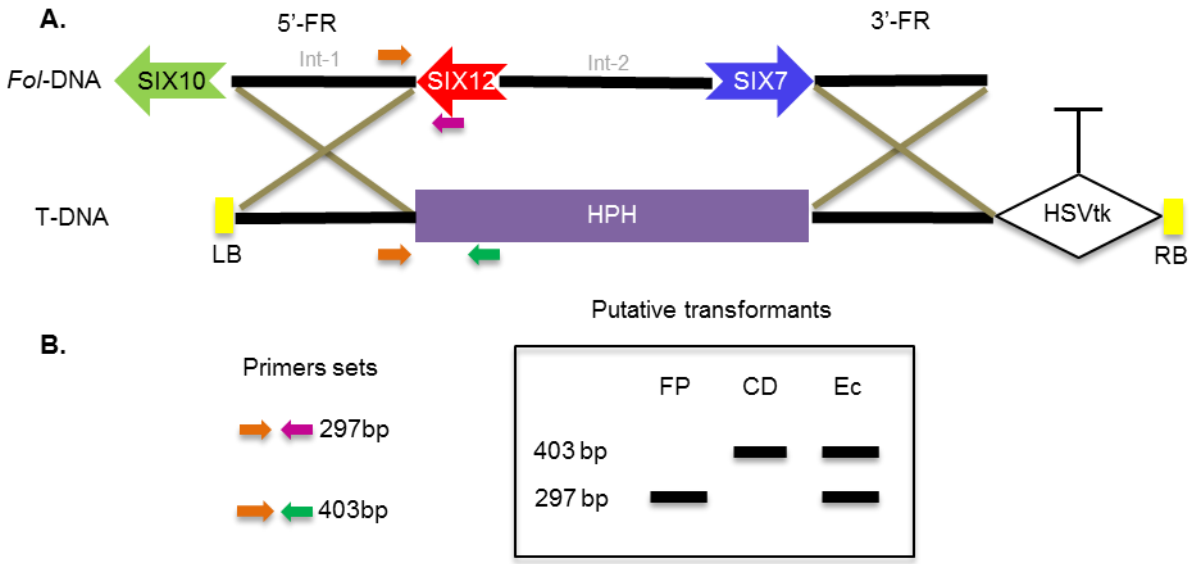


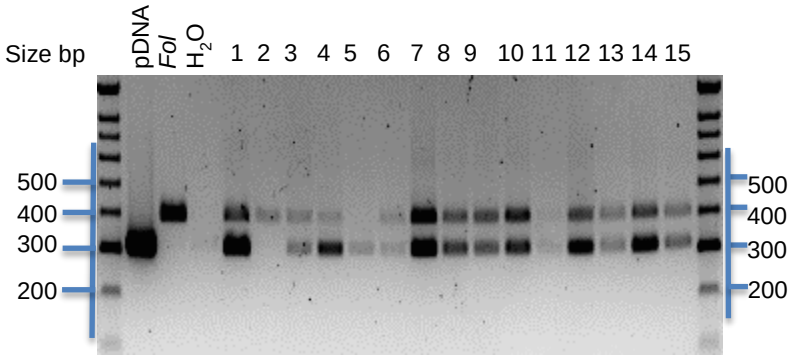
Figure 4.3 A. Schematic representation of SIX12/7 gene replacement by the *hph* cassette in *Fol* transformants. Arrows indicate primer-binding sites, T-DNA = transfer DNA, Int-1= SIXcl intergenic region 1 between SIX10 and SIX12, Int-2= SIXcl Intergenic region 2 between SIX12 and SIX7, LB = left border of the T-DNA, RB = right border of the T-DNA, HSV*tk* = Herpes Simplex Virus thymidine kinase cassette. **B.** Arrows indicate the combination of primers pairs used for the PCR screening of SIXcl or *SIX10* knockouts. The putative transformants are predicted to be false positives (FP) with no insertion of the *hph* cassette, candidate deletion (CD) mutants, or mutants with ectopic (Ec) insertions of the *hph* cassette.

4.3 Results

4.3.1 *Fol* transformants generated with no counter-selection against ectopic *hph* insertions

Fol transformation was carried out by ATMT using the binary vector pPK2HPH::ΔSIXcl. A total of 128 putative transformants were observed as filter outgrowths after five days of incubation on CDA media containing 75 µg/mL hygromycin. Multiplex PCR screening of these putative transformants showed three candidate SIXcl deletion mutants, which generated a 297 bp product but not a 403 bp product corresponding to the intact *SIX10* coding region (Figure 4.4A). However, subsequent screening with purified DNA showed the presence of both products (Figure 4.4B), indicating ectopic insertion of the HPH cassette and no deletion of the SIXcl region.

A.



B.

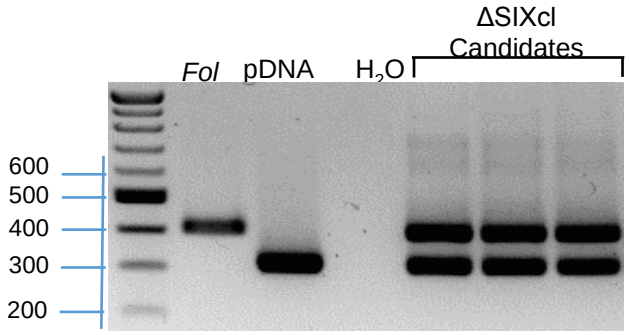


Figure 4.4 PCR screening of 15 *Fol* race 3 transformants for SIXcl genes knockouts. **A**. Initial multiplex PCR screening with crude DNA preparations showed transformant 5 generated only the 297 bp product corresponding to the HPH cassette. **B**. Multiplex PCR screening of high quality DNA from monosporic cultures of the three candidate gene deletion transformants (ΔSIXcl candidates) showed amplification of the both the *SIX10* and HPH fragments suggesting ectopic insertion of the HPH cassette and no deletion of the SIXcl genes by HPH cassette replacement. PCR product visualization was carried out following electrophoresis in 1.5% agarose gels. Controls included genomic DNA from untransformed *Fol* (*Fol*), binary vector pPK2HPH::ΔSIXcl DNA (pDNA) and no template (H_2O).

4.3.2 *Fol* transformants generated with counter-selection against ectopic HPH insertions

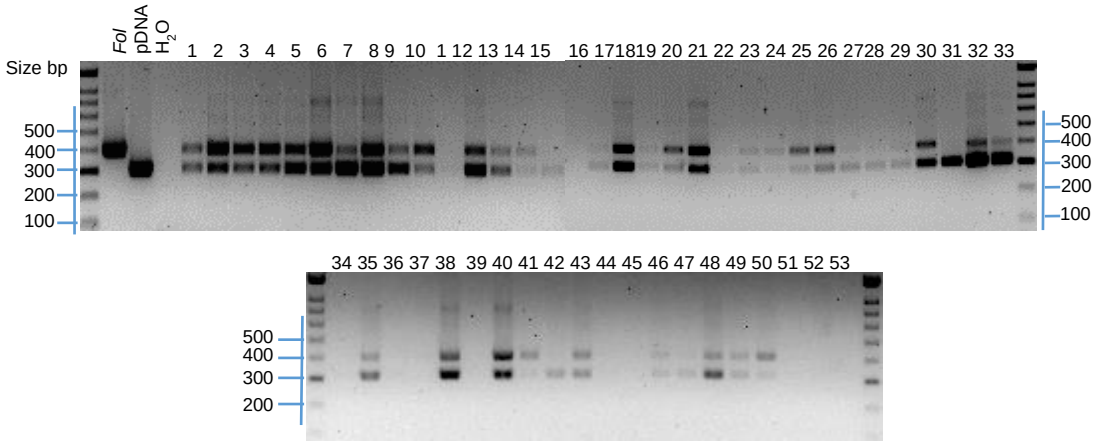
To increase the opportunity to recover gene deletion transformants, subsequent *Fol* transformations were performed with vectors carrying the HSV*tk* cassette enabling selection against ectopic insertions of the *hph* cassette i.e. as T-DNA insertions (Figures 4.2 and 4.3, Appendix 4.D, E and F). A total of 237 putative *Fol* transformants were obtained using the vector pPK2HPH::ΔSIXcl::HSV*tk* after five days of incubation on CDA media containing antibiotics and F2dU (Table 4.4; Figure 4.5A).

Table 4.4 Transformation and homologous recombination efficiency of the *SIX* gene deletion vectors in *Fol.*

Targeted gene	No. Filters	Positive transformants[1]	Multiplex PCR screening		Gene deletion transformants[2]	False positives
			Double band	Single HPH band		
SIXcl[3]	240	128	125	3	0	3
SIXcl[4]	840	237	221	16	0	16
SIX10[4]	190	80	34	7	0	7
SIX12-7[4]	140	35	11	0	0	-
Total	**1410**	**480**	**391**	**26**	**0**	**26**

[1] Fungal strains insensitive to selection media. [2]Confirmation by PCR screening of high quality genomic DNA samples. [3] Vector with no HSV*tk* cassette. [4] Vector with HSV*tk* cassette.

A.



B.

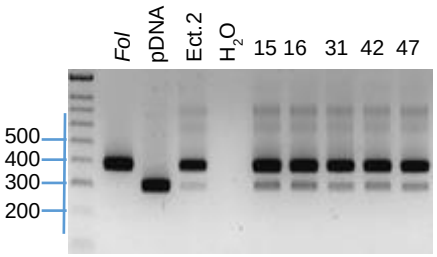
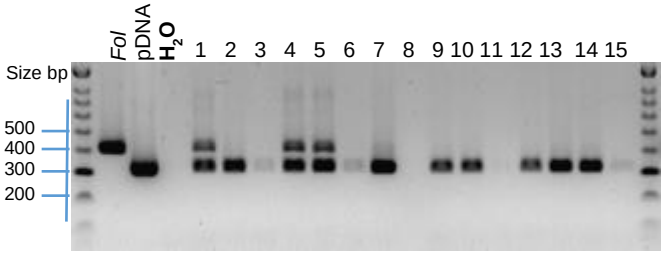


Figure 4.5 PCR screening of 53 *Fol* race 3 transformants for SIXcl gene knockouts. **A**. Initial multiplex PCR screening with crude DNA preparations showed four transformants (15, 31, 42 and 47) that generated only the 297 bp product corresponding to the HPH cassette. **B.** Multiplex screening with high quality DNA of transformants 15, 16, 31, 42 and 47, showed amplification of both fragments, suggesting ectopic insertion of the HPH cassette and no deletion of the SIXcl genes. Transformant 16 was included due to previous non-amplification of the upper band in the multiplex PCR while transformant 2 was included as an ectopic insertion control (Ect2). PCR product visualization was carried out following electrophoresis in 1.5% agarose gels. Controls included genomic DNA from untransformed *Fol* (*Fol*), binary vector pPK2HPH::ΔSIXcl::HSV*tk* DNA (pDNA) and no template (H_2O).

Multiplex PCR screening for gene deletion transformants identified four transformants 15, 31, 42 and 47 that failed to generate a PCR product corresponding to an intact *SIX10* gene. However, further screening with purified DNA showed that the *SIX10* gene was not disrupted in these transformants (Figure 4.5B).

For the construct pPK2HPH::ΔSIX10::HSV*tk*, a total of 34 transformants were confirmed to carry the HPH cassette (Table 4.4). Although, no PCR product corresponding to intact *SIX10* gene was observed for seven transformants, further screening with purified DNA showed the *SIX10* gene was not disrupted in these transformants (Figure 4.6).

A.



B.

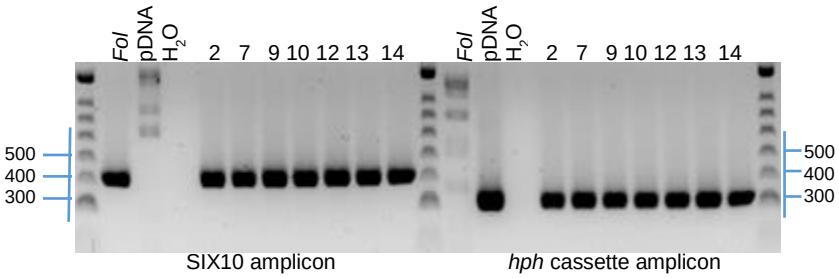
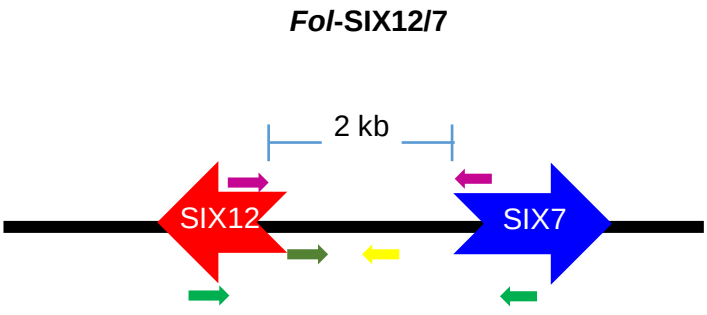


Figure 4.6 PCR screening of 15 out of 35 *Fol* race 3 transformants for *SIX10* gene knockouts. **A**. Initial multiplex PCR screening with crude DNA preparations showed seven transformants (2, 7, 9, 10, 12, 13 and 14) that generated only the 297 bp product corresponding to the HPH cassette. **B** Separate PCR screening with high quality DNA of transformants 2, 7, 9, 10, 12, 13 and 14 showed amplification of both the *SIX10* and HPH fragments suggesting ectopic insertion of the HPH cassette and no deletion of the *SIX10* gene by HPH cassette replacement. PCR product visualization was carried out following electrophoresis in 1.5% agarose gels. Controls included genomic DNA from untransformed *Fol* (*Fol*), binary vector pPK2HPH::ΔSIX10::HSV*tk* DNA (pDNA) and no template (H_2O).

To generate separate gene deletion vectors for the *SIX12* and *SIX7* effectors of *Fol,* the downstream region of *SIX12* was amplified with the primers SIX12_3′FR1 and SIX12_3′FR2 (Table 4.1) and cloned into the pPK2HPH backbone vector at the 3′ end of the HPH cassette for *SIX12* deletion (Appendix 4.E) and cloned at the 5′ end for *SIX7* deletion (Appendix 4.F). The upstream 2 kb region of *SIX12*, which is also the downstream region of *SIX7* (named Int-2, Figure 4.3A), could not be amplified despite several modifications of the PCR conditions, primer combinations and DNA samples used (Figure 4.7).

Genomic DNA samples from *Fol* race 1, 2 and 3 were also used to rule out a lack of primer annealing due to sequence differences compared to the reference genome (*Fol* race 2 isolate 4287). Extension times were also modified to allow for any difference in size of the PCR products, due to the possible insertion of transposons. However, no PCR amplicons of expected size were observed in any of the three *Fol* races tested (Figure 4.7). Further analysis of the Int-2 sequence revealed multiple inverted repeats that could have interfered with primer binding. However, this hypothesis was not verified in this study. Instead, a strategy to generate a double gene (*SIX12-SIX7*) deletion vector was adopted based on the 3′ flanking region of *SIX12* and the 3′ flanking region of *SIX7* (Figures 4.3A and 4.7A). The resulting vector was also engineered to carry the HSV*tk* cassette at the right border, generating pPK2HPH::ΔSIX12/7::HSV*tk*.

A.



B.

Primers sets		
	1737 bp	Inter2-F/SIX12_5'FR-R
	1289 bp	SIX7_5'FR-F/Inter2-R
	2973 bp	SIX12_F/SIX7-R

C.

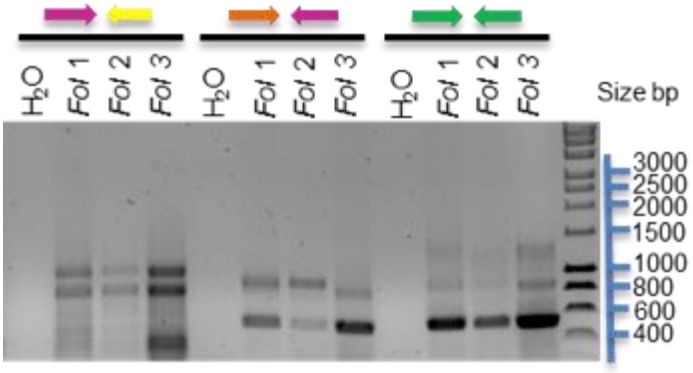


Figure 4.7 A. Schematic representation of the *SIX12-SIX7* genomic region and primer binding sites (shown by the arrows). **B**. Primer combinations used in the attempt to amplify the intergenic region between *SIX12* and *SIX7* **C.** Attempted PCR amplification of the intergenic region between *SIX12* and *SIX7* in *Fol* races 1 (*Fol* 1), 2 (*Fol* 2) and 3 (*Fol* 3) generated no product of the expected size for any primer combination shown in B. PCRs were performed with Taq polymerase and supplemented with 1% dimethyl sulfoxide (DMSO). PCR product visualization was carried out following electrophoresis in 1% agarose gels. Water was used as a no template control.

Using the construct pPK2HPH::ΔSIX12/7::HSV*tk*, a total of 11 transformants were identified that carry the HPH cassette (Table 4.4). However, all of these transformants generated the 297 bp product corresponding to the *SIX12* coding region, suggesting ectopic insertion of the HPH cassette and no disruption of the *SIX12*/*7* region (Figure 4.8).

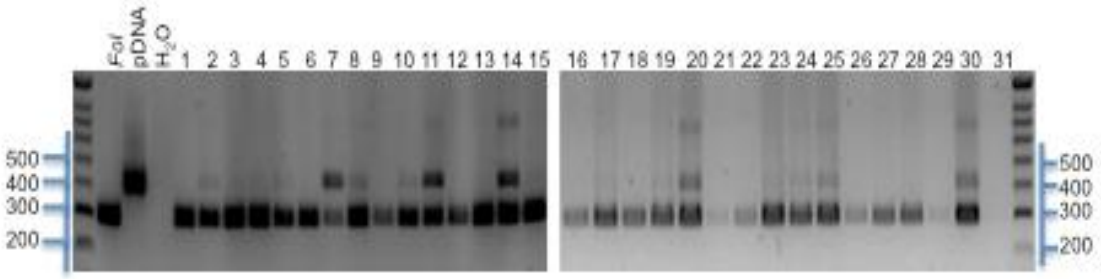


Figure 4.8 Multiplex PCR screening of 31 out of 35 *Fol* race 3 putative transformants for *SIX12* and *SIX7* double gene knockouts. All candidates showed amplification of the *SIX12* gene product (297 bp), while several showed amplification of the product corresponding to the HPH cassette (403 bp). PCR product visualization was carried out following electrophoresis in 1.5% agarose gels. Controls included genomic DNA from untransformed *Fol* (*Fol*), binary vector pPK2HPH::ΔSIX12/7::HSV*tk* DNA (pDNA) and no template (H_2O).

4.3.3 The effectiveness of counter-selection against ectopic *hph* insertions

To test whether the HSV*tk* cassette was able to provide effective counter selection, two *Fol* transformants with T-DNA insertions carrying the HSV*tk* cassette were tested on media containing 0, 5 or 50 µM F2dU. No growth of these transformants was observed on media containing F2dU (Figure 4.9) despite the fact that these two transformants were initially selected on media containing 75 µg/ml hygromycin and 5 µM F2dU. Although this result suggests that the HSV*tk* cassette is capable of providing effective counter selection at 5 µM F2dU, no *SIX* gene deletion transformants were recovered for any of the vectors generated in this study.

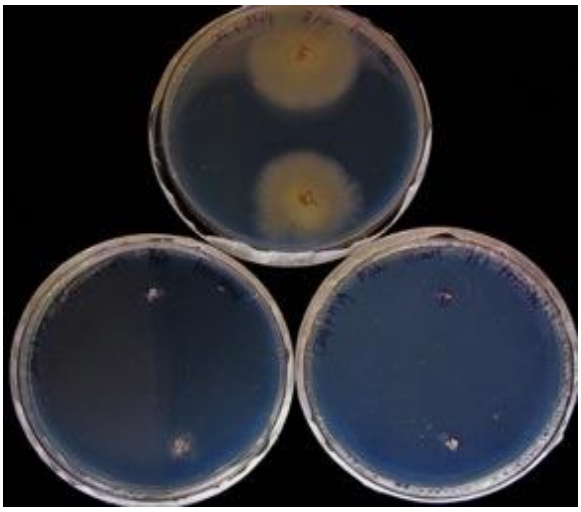


Figure 4.9 Effect of F2dU against *Fol* transformants with T-DNA insertions carrying the HSV*tk* counter-selectable marker. Mycelial growth of two transformants was observed on CDA containing 75 µg/ml hygromycin but not F2dU (upper plate). In contrast, mycelial growth of the transformants was inhibited on CDA containing 75 µg/ml hygromycin and 5 µM or 50 µM F2dU (lower plates).

4.4 Discussion

Homologues of the *Fol* candidate effector genes *SIX7*, *SIX10* and *SIX12* (Houterman et al., 2007; Schmidt et al., 2013) have been identified in the cape-gooseberry-infecting *F. oxysporum* f. sp. *physali* (*Foph*, Chapter 2). Although their role in pathogenicity remains unknown, their identity (93 to 99%) at the protein level between *Fol* and *Foph*, suggests that any role in pathogenicity might be conserved. Therefore, disruption of the members of the conserved SIX gene cluster (SIXcl) through gene knockout methods in *Fol*,

represented a useful alternative to analyse their function which can be extrapolated to *Foph*.

In order to obtain a deletion of the entire SIXcl or a single gene deletion in *Fol*, a knock out approach was used in this study. However, no SIXcl, *SIX10* or *SIX12/7* gene deletions were obtained by targeted gene replacement using ATMT. This is despite numerous transformation attempts (1360), the inclusion of the counter selectable HSV*tk* cassette for selection against ectopic insertions, the recovery and analysis of 352 putative transformants selected on hygromycin and F2dU (Table 4.4), and the demonstrated effectiveness of the counter selection (Figure 4.9). In contrast, Khang et al. (2005), who developed the dual selection method for use in *F. oxysporum*, successfully obtained targeted replacements of the *FoSNF1* (FOXG_05528) gene, with a frequency of 15% of all transformants selected on hygromycin (75 µg/ml) and F2dU (5 µM). Nevertheless, when ATMT coupled with the HSV*tk* cassette was used to disrupt another candidate effector gene (*RALF*), four gene deletion transformants were obtained out of 44 transformants (9%; Chapter 5 and reported in Thynne et al., 2016).

Khang et al. (2005) also found a large number of false positives (71%) when transformants were initially selected on hygromycin and 5 µM F2dU, but subsequently showed growth inhibition when transferred to new media containing the same concentration of F2dU, a problem with leakiness that was not solved during the development of the method. Khang et al. (2005) proposed that expression of the HSV*tk* gene might be suppressed during the fungal regeneration stage, resulting in the observed leakiness, and that the use of a different promoter may help solve this problem. As with Khang et al. (2005), in this study two false positives also showed growth inhibition in fresh media (Figure 4.9). However, the leakiness of this method was not solved by the use of a different fungal promoter (*trpC* in this study cf. *gpdA* in Khang et al., 2005). The poor performance of the counter selection strategy in this study, as indicated by the number of transformants obtained with ectopic T-DNA insertion events, remains unexplained and was not investigated further.

Targeted gene deletion by ATMT has been used extensively in fungi to test gene function (Michielse et al., 2005). However, fungi often exhibit short flanking regions of the targeted gene, which decreases the efficiency of T-DNA recombination at the homologous site (Hynes, 1996; Michielse et al., 2005). In *Fol,* ATMT has been used to disrupt the *SIX1*, *SIX3*, *SIX4*, *SIX5* genes with low homologous-recombination frequencies (e.g. one to three gene deletion transformants out of 100 putative transformants, approximately) (Rep et al., 2004; Houterman et al., 2009; Schmidt et al., 2013; Ma et al., 2013, 2015).

Fol SIX6 has been the only member of the SIX effectors that showed a high frequency of gene deletion transformants (25%) during ATMT (Gawehns et al., 2013). Homologues of *Fol SIX4* and *SIX6* have also been disrupted by ATMT in the ff. spp. *conglutinans* and *niveum* (Thatcher et al., 2012; Niu et al., 2016). However, the efficiency of gene deletion was not reported.

The majority of the *SIX* effector genes identified to date in *Fol* are located on the pathogenicity chromosome 14 with the exception of *SIX4*, which is only present in *Fol* race1 and no assembled genome is available for comparison, and *SIX8b* with four copies located at the LS chromosomes 3 and 6 of *Fol* race 2 (reference genome; Schmidt et al., 2013). The low homologous recombination frequency observed during generation of *SIX1*, *SIX3* and *SIX5* knockouts (Rep et al., 2004, 2005; Houterman et al., 2009; Ma et al., 2015), could be consistent with their location on a lineage-specific chromosome as opposed to a core chromosome (Ma et al., 2010). The difficulty obtaining gene deletion transformants for the *SIX10*, *SIX12* and *SIX7* gene cluster found in this study may indicate that targeted homologous recombination events in *Fol* lineage-specific regions are less frequent, possibly due to the high content of transposable elements that might interfere with recombination.

Knockouts of the *SIX7*, *SIX10* and *SIX12* genes, is necessary not only to unravel their role in *Fol* pathogenicity, but also in other economically important ff. spp. where homologues have been identified (Chapter 2; Taylor et al., 2016). However, the poor performance of the homologous recombination-ATMT methods used to knockout the *Fol*-SIXcl region in this study and the low frequency of gene deletions observed for other *SIX* genes located on chromosome 14, indicate that more efficient gene deletion methods are needed. Potentially, ATMT with genome-editing (CRISPR-*Cas9*) constructs designed for fungal genomes (Arazoe et al., 2015; Liu et al., 2015; Katayama et al., 2016) could be used to target the *SIX* genes with greater efficiency.

Chapter 5.

Analysis of the *Fol* RALF gene in pathogenicity

5.1 Introduction

Plant rapid alkalinisation factor (*RALF*) genes encode secreted peptides that were first identified through their ability to trigger a rapid increase in extracellular pH when added to plant cell suspensions (Pearce *et al.*, 2001). In the *Arabidopsis thaliana* genome, 34 genes have been identified within the *RALF* gene family. These genes were named *RALF1* to *RALF34*, and all homologues in other species have been named according to their similarities to members of the *A. thaliana* family (Thynne et al., 2016). Generally, plant RALF peptides are involved in the alkalinisation of the extracellular space and cell expansion during plant growth (Murphy and De Smet, 2014). In tomato plants, RALFs inhibit the emergence of the primary root during seed germination and negatively regulate the elongation of pollen tubes (Pearce *et al.*, 2001, 2010).

Genes encoding RALF homologues are widely distributed amongst a diverse range of plant pathogenic fungal species including *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), suggesting that these peptides might have a role during plant infection (Thynne et al., 2016). Recently, it was demonstrated that invasive growth of *Fol* hyphae in tomato roots is mediated by a pathogenicity-related mitogen-activated protein kinase (MAPK) signalling cascade, which is activated upon an increase of extracellular pH (Masachis et al., 2016). This boost has been associated with a functional RALF (FOXG_21151) homologue in *Fol* with alkalinizing and growth-regulatory activity towards plants.

Chemically synthesised *Fol* RALF peptide triggered extracellular alkalinisation in tomato cell suspensions, similar to that produced by *Nicotiana tabacum* RALF (Masachis et al., 2016). Moreover, leaf discs of *Nicotiana benthamiana* and tomato treated with synthesised *Fol* RALF and tomato RALF peptides, showed a burst of ROS (reactive oxygen species) production, strong activation of MAP kinases and a significantly greater increase in extracellular alkalinisation in both plant species treated with *Fol* RALF as compared with tomato RALF (Thynne et al., 2016). The synthetic *Fol* RALF peptide also caused a marked inhibition of root elongation and root hair growth in seedlings of tomato and *Arabidopsis* (Masachis et al., 2016; Thynne et al., 2016).

Mutant derivatives of *Fol* with deletions of the *FoRALF* gene (*Fol*-ΔRALF) showed reduced extracellular alkalinisation in roots of inoculated tomato plants compared with

wild type *Fol* (*Fol*-WT). Additionally, plants inoculated with *Fol*-ΔRALF, exhibited significantly greater survival than those inoculated with *Fol*-WT as a consequence of a supposedly reduced capacity to colonise the host plant (Masachis et al., 2016). This result suggested that the RALF contributes to *Fol* virulence in tomato plants. However, no significant differences were observed in pathogenicity when *Fol*-ΔRALF strains were tested under different experimental conditions using a different methodology to score disease symptoms (Thynne et al., 2016). This difference could perhaps be explained by a subtle role for the RALF in pathogenicity not evident under the experimental conditions used in this study.

In this chapter, I describe use of the strategy described in Chapter 4 to obtain the *Fol* race 3 *RALF* deletion mutants reported by Thynne et al., 2016, as well as experiments to assess their effect on pathogenicity. This chapter extends this analysis and also describes the identification of the *Foph RALF* gene and compares it to other *F. oxysporum RALF* genes including *Fol RALF*.

5.2 Materials and Methods

5.2.1 Tomato cultivar and fungal strains used

The tomato M82 cultivar susceptible to *Fol* race 3 was used for pathogenicity tests. *Fol* race 3 isolate #1943 provided by Mr Des McGrath (Agri-Science Queensland, Australia) was used for reverse transcriptase (RT)-PCR analysis and to generate the knockout mutants.

5.2.2 Reverse transcriptase PCR analysis of *RALF* gene expression during infection

Fol RALF expression analysis was performed using the RT-PCR methods described in Section 3.2.4 with the Primers 5’ GCTGAAGCCAACCCCTATAA 3’ and 5’ TTACGATCCGGTTACCAAGC 3’ for the *RALF* gene. The primers described in Section 3.2.4 for the *F. oxysporum* Extracellular Matrix 1 gene (*FEM1*) were used as a positive control for fungal gene expression. RNA and genomic DNA (50 ng) from non-inoculated tomato plants and a five-day-old culture of *Fol* race 3 were used as a control.

5.2.3 Gene deletion vector construction

The binary vector pPK2HPH described in Section 4.2.1 was used as a back-bone for assembly of the *RALF* gene deletion cassette. The upstream flanking region of the *RALF* gene was amplified with the primers RALF_FR1/RALF_FR2 (Table 5.1) and cloned via *Kpn*I and *BsrG*I restriction sites into the 5' end of the hygromycin-resistance (HPH) cassette in pPK2HPH and the downstream flanking region was amplified with the primers RALF_FR3/RALF_FR4 (Table 5.1) and cloned via *Xba*I and *Hin*dIII sites into the 3' end of the HPH cassette, to generate the pPK2HPH:ΔRALF vector Appendix 5.1).

To increase the frequency of recovery of homologous recombination events following *Agrobacterium tumefaciens* mediated transformation (ATMT) of *Fol*, the *HSVtk* cassette described in the Section 4.2.2 was cloned into the pPK2HPH:ΔRALF vector (Appendix 5.2), next to the *RALF* downstream flanking region, using the *Hin*dIII and *Pme*I sites.

Table 5.1. Primers used for gene deletion vector construction

Primer Name	Sequence	Restriction site
RALF_FR1	catggtacCAATTTCAGTCTTCTACATA	*Kpn*I
RALF_FR2	gactgtacaACTGACTGAATAAGGTTGAT	*Bsr*GI
RALF_FR3	agtctagaGCTTGGTAACCGGATCGTAA	*Xba*I
RALF_FR4	tgtaagcttCTACCCAAGCAGTCGGTAGC	*Hin*dIII

5.2.4 Transformation of *F. oxysporum* with gene deletion vectors

Fol transformation was performed using the ATMT methods described in Sections 3.2.2 and 4.2.3

5.2.5 Screening for *Fol* race 3 ΔRALF transformants

Fol transformants were screened for deletion of the *RALF* gene by multiplex PCR as described in Section 4.2.4, using primers scRALF_5'-F and scGPD-R, which amplify a 297 bp region corresponding to part of the *RALF* 5' flanking region and gpdA promoter in the pPK2HPH:ΔRALF:HSVtk vector, together with primer scRALF_int-R, which amplify a region of 404 bp corresponding to part of the *Fol RALF* promoter and coding sequence (Table 5.2, Figure 5.1). Presence of the 297 bp PCR product indicates insertion of the gene replacement cassette. *RALF* gene replacement by homologous recombination was indicated the presence of this band alone, while the additional presence of 404 pb product indicated no disruption of the *RALF* gene i.e. ectopic insertion of the T-DNA (Figure 5.1). Transformants carrying a knockout of the *RALF* gene

were also screened for ectopic insertions using the primers scRALF_5'-R and LB_Ko-F, which amplified a region of 727 bp corresponding to left border insertion of the T-DNA (Table 5.2, Figure 5.1).

Table 5.2. Primers used to screen for *RALF* gene deletion mutants

Primer	Sequence	Screening for
scRALF_5'-R	TTGGAGGGCTCCCTTATTCT	Ectopic T-DNA insertion
LB_Ko-F	TAATAACACATTGCGGACGT	
scGPD-R	AATCATCCACTGCACCTCAG	
scRALF_5'-F	CTTTCCTCTGCCGGTATTTTC	RALF gene deletion
scRALF_int-R	TTGAACTTACGTTGCCACCA	

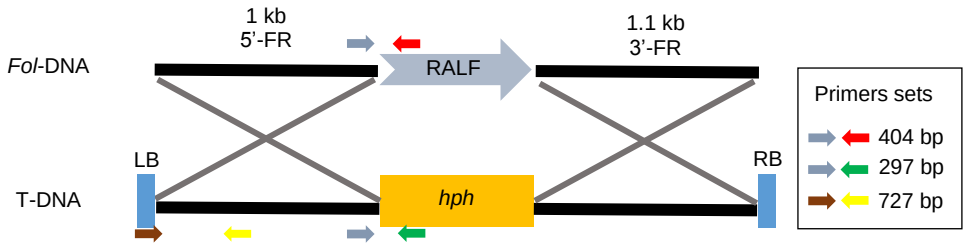


Figure 5.1 Schematic representation of *RALF* gene replacement by the *HPH* cassette. Coloured arrows indicate priming sites. Blue; scRALF_5'-F, red; scRALF_int-R, green; scGPD-R, brown; LB_Ko-F and yellow; scRALF_5'-R primers (Table 5.2), used for screening.

5.2.6 Pathogenicity tests on tomato plants

Pathogenicity tests were performed using the methods described in Section 3.2.3. Four independent *Fol* race 3 *RALF* deletion transformants were tested for the effect of the *RALF* deletion on pathogenicity. Ten 3-week-old M82 plants were used per each *Fol* transformant. Wild-type *Fol*, a transformant with an ectopic T-DNA insertion and mock-inoculated plants were used as controls.

5.2.7 Phylogenetic analysis

Using Blastp searches, the predicted protein sequence of the *RALF* homologue identified in *Foph* was compared with homologues reported for other *ff. spp.* in the UniProt database (The UniProt Consortium, 2015). Protein sequences that showed more than 80% identity with the corresponding *Foph* homologue were retrieved for subsequent analysis as described in section 2.2.5.

5.3 Results

5.3.1 The *Fol RALF* gene is expressed during infection of tomato roots

A RT-PCR analysis using the *RALF* and *FEM1* primers described in Section 5.2.2 above was performed on roots of the susceptible tomato cultivar M82 infected with *Fol* race 3, at 3 and 6 days post inoculation (dpi) (Figure 5.2). No *RALF* gene expression was detected in samples from roots of mock-inoculated tomato plants or five-day-old mycelia grown *in vitro*, but expression was detected in the 3 and 6 dpi samples. The *RALF* and *FEM1* primers flanked introns and therefore yielded different-sized PCR products from cDNA and genomic DNA. No *RALF* or *FEM1* PCR products were detected in samples prepared without reverse transcriptase, indicating that the *RALF* and *FEM1* bands observed at 3 and 6 dpi are authentic RT-PCR products. The absence of *RALF* gene expression in mycelia grown *in vitro* suggests that *RALF* gene expression is induced during plant infection, consistent with a potential role as an effector.

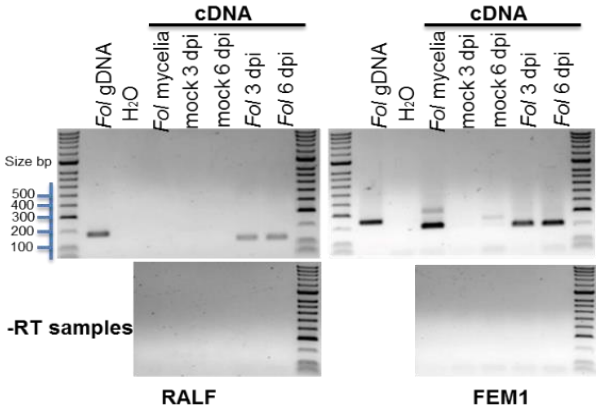


Figure 5.2 RT-PCR analysis shows that *RALF* is expressed during infection of tomato roots by *Fol*. Upper gel images show bands (expected size 123 bp) consistent with RALF gene expression in *Fol*-infected roots at 3 and 6 dpi but not in mock-inoculated tomato plants or five-day-old mycelia grown *in vitro*. Analysis of *FEM1* shows bands (expected size 201 bp) consistent with *FEM1* gene expression in infected roots at 3 and 6 dpi and in five-day-old mycelia grown *in vitro.* The *FEM1* control shows that cDNA synthesis was successful for the mycelial RNA sample. PCR analysis of a *Fol* genomic DNA sample allowed detection of *RALF* and *FEM1* in *Fol*. The *RALF* and *FEM1* PCR products amplified from genomic DNA and cDNA differ in size (expected sizes 179 bp versus 123 bp for *RALF* and 250 bp versus 201 bp for *FEM1*), allowing the detection of genomic DNA contamination in cDNA samples. No *RALF* or *FEM1* products arising from genomic DNA were detected in the cDNA samples. No bands were observed from the samples prepared without reverse transcriptase (lower gel images), indicating that the products observed for the samples prepared using reverse transcriptase (upper gel) images) were authentic RT-PCR products. gDNA = genomic DNA, H_2O = template free PCR amplification control.

5.3.2 Deletion of the *RALF* gene in *F. oxysporum* race 3

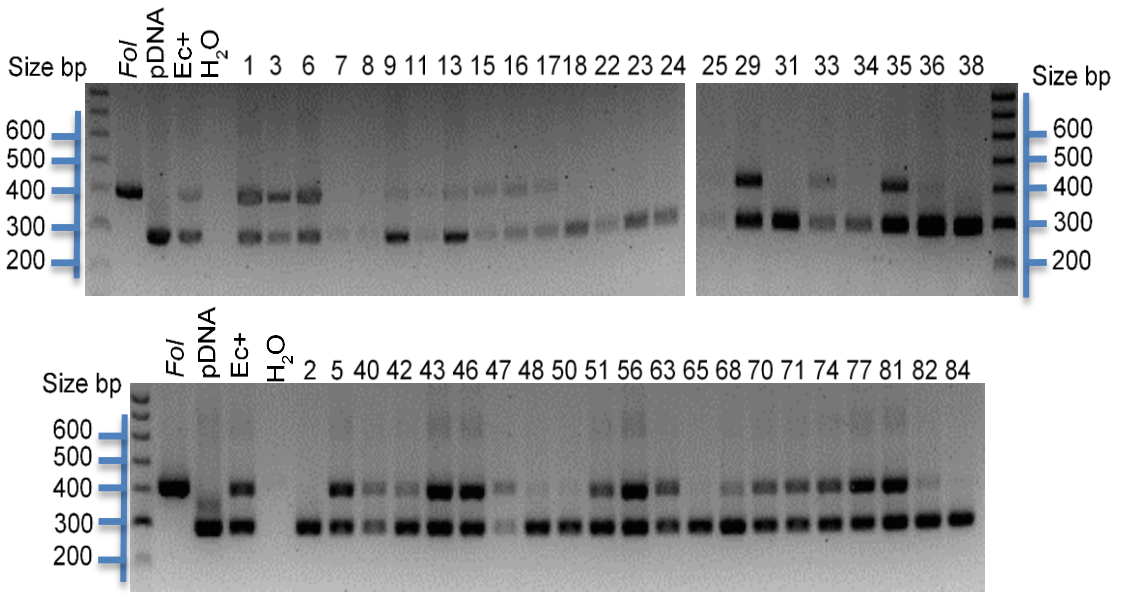
To discriminate between *Fol* transformants that carried a replacement of *RALF* gene sequence by the *HPH* cassette and those with an ectopic T-DNA insertion carrying the *HPH* cassette, a multiplex PCR using the primers scRALF_5'-F, scRALF_int-R and scGPD-R (Table 5.2, Figure 5.1), was performed for 44 transformants that grew on selection media containing 75 µg/ml hygromycin and 50 µM F2dU (Table 5.3). A single band of 297 bp, corresponding to the *HPH* cassette was observed in all transformants while a PCR product of 404 bp corresponding to the *RALF* gene was absent in eleven transformants (Table 5.3, Figures 5.1, 5.3A).

Table 5.3. Transformation efficiency of the ΔRALF vector in *F. oxysporum*

No. Filters placed	Hyg/F2dU insensitive	Multiplex PCR screening		Gene deletion transformants confirmed	False positives
		Double band	Single HPH band		
140	44	33	11	4 (9%)	40 (91%)

To confirm loss of the *RALF* gene amplicon, DNA was extracted from mycelia of these eleven transformants and screened in separate PCRs with primers scRALF_5'-F and scRALF_int-R (i.e. 404 bp *RALF* PCR product) in one reaction and primers scRALF_5'-F and scRALF_5'-R (i.e. 297 bp *HPH* PCR product) in the other (Table 5.2 and Figures 5.1 and 5.3B). *RALF* gene products were amplified from seven transformants, indicating that they carried an ectopic insertion of the T-DNA. For transformants 2, 23, 24 and 31, no *RALF* gene product was observed, while an *HPH* product was amplified from all transformants (Figure 5.3B). The absence of the *RALF* gene amplicon in transformants 2, 23, 24 and 31 suggested that the *RALF* gene had been successfully disrupted and replaced by the *HPH* cassette.

A.



B.

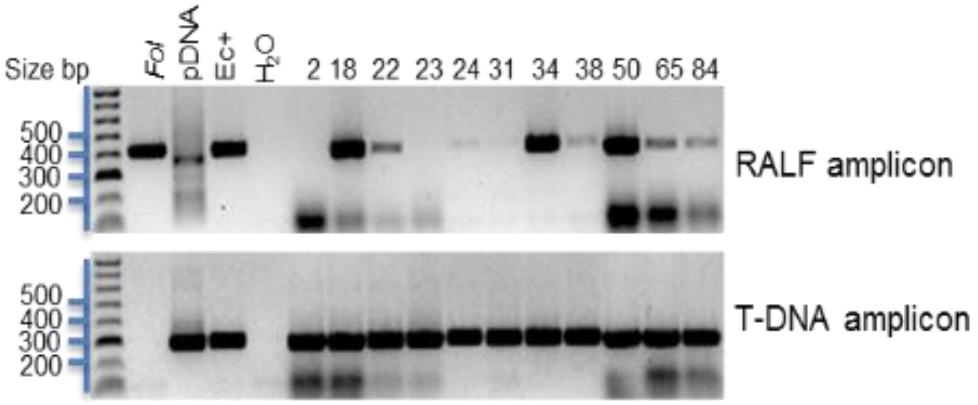


Figure 5.3. PCR screening of 44 *Fol* race 3 transformants for knockouts of the *RALF* gene. **A**. Initial multiplex screening with crude DNA preparations. Eleven candidates (2, 18, 22, 23, 24, 31, 34, 38, 50, 65 and 84) showed only a PCR product of 297 bp suggesting a possible replacement of *RALF* gene sequence by the *HPH* cassette. B. Separate PCR screening with better quality DNA minipreps of the candidates with the primers for the *RALF* gene and *HPH* cassette. Transformants 2, 23, 24 and 31 showed no *RALF* product suggesting that the *RALF* gene was disrupted. PCR product visualization was carried out following electrophoresis in 1.5% agarose gels. *Fol* = *Fusarium oxysporum* f. sp. *lycopersici* genomic DNA, pDNA = binary vector plasmid DNA, H_2O = template free PCR amplification control, Ec^+ = DNA from a transformant with an ectopic T-DNA insertion, bp = base pairs.

To confirm deletion of *RALF* coding sequence in the four transformants (named ΔRALF2, ΔRALF23, ΔRALF24 and ΔRALF31) and replacement by the *HPH* cassette, a third PCR screening was performed on genomic DNAs extracted from monosporic cultures, using the primers LB_KO-F located on the left border (LB) of the T-DNA and scRALF_5'-R located in the RALF 5' flanking region adjacent to the HPH cassette of pPK2HPHΔRALF:HSVtk (Table 5.2, Appendix 5.3), with a predicted PCR product of 727 bp (Figures 5.1 and 5.4).

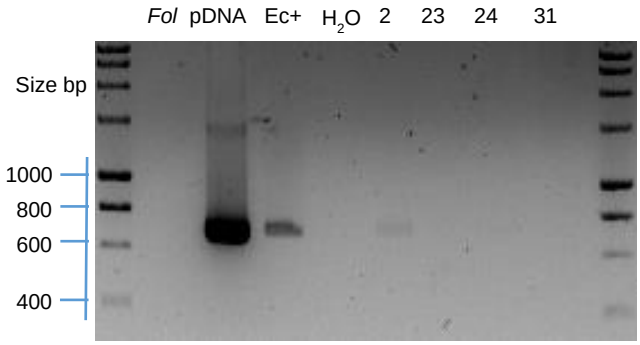


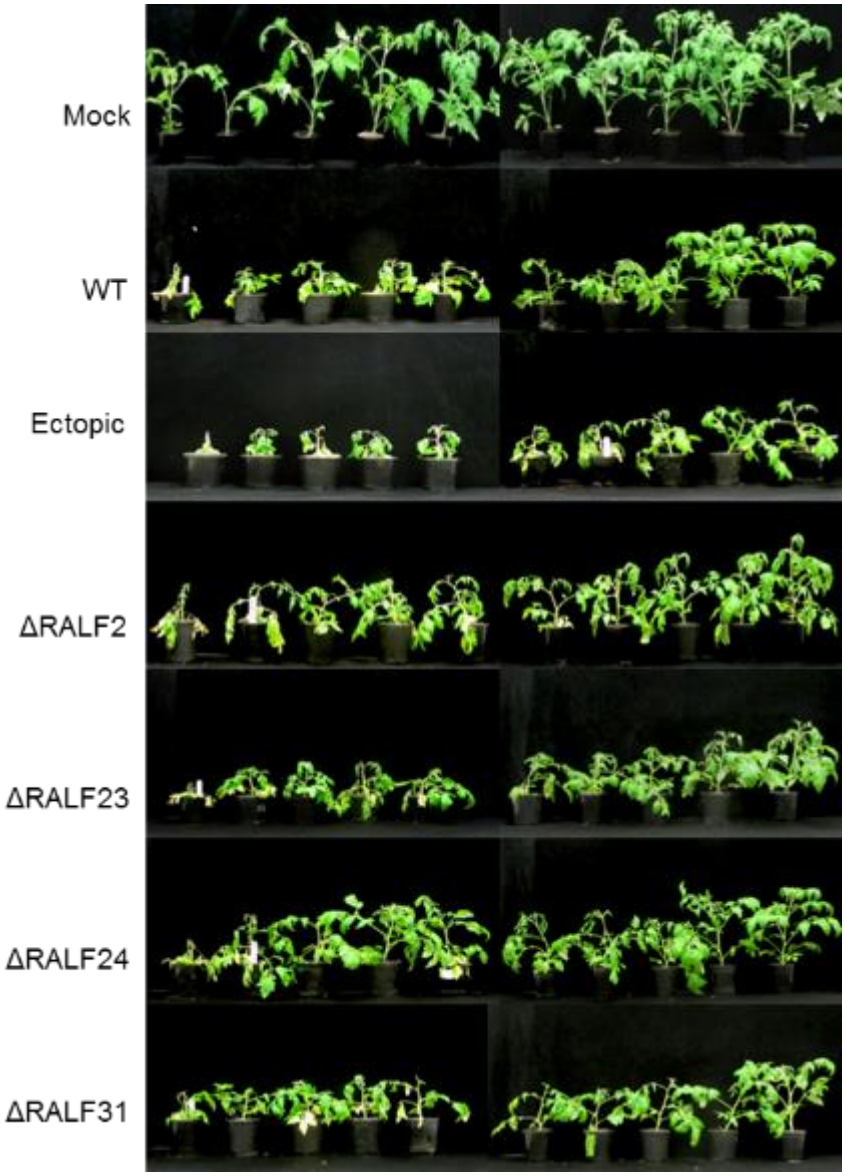
Figure 5.4. Screening of the T-DNA insertion in the ΔRALF transformants with LB and 5' *RALF* flanking region primers (Table 2). The PCR fragment of 727 bp was not observed in the ΔRALF transformants 23 or 31 but faint products were observed in ΔRALF transformants 2 and 24, indicating possible T-DNA insertion in these two transformants. PCR product visualization was carried out following electrophoresis in 1.5% agarose gels.

No PCR fragment corresponding to the T-DNA insertion was observed for transformants 23 and 31, which suggests that these transformants may have undergone gene replacement by homologous recombination. The faint bands observed for transformants 2 and 24 suggest that they may have an ectopic insertion of the T-DNA in addition to the gene replacement, although other explanations are possible, such as retention of T-DNA-carrying *Agrobacterium* despite selection against *Agrobacterium*.

5.3.3 Effect of the RALF gene knockout on *Fol* race 3 pathogenicity

Four *Fol*-ΔRALF transformants were used to evaluate the role of the *RALF* gene in virulence. Three-week-old tomato plants (M82 cultivar susceptible to *Fol* race 3) were inoculated with *Fol*-WT, Ect 50 (transformant with an ectopic T-DNA insertion) and the ΔRALF transformants No. 2, 23, 24 and 31. Disease symptoms were measured according to the disease scoring index described by Rep et al., 2004, with 0 = healthy plant to 4 = severely wilted plant (Figure 5.5).

A.



B.

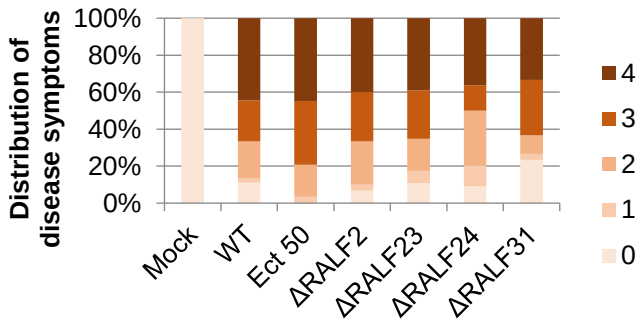
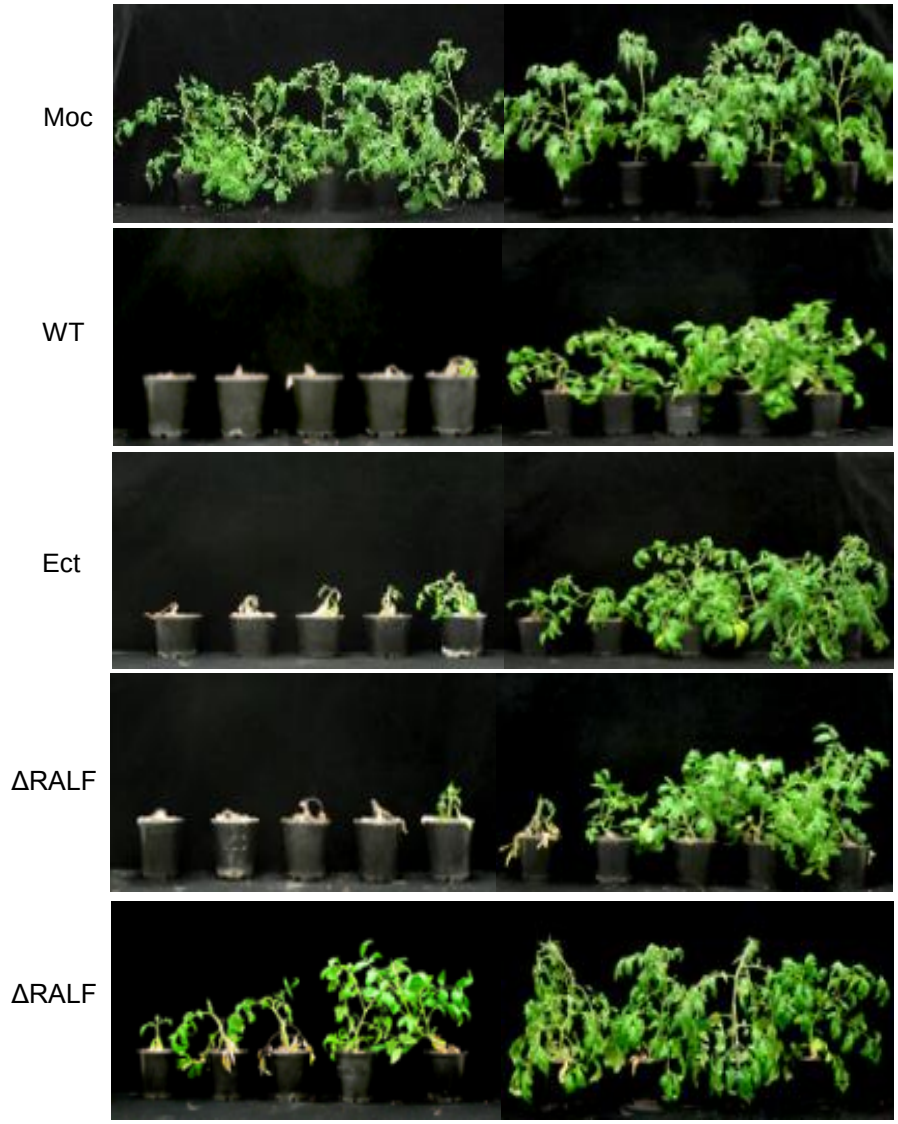


Figure 5.5. Pathogenicity test on M82 plants with either *Fol* race 3 or *Fol* transformants as follows: wild type *Fol* (WT), Ectopic control (Ect 50), ΔRALF2, ΔRALF23, ΔRALF24 and ΔRALF31, **A**. Photographs were taken 21 days post infection (dpi), showing ten plants per treatment with disease symptoms representative of three replicate experiments. **B**. Disease scoring of the symptoms observed at 21 dpi from three replicate experiments (n = 30). Probability values were obtained using the pairwise non-parametric Mann Whitney test (IBM SPSS statistics for Macintosh, version 22.0, 2013), comparing the disease scores between each of the transformants and WT (n=30 plants per treatment). No significant difference was found at p = 0.05 in the distribution of symptoms among the various *Fol* transformants compared with WT *Fol.*

The pathogenicity test was repeated three times and no significant differences in virulence were observed between *Fol*-WT and the transformants (Ect 50 and ΔRALF), despite a trend towards reduced symptom development for ΔRALF24 (Figure 5.5B). The results indicate that the ΔRALF strains obtained in this study were all still able to infect the host and develop disease symptoms typical of wild type *Fol* infection in tomato.

Additional pathogenicity tests were performed in order to evaluate plant survival as an alternative criterion for comparison of virulence between the different *Fol* strains in tomato plants. In one experiment, three-week old M82 tomato plants (susceptible to *Fol* race 3) were inoculated with *Fol*-WT, Ect 50 and two ΔRALF transformants. The scoring of plant survival after inoculation was measured up to 35 dpi as described by Masachis et al. (2016) (Figure 5.6).

A.



B.

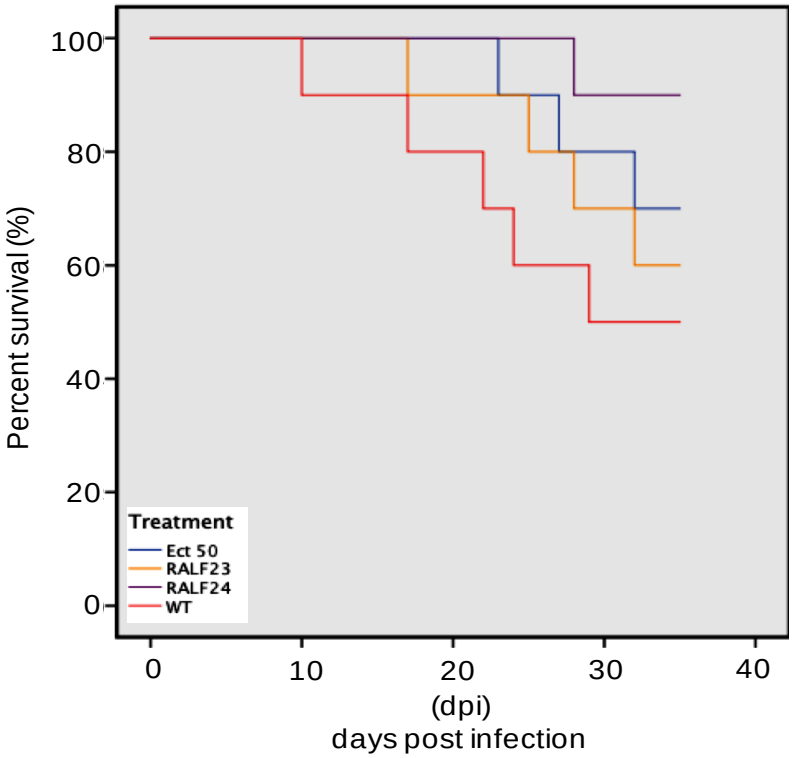
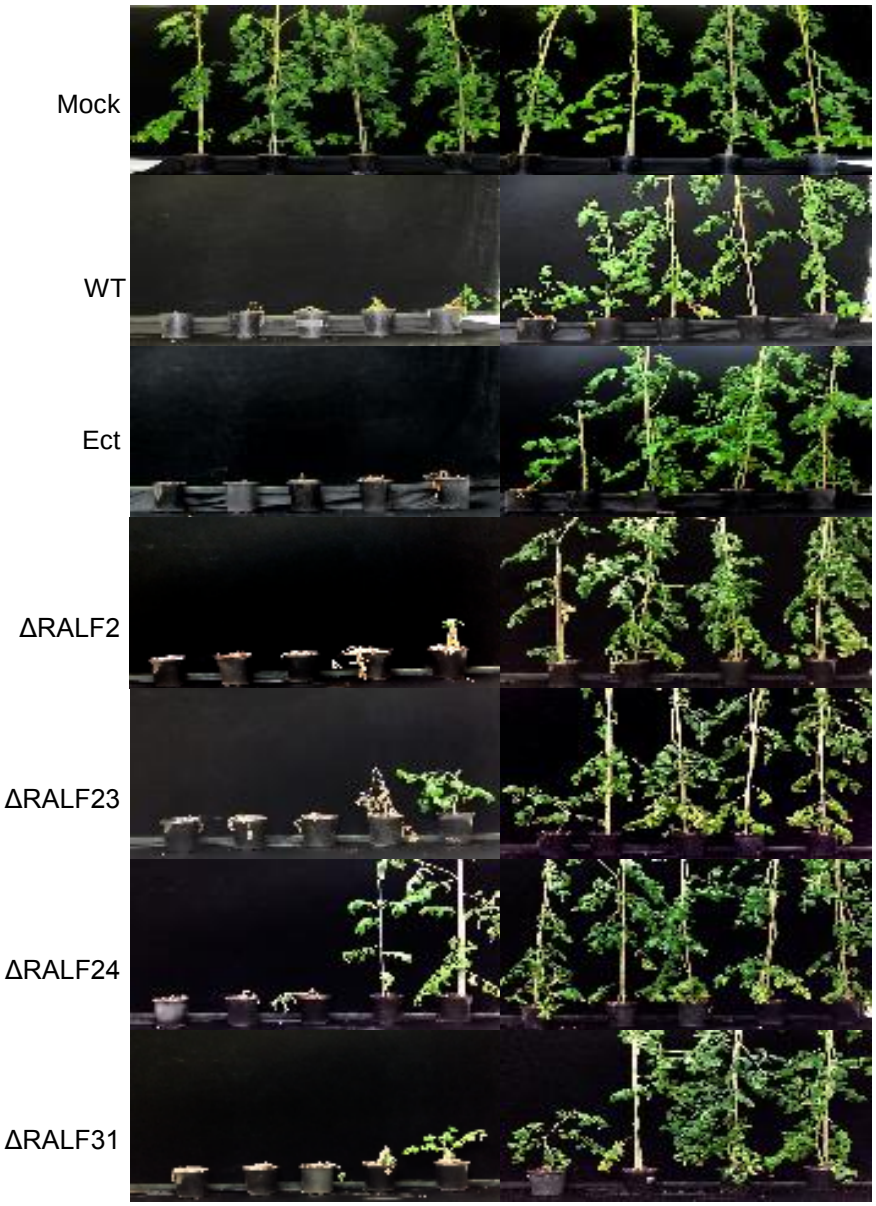


Figure 5.6. Survival of M82 tomato plants following inoculation with either *Fol* race 3 (WT) or *Fol* transformants Ect 50 (carrying an ectopic T-DNA insertion), ΔRALF23 or ΔRALF24. **A**. Photographs of inoculated plants taken at 35 dpi, showing ten plants per treatment corresponding to one replicate of the experiment. **B**. Survival of tomato plants following *Fol* inoculation from one replicate experiment (n=10). A Kaplan-Meier analysis (IBM SPSS statistics for Macintosh, version 22.0, 2013), revealed no significant difference in plant survival at p = 0.05 between the treatments overall. However, a significant difference (p = 0.045) between WT and ΔRALF24 strains was found using the pairwise Mantel-Cox test (IBM SPSS statistics for Macintosh, version 22.0, 2013).

No significant differences in survival were observed between plants infected with *Fol*-WT and ΔRALF23 (Figure 5.6B). However, plants inoculated with ΔRALF24 showed significantly less mortality compared with plants inoculated WT, Ect 50 and ΔRALF23. Therefore, the plant survival test was repeated with all four ΔRALF strains obtained in this study, to test whether the remaining two ΔRALF strains show a lower mortality similar to that observed for ΔRALF24. In this experiment, scoring of plant survival was measured up to 54 dpi to increase the chance of observing differences in plant mortality across the treatments (Figure 5.7).

A.



B.

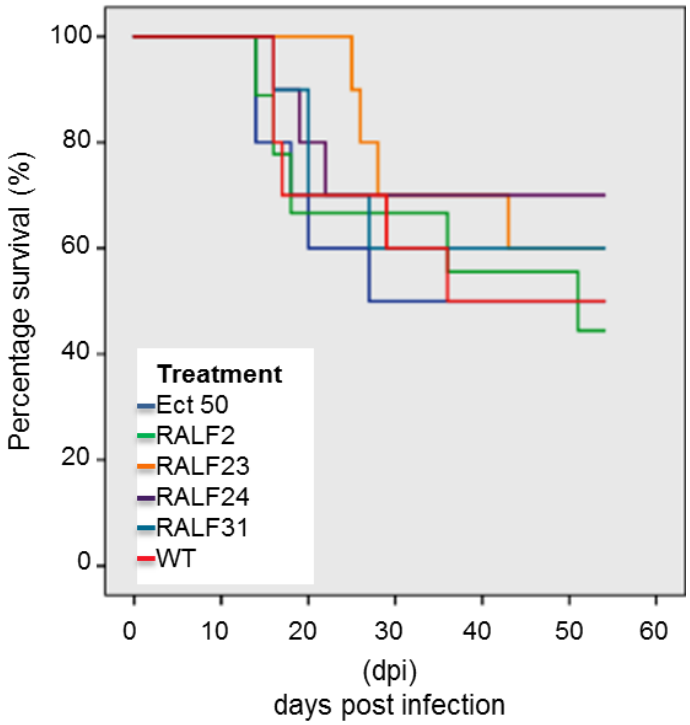


Figure 5.7. Survival of tomato M82 plants following inoculation with either *Fol* race 3 (WT), Ect 50 or *Fol* transformants ΔRALF2, ΔRALF23, ΔRALF24 or ΔRALF31. **A**. Photographs of inoculated plants taken at 54 dpi, showing ten plants per treatment. **B**. Survival of tomato plants following *Fol* inoculation from one replicate experiment (n=10). A Kaplan-Meier analysis revealed no significant difference in plant survival at p = 0.05 between the treatments overall (IBM SPSS statistics for Macintosh, version 22.0, 2013). No significant difference between WT and ΔRALF strains was found using the pairwise Mantel-Cox test (IBM SPSS statistics for Macintosh, version 22.0, 2013).

In this second survival test, no significant differences in survival were observed between plants infected with *Fol*-WT and ΔRALF transformants (Figure 5.7B). This contrasts with the previous observation of significantly less mortality on plants inoculated with ΔRALF24 compared to the plants inoculated with *Fol*-WT and Ect 50 controls (Figure 5.6B). Overall, these results indicate that a knockout of the *RALF* gene does not have an effect the development of virulence symptoms, compared to the symptoms observed on plants inoculated with *Fol*-WT or Ect 50 controls, suggesting that RALF may not be involved in *Fol* pathogenicity.

5.3.4 Identification of a homologue of the *RALF* gene in *Foph*

No *RALF* homologue was detected during the mapping of the RNAseq data generated from *Foph*-infected cape gooseberry (Chapter 2), despite the presence of three RALF-like genes in the candidate effector database generated by van Dam et al. (2016). Nevertheless, *Foph* was expected to possess a *RALF* gene based on the observation that all plant pathogenic isolates of *F. oxysporum* appear to possess a RALF gene (Thynne et al., 2016). Therefore, primers RIB-F (5' TTCACCTATCAACCTTATTCAGTCA 3') and RIB-R (5' TTACGATCCGGTTACCAAGC 3') based on sequences flanking the *Fol RALF* gene were synthesised and sent to CORPOICA for amplification and sequencing of the *Foph* RALF gene. A *RALF* gene homologue was identified from *Foph* genomic DNA by PCR and sequencing (Appendix 5.4). The predicted *Foph* RALF protein shares 99% identity with the *Fol* RALF protein (Figure 5.8).

```
            -Signal peptide-
Foph_RALF   MKFSIITLSLITLASAAPAAKPQSGEISYGALNRDHIPCSVKGASAANCRPGAEANPYNR 60
Fol_RALF    MKFSIITLSLITLASAAPAAKPQSGEISYGALNRDHIPCSVKGASAANCRPGAEANPYNR 60
            ************************************************************

Foph_RALF   GCNAIEKCRGGVGDN 75
Fol_RALF    GCNAIEKCRGGVGGN 75
            ************** *
```

Figure 5.8. Multiple sequence alignment of *Foph* and *Fol* RALF proteins generated using Clustal O (1.2.3). Arrow indicates cleaved site for the mature protein.

5.3.4 Phylogenetic analysis of *Foph* RALF

The *Foph* RALF protein sequence was compared using a Blastp search with homologues from other *ff. spp.* present in the UniProt database (The UniProt Consortium, 2015). A phylogenetic analysis based on RALF homologues with more than 80% identity to *Foph* RALF showed it is more closely related to the RALFs of other *ff. spp*. than *Fol* (Figure 5.9), although this finding should be viewed with caution because it is based on only a single amino acid difference between *Foph* and *Fol* RALFs.

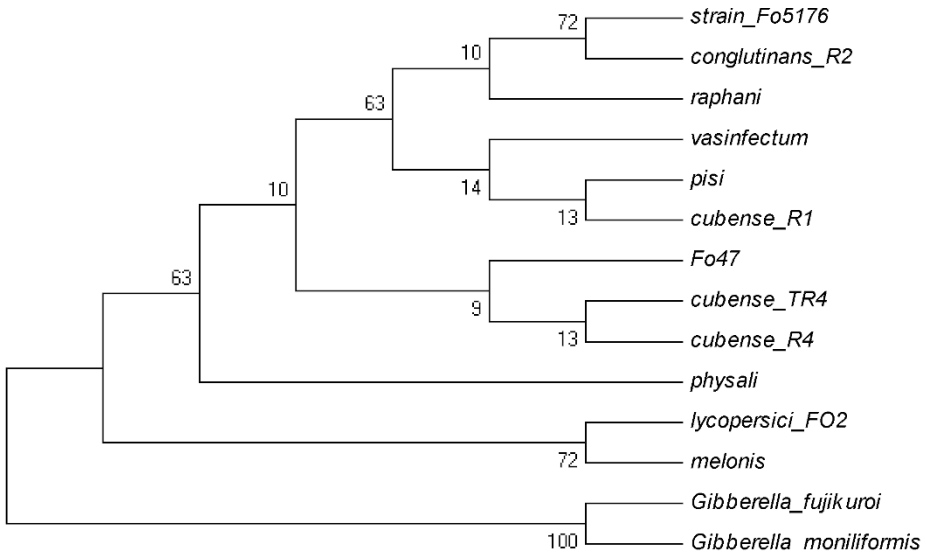


Figure 5.9 Phylogenetic analysis of predicted RALF proteins from various ff. spp. of *Fusarium oxysporum*. Phylogenetic analysis was performed using the Maximum Likelihood Method in MEGA. Numbers on the branches represent the percentage of 1000 bootstrap replicates grouping the associated ff. spp. together. The protein sequences were retrieved from the Uniprot database (The UniProt Consortium, 2015),

5.4 Discussion

Expression of the *Fol RALF* gene in *Fol*-infected tomato plants was observed at 3 and 6 dpi (Figure 5.2; published in Thynne et al., 2016), which, along with functional evidence for extracellular alkalinisation and production of ROS in tomato and *N. benthamiana* treated with synthetic *Fol* RALF peptide, is consistent with a potential role for the *Fol* RALF peptide in pathogenicity.

In the present study, the similar disease symptoms developed by *Fol*-WT and ΔRALF strains in pathogenicity tests indicate that the *RALF* gene is not involved in the pathogenicity of *Fol* race 3 on tomato. In contrast, Masachis et al. (2016) reported significantly lower mortality in tomato plants inoculated with two ΔRALF strains, compared with WT *Fol* race 2, transformants with ectopic insertions of the knockout construct and several ΔRALF strains complemented with the *Fol RALF* gene, suggesting that the RALF protein might contribute to the virulence of *Fol* in tomato plants. The differing findings regarding the role in pathogenicity of the *Fol* RALF gene may be explained by the different experimental conditions and scoring criteria used by Masachis et al. (2016) compared to the findings presented in this chapter.

The different parameters scored in the two studies could also explain the different effect on pathogenicity observed. Masachis et al. (2016), scored plant survival up to 35 dpi, as opposed to scoring of disease symptoms up to 21 dpi as done in the present study, in which scoring was based on the gradual wilting of plants during the assay as opposed to plant death. Plant death was not frequently observed at 21 dpi (Figure 5.5A) and compared to the results of Masachis et al. 2016, the extent of plant death was similar in both studies at 21 dpi and did not differ markedly between *Fol*-WT and ΔRALF strains. The methodology of the present study involved dipping of trimmed roots of tomato seedlings into a spore suspension as a routine inoculation technique to facilitate *Fol* infection. Inoculated plants were then replanted in potting mix. In contrast, Masachis et al. (2016) dipped the roots of tomato seedlings into a spore suspension and grew plants for inoculation in vermiculite with no plant nutrients provided. Less severe disease symptoms have been observed on inoculated plants supplied with fertilizers compared to those without (Nordzieke, 2016, Molecular Genetics of Fungal Pathogenicity group, Cordoba University, personal communication). Therefore, it is possible that the effect observed in the nutrient-deprived (i.e. stunted and stressed) plants used in the Masachis et al. (2016) assays was not evident in healthy plants grown in potting mix well supplied with nutrients.

Masachis et al. (2016) showed that the pH of the plant growth media could complement the loss of *RALF* gene function. When the substrate used for growth of tomato plants inoculated with a Δ*RALF* mutant was buffered to pH 7.0, a significantly higher mortality was observed compared with inoculated plants grown on non-buffered substrate. This result suggests that the pH of the plant growth media could affect the pathogenicity phenotype displayed by a Δ*RALF* mutant. In this study, the pH of the substrate (potting mix) used during the pathogenicity tests in tomato with *Fol*-WT and ΔRALF strains was measured to determine whether the substrate was alkaline and therefore could interfere with the pathogenicity tests. However, the pH of the potting mix used was found to be 6, suggesting that the pH of the potting mix could not have masked any effect of the Δ*RALF* knockout on *Fol* pathogenicity in the experiments described in this chapter.

The trimming of roots prior to inoculation may also have contributed to the different outcomes observed if Masachis et al. (2016) did not do the same. It is possible that *Fol* RALF might be necessary for fungal penetration of the root cortex as the alkalinisation promoted by RALF, could regulate the expression of the *Fmk1* gene, which is involved in virulence and chemotropism of infecting hyphae towards host root tissue (Di Pietro et al., 2001; Perez-Nadales et al., 2014; Turra et al., 2014, 2015; Masachis et al. 2016). However, the role of RALF in host colonisation is a matter for further investigation, which

could, for example, include an analysis of fungal biomass during host infection by measuring chitin content or by measuring the proportion of *Fol* DNA (WT or ΔRALF mutant) during the early stages of infection.

To perform a fairer comparison of the results observed with the *Fol* ΔRALF strains obtained in this study and the observations reported by Masachis et al. 2016, two pathogenicity tests were performed focusing on the scoring of plant survival up to 35 and 54 dpi, respectively. However, no significant difference in plant survival was observed between *Fol*-WT and the ΔRALF transformant strains (Figures 5.6B and 5.7B), apart from ΔRALF24 in the 35 dpi experiment (Figures 5.6). Although plants inoculated with ΔRALF24 showed wilting symptoms similar to those observed for plants inoculated with the other *Fol* strains (Figure 5.6A), ΔRALF24 inoculated plants showed significantly lower mortality ($p = 0.04$) compared with plants inoculated with *Fol*-WT, when a pairwise comparison was performed with the Mantel-Cox test (Figure 5.6B).

However, no significant difference in plant survival was observed between *Fol*-WT and the ΔRALF transformant strains including ΔRALF24 in the 54 dpi experiment (Figure 5.7). This second result supports the overall conclusion presented in this study that the RALF gene is not involved in the pathogenicity of *Fol* race 3 on tomato and contradicts the results of Masachis et al. (2016), where the capacity to colonise the host plant was reduced in *Fol*-ΔRALF strains compared to the ectopic and WT controls. Nevertheless, as these two survival tests were not replicated, further replicates need to be performed to determine whether or not ΔRALF transformants have a differential effect on plant mortality compared with those inoculated with WT and ectopic strains.

The *Fol* genome is organised into core chromosomes or regions, which are thought to be mostly associated with structural and vital functions of the species, and lineage-specific (LS) chromosomes or regions, which are present only in pathogenic strains and harbor the majority of genes that are predicted to be involved in fungal–host interactions (Ma et al., 2010). Chromosome 12 is considered to be part of the *Fol* core genome. However, in a recent study, Vlaardingerbroek et al. (2016a) generated *Fol* strains carrying deletions in chromosome 12 or a total loss of the chromosome, and tested their pathogenicity by measuring the severity of disease symptoms in the same way as performed in the present study. The deletion strains showed a similar distribution of disease scores compared to the control strains, indicating that chromosome 12 does not encode vital functions and is dispensable for pathogenicity on tomato. The findings of the present study are in agreement with the findings of Vlaardingerbroek et al. (2016a), given that the *RALF* gene (FOXG_21151) is located on chromosome 12.

Although no significant differences were observed when ΔRALF strains were compared with the control strains, the *Fol RALF* gene may have a role in *Fol* pathogenicity since it is induced during infection. The alkalinizing function of RALF may be important for the expression of other genes involved in host colonisation such as genes involved in the growth of infecting hyphae (Di Pietro et al., 2001; Perez-Nadales et al., 2014; Turra et al., 2014, 2015). Interestingly, phylogenetic analysis suggests that *Foph*-RALF is more related to other *ff. spp.* than *Fol*, although as already noted this finding should be viewed with caution as it is only based on a single amino acid difference between *Foph* and *Fol* RALFs. Further analysis of the *Foph* RALF might include testing to see if it can trigger extracellular alkalinisation in tomato and cape gooseberry. Gene knock-outs could also be tested to see if, unlike its counterpart in *Fol*, *Foph* RALF could play a role in *Foph* pathogenicity.

Chapter 6

General Discussion

Physalis peruviana, known as cape gooseberry, is an economically important crop plant in Colombia that is under threat from a vascular wilt disease caused by a newly-described *forma specialis* of *Fusarium oxysporum,* (here designated f. sp. *physali* [*Foph*]). Little is known about the interaction between *Foph* and cape gooseberry. Therefore, in this study, RNAseq data from *Foph*-infected cape-gooseberry plants were used to identify *Foph* homologues of known effectors or candidate effectors from other *F. oxysporum* pathosystems, especially those from the well-characterised tomato/*Fol* pathosystem. Owing to a lack of access to *Foph,* functional analysis of these candidate effectors was attempted using the tomato/*Fol* pathosystem. This chapter discusses the identification of eight candidate effectors in *Foph*, the presence and possible horizontal acquisition of a region of *Fol* chromosome 14 carrying four of these effectors, the results of attempts to analyse the function of several of these candidate effectors using the tomato/*Fol* pathosystem and the possible implications of these findings with respect to *F. oxysporum* pathogenicity in cape gooseberry and other solanaceous crop plants with overlapping distributions.

6.1 Identification of putative effector transcripts in *Foph*

As described in Chapter 2, seven putative effector transcripts were identified in *Foph* based on the mapping of RNAseq data from infected cape gooseberry plants against the *Fol* LS transcriptome, and a database of putative effectors identified in other *F. oxysporum* ff. spp. Six of them corresponded to homologues of *Fol* genes (*SIX1, SIX7, SIX10, SIX12*, *SIX15* and *Ave1*). Interestingly this included *Ave1*, and is the first report of an *Ave1* homologue in *F. oxysporum* other than *Fol*. A homologue of a candidate effector (FOXM_16306) from legume-infecting *F. oxysporum* ff. spp. (Williams et al., 2016), was also identified in *Foph* and intriguingly, a BLAST search against *F.oxysporum* genomes at the Ensembl fungi database (Kersey et al., 2016), revealed a homologue of this gene also present in the banana-infecting f. sp*. cubense* race 1. Future gene knockout studies to determine the function of these seven putative effectors in *Foph* pathogenicity will need to be performed.

Additionally, a homologue of the *Fol* RALF gene was identified in *Foph* (Chapter 5). In plants, RALF proteins are involved in cell expansion during plant growth (Murphy and De Smet, 2014). In fungi, RALF homologues are present in

phytopathogens including *Fol*, and are perhaps involved in pathogenicity (Masachis et al., 2016; Thynne et al., 2016). The RALF homologue present in *Foph* is 99% identical at the protein level to its counterpart in *Fol (*Chapter 5), suggesting that RALF function might be highly conserved between *Fol* and *Foph. Fol* RALF plays a role in extracellular alkalinisation in the host root (Thynne et al., 2016). High extracellular pH activates a conserved fungal mitogen-activated protein kinase (MAPK), named Fmk1, which promotes fungal pathogenicity (Masachis et al., 2016). Despite the suggested role of *Fol* RALF in promoting fungal colonisation of host roots, pathogenicity tests on tomato plants using four Fol-ΔRALF strains, described in Chapter 5 and published by Thynne et al. (2016), did not support this hypothesis. Nevertheless, *Foph* RALF could play a role in *Foph* pathogenicity and should therefore be tested for ability to trigger extracellular alkalinisation in tomato and cape gooseberry. Gene knock-outs could also be tested to see if, unlike its counterpart in *Fol*, *Foph* RALF has a role in *Foph* pathogenicity on cape gooseberry.

6.2 Evidence of a highly conserved pathogenicity chromosomal segment between *Fol* and *Foph*

F. oxysporum has a compartmentalised genome, divided into core chromosomes which contain genes with metabolic and structural functions conserved between species, and lineage-specific (LS) or dispensable chromosomes containing genes determining pathogenicity and host specificity (Ma et al., 2010; Schmidt et al., 2013). Horizontal acquisition of LS chromosomes has been proposed as an evolutionary mechanism for emergence of pathogenic strains and diversification of host range (Ma et al., 2010). In *Fol*, horizontal transfer of LS chromosome 14 was demonstrated when a pathogenic strain was co-cultivated *in vitro* with a non-pathogenic one. The recipient strain (non-pathogenic) also acquired virulence on tomato plants, confirming that *Fol* chromosome 14 contains genes involved in pathogenicity (Ma et al., 2010).

Homologues of *Fol* SIX genes have been identified in other ff. spp. of *F. oxysporum* (Chapter 2; Thatcher et al., 2011; Meldrum et al., 2012; Rocha et al., 2015; Schmidt et al., 2015; Taylor et al., 2016; Williams et al., 2016; Li et al., 2016). The presence of these homologues might be a consequence of horizontal transfer of genes or segments of pathogenicity chromosomes between different strains of *F. oxysporum*. In this study, the identification of a highly-conserved cluster of physically-linked genes (the putative effectors *SIX7*, *SIX10*, *SIX12* and *SIX15,* and the transcription factor αTF1) shared

between *Foph* and *Fol* (Chapter 2), suggests horizontal acquisition of an entire chromosomal segment from an ancestor of *Fol* or *Foph.* Furthermore, the presence of less conserved homologues of *Fol SIX1* and *Ave1*, which are also located on *Fol* chromosome 14, suggests that these effector genes may have a different ancestry, perhaps via acquisition of different segments of the pathogenicity chromosome at different times in the evolution of *Fol* or *Foph*. Future phylogenetic analysis comparing an assembled *Foph* genome sequence with the assembled genomes of other ff. spp. of *F. oxysporum* may help to support these hypotheses.

In *Fol*, races 2 and 3 do not contain the *SIX4* effector gene (located close to *SIX6* in *Fol* race 1), but remain pathogenic on susceptible tomato cultivars (Houterman et al., 2008; Chellappan et al., 2016; Kashiwa et al., 2016). In addition, mutant strains with a large deletion (0.9 Mb) of chromosome 14, including the candidate effector genes *SIX6*, *SIX9* and *SIX11* did not show any loss of virulence compared to wild type *Fol* on tomato plants (Vlaardingerbroek et al., 2016). These findings indicate that the genes located in the remainder of chromosome 14 (including the *SIX* genes with homologues in *Foph* identified in Chapter 2) are sufficient for tomato infection (Vlaardingerbroek et al., 2016). Nevertheless, the role in pathogenicity of SIX7, SIX10, SIX12 and SIX15 is unknown. It might be possible that they have a role in *Fol* pathogenicity but not *Foph* pathogenicity or vice versa or that they are undergoing adaptation to another environment (i.e. a different host plant) due to the recent horizontal acquisition in *Fol* or *Foph*. It is also possible that, like SIX4, they do not have a direct role in *Fol* or *Foph* pathogenicity. Although the attempt to knock out the *SIX7, SIX10, SIX12* gene cluster did not work in this study (Chapter 4), further characterisation studies are needed to investigate the role of these candidate effectors (and *SIX15*) in *F. oxysporum* pathogenicity. *Foph* genomic sequencing is under way (Gonzalez, 2016, personal communication). Assembly and annotation of a *Foph* genome sequence will be useful to better define the highly conserved segment of the *Fol* chromosome 14 found in this study. Additional *Foph* strains also need to be analysed for the presence of this segment of chromosome 14 to determine whether it is a general feature in *Foph* or unique to this particular isolate.

6.3 The role in pathogenicity of *Foph*-SIX1a and SIX1b appears to be specific for cape gooseberry infection

In this study, two homologues of *Fol*-SIX1 (named *SIX1a* and *SIX1b*) were identified in *Foph* (Chapter 2). In *Fol*, *SIX1* is located on the LS chromosome 14, which carries several other *SIX* genes associated with pathogenicity (Ma et al., 2010; Schmidt et al., 2013; Kashiwa et al., 2016; Vlaardingerbroek et al., 2016). Furthermore, another homologue (*FoAve1*), located on *Fol* chromosome 14 at a distance of 212 kb from *SIX1,* was identified in *Foph* raising the possibility of further conservation of *Fol* chromosome 14 in *Foph*. Nevertheless, it might also be possible that *Foph SIX1a, SIX1b* and *Ave1* are located on different chromosomes. The ongoing *Foph* genome sequencing, assembly and annotation will enable this relationship to *Fol* chromosome 14 to be examined in more detail.

In *Fol*, the *SIX1* effector is involved in pathogenicity (Rep et al., 2002, 2004). Similarly, the *SIX1* homologue present f. sp. *conglutinans* R2 has also been shown to play a role in host virulence in the *Brassicaceae* (Li et al., 2016). *Fol SIX1* has also been characterised as an avirulence gene (*Avr3*), since its protein product is recognised by the I-3 protein, thereby conferring resistance to *Fol* races 2 and 3 (Rep et al., 2004, 2005, Catanzariti et al., 2015). Gene expression analysis suggests that *SIX1* may have a role in the biotrophic stage of *Fol* infection, as it is expressed during penetration of the root cortex (van der Does et al., 2008). Homologues of the *Fol SIX1* gene have been identified in other ff. spp. of *F. oxysporum* (Meldrum et al., 2012; Thatcher et al., 2012; Laurence et al., 2015 Nino-Sanchez et al., 2015; Rocha et al., 2015; Schmidt et al., 2016 and Williams et al., 2016). These homologues encode proteins with 67 to 80% identity to *Fol* SIX1. They share highly similar signal peptide and prodomain sequences, but show highly diverse mature protein sequences, with some residues showing low variation, while others are highly variable (Figure 1) and possibly under diversifying selection to avoid recognition by their corresponding host resistance proteins.

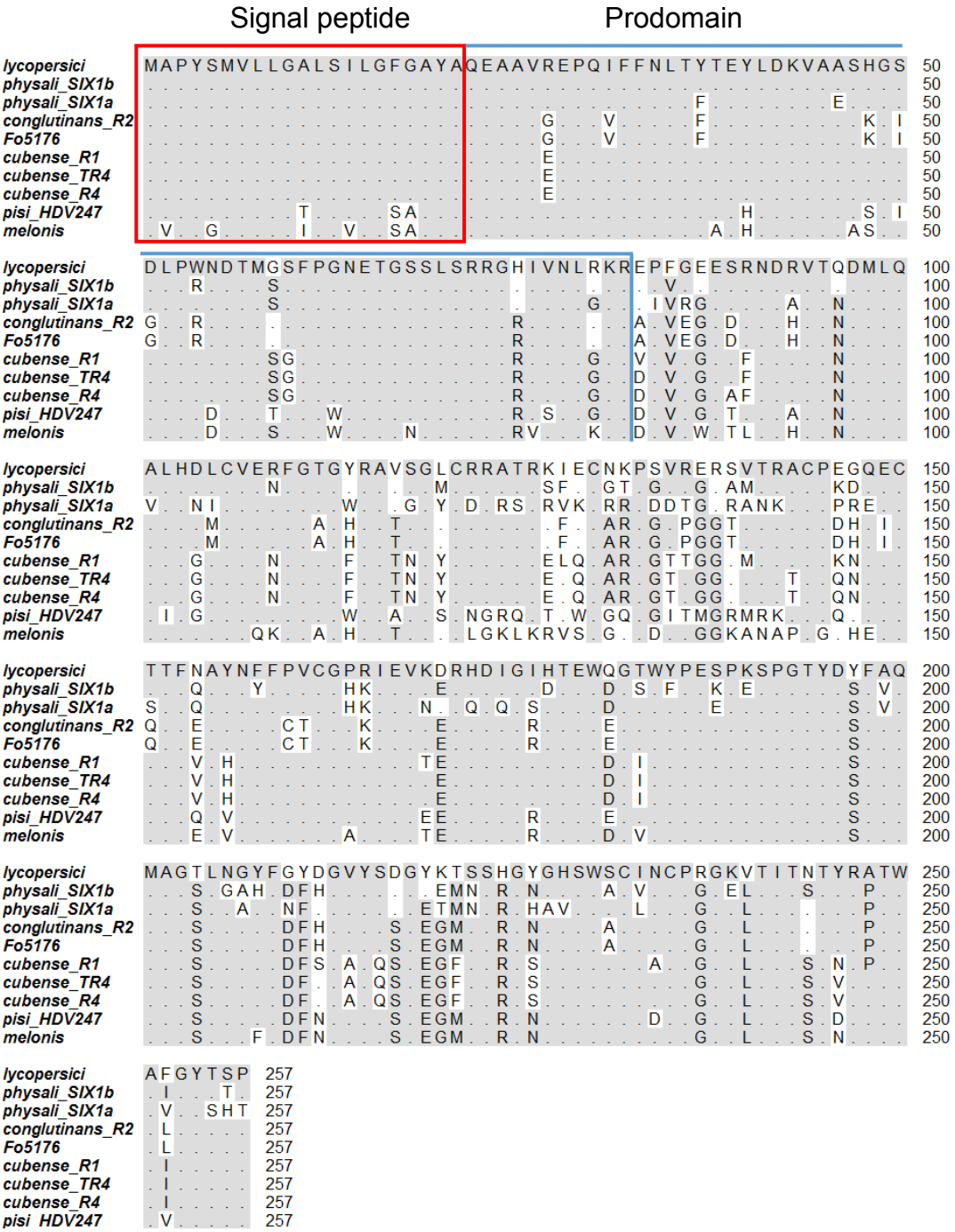


Figure 6.1. Protein sequence alignment of SIX1 *homologues in Foph and other ff. spp. of F. oxysporum*, generated using Clustal-Omega (Goujon et al., 2010). Signal peptide highlighted in red indicates the signal sequence for protein secretion; Prodomain highlighted in blue, indicates the region cleaved off the mature protein (Rep et al., 2004; Houterman et al., 2007). Dots represent amino acids identical to *Fol* SIX1; Grey shaded regions indicate identical residues.

Transformation of a *Fol-ΔSIX1* strain with *Foph SIX1a* or *SIX1b* showed that neither *SIX1a* nor *SIX1b* complemented the loss of *SIX1* pathogenicity function in *Fol* (Chapter 3). A similar result was observed when *Fol SIX1* was tested for its ability to complement a loss of *SIX1* function in the cabbage-infecting *F. oxysporum* f. sp. *conglutinans* (Li et

al., 2016). No restoration of pathogenicity was observed in susceptible cabbage plants inoculated with *Foc-ΔSIX1* transformed with *Fol-SIX1*. These results suggest that *SIX1* is associated with host specificity (Li et al., 2016; Chapter 3). The *SIX1* gene has homologues in many ff. spp. of *F. oxysporum* (Chapter 1), suggesting that it may play a prominent role in the diversification of host range in *F. oxysporum*.

6.4 *Foph SIX1b* can act as an avirulence factor

Although *SIX1a* and *SIX1b* may have a specific role in *Foph* pathogenicity, this study also showed that SIX1b is recognised by the tomato I-3 protein and is therefore able to trigger resistance to *Fol* race 3 in tomato (Chapter 3). The tomato I-3 protein is a membrane-bound S-receptor like kinase (SRLK), consisting of a cytoplasmic kinase domain and an extracellular S-domain (Catanzariti et al., 2015). The SRLK proteins represent a large subfamily of the receptor-like kinase (RLK) protein family in plants (Xing et al., 2013). It is not yet known how I-3 recognises *Fol* SIX1, but the membrane location and structural similarity of its ectodomain to protein domains involved in ligand recognition, suggest that the tomato I-3 / *Fol* SIX1 interaction may take place either directly or indirectly in the apoplast of xylem parenchyma cells (Catanzariti et al., 2015). A similar mode of recognition may occur during the interaction of I-3 with *Foph* SIX1b. The mature *Foph* SIX1b protein has more similarity to *Fol* SIX1 compared to *Foph* SIX1a and the SIX1 homologues from other ff. spp. (Figure 6.1), suggesting that the additional residues conserved between *Fol* SIX1 and *Foph* SIX1b might be important for recognition by I-3. Future studies of these conserved residues by mutations, domain swaps and/or deletions, need to be carried out to further investigate the specificity of SIX1 recognition.

Besides the *I-3* gene (encoding an SRLK protein), several other genes for resistance to *Fol* (*I*, *I-2,* and *I-7*) have been identified in tomato (Ori et al., 1997; Simons et al., 1998; Gonzalez-Cendales et al., 2016; Catanzariti et al., 2017. The proteins encoded by these *R* genes recognise other specific SIX effector proteins. SIX3 (Avr2) is recognised intracellularly by I-2 (CC-NBS-LRR protein), conferring resistance to *Fol* race 2 (Houterman et al., 2009; Ma et al., 2015), while SIX4 (Avr1) is recognised at the plasma membrane by the LRR-receptor like protein (LRR-RLP) encoded by the *I* gene, which confers resistance to *Fol* race 1 (Catanzariti et al., 2017). Furthermore, in the melon-infecting f. sp. *melonis* (*Fom*), the AvrFom2 effector protein is recognised by an NB-LRR protein encoded by the *Fom-2* gene in melon plants resistant to *Fom* race 2 (Joobeur et al., 2004), suggesting that AvrFom2 may also be translocated into the cytoplasm as occurs with the SIX3 effector of *Fol*. Furthermore, another *R* gene named *Fom-1,* which

encodes a Toll/Interleukin-1 receptor (TIR) NB-LRR protein, confers resistance to *Fom* races 0 and 2 (Brotman et al., 2013). However, no avirulence counterpart has yet been identified in *Fom.* In Arabidopsis, six quantitative trait loci (QTL) involved in the resistance to *F. oxysporum* f. sp. *matthioli* have been identified (named *RFO1* to *6*) *RFO1*, *2* and *3* encode a wall-associated kinase-like (WAKL) protein, a LRR-RLP and an SRLK, respectively (Shen and Diener, 2013; Cole and Diener, 2013), with the two latter proteins similar to those encoded by the tomato *I, I-3* and *I-7* genes in tomato.

The identification of at least five different types of receptor proteins (LRR-RLPs, SRLKs, WAKLs, TIR-NB-LRRs and CC-NB-LRRs) including three different types of membrane receptors able to trigger resistance against *F. oxysporum* in different host plants, suggests that effector recognition at the apoplast by membrane receptor-like proteins might be a common defence mechanism against *F. oxysporum*. However, in cape gooseberry, a gene that encodes a CC-NBS-LRR protein was found to be highly correlated with *Foph* resistance, suggesting that this protein might have a similar function to that of tomato I-2 (Enciso-Rodriguez et al., 2013). However, no *Foph* homologue of *SIX3* was identified in this study (Chapter 2), which suggests that the CC-NB-LRR gene in cape gooseberry most likely confers resistance through recognition of a different *Foph* effector or perhaps *Foph* has a homologue of *Fol-SIX3*, which is absent in the isolate used to obtain the RNAseq data and therefore not identified in this study. Future studies including the screening of more *Foph* isolates are required to identify the corresponding avirulence protein in *Foph*.

Conversely, the functional evidence for *Foph* SIX1b recognition by the tomato I-3 (SRLK) resistance protein observed in this study (Chapter 3), suggests that SIX1b could also function as an avirulence protein in *Foph* and could possibly be recognised by an SRLK protein in cape gooseberry (i.e. a putative homologue of tomato I-3), thereby triggering resistance to *Foph*. Alternatively, cape gooseberry plants could be transformed with the tomato *I-3* gene to see if it can recognise SIX1b and mediate resistance to *Foph* in cape gooseberry, thereby providing plant breeding programs with an alternative source of resistance for development of new cape gooseberry cultivars.

6.5 Concluding remarks

This thesis describes the identification of *Fol* effector homologues in *Foph* and represents the first analysis of the *Foph* transcriptome involved in pathogenicity. The chromosomal segment conserved between *Fol* and *Foph* suggests a horizontal transfer event, indicating that the effectors located in this segment could have similar functions

in *Fol* and *Foph*. Future work on other *Foph* isolates (i.e. comparative genomics) is needed to corroborate these findings. Vascular wilt diseases caused by *F. oxysporum* also negatively affect production in other important solanaceous plants, (e.g. *Solanum quitoense*, *Solanum betaceum* and *Physalis philadelphica*), causing a negative socio-economic impact, principally in the Andean regions of Bolivia, Colombia, Ecuador and northern Chile. Therefore, it will be important to determine whether presence of the chromosomal segment conserved between *Fol* and *Foph* might be extended to other solanaceous ff. spp. The effectors located in this segment could then be used for identification of pathogenic strains of *F. oxysporum* and resistance genes in the host and also as diagnostic tools for inclusion in *Fusarium*-wilt disease management programs in these crops. Additionally, further analysis of *Foph SIX1b/I-3* interaction in cape gooseberry may provide an efficient breeding option to generate *Foph* resistant cultivars.

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Appendix 1

1.1 Identification of *Fol* homologue effector transcripts in *Foph*

LS region of chromosome 1, chromosome 15 and unpositioned transcripts

ID	Annotation	Gene length	Unique gene reads	SignalP
FOXG_00133-chr1	hydrolase	2549	1522	n
FOXG_14350-Chr15	hypothetical protein	573	197	n
FOXG_16939		987	12	
FOXG_16941	Actelyl-transferase	726	36	y
FOXG_16942	lyase	1455	23	n
FOXG_16943	glycosyl-hydrolase/enzyme	906	43	y
FOXG_16944		1569	14	
FOXG_16958		717	18	
FOXG_16962		1413	62	
FOXG_16979	dehydrogenase	1041	18	n
FOXG_17013	glycosyl-hydrolase/enzyme	1092	20	n
FOXG_17029		690	29	
FOXG_17143		654	29	
FOXG_17152	major facilitator/transport	1383	24	n
FOXG_17172		804	11	
FOXG_17180	Catalase-peroxysdase	2331	304	y
FOXG_17187	peptidase/enzyme	1617	38	n
FOXG_17269	Zinc-Finger/Transcription-F	1236	30	n
FOXG_17299		1614	941	
FOXG_17305		513	8	
FOXG_17342	hypothetical protein	1158	15	n
FOXG_17353		1929	9	
FOXG_17357		2085	8	
FOXG_17359	hypothetical protein	1113	18	y
FOXG_17368		738	42	
FOXG_17382	oxydoreductase/enzyme	1248	17	n
FOXG_17403	hydrolase/carboxylase	828	23	y
FOXG_17404	Endoglucanase-precursor	714	35	y
FOXG_17413		1545	8	
FOXG_17414	hypothetical protein	1632	16	y
FOXG_17418		1161	8	
FOXG_17419	Terpenoid-synthase	1095	31	n
FOXG_17420	Cytochrome P450	1467	20	
FOXG_17421	1.4beta xylanase/enzyme	840	41	n
FOXG_17425		1917	9	
FOXG_17466		576	32	
FOXG_17483	Amine-oxydase	1581	33	y
FOXG_17496	glycosyl-hydrolase/enzyme	1572	37	n

FOXG_17498	glycosyl-hydrolase/enzyme	1185	240	y	
FOXG_17514	glycosyl-hydrolase/enzyme	1254	65	n	
FOXG_17519		1713	16		
FOXG_17523	metallopeptidase	762	23	n	
FOXG_17579		1326	18		
FOXG_17581		2832	10		
FOXG_17592		384	20		
FOXG_17607	sugar-inositol transporter	1617	55	n	
FOXG_17608	glycosyl-hydrolase/enzyme	1647	14	y	
FOXG_17619	hydrolase/oligopeptidase	693	9	n	
FOXG_17637		1224	88		
FOXG_17645	hypothetical protein	318	20	y	
FOXG_17671		1800	10		
FOXG_17695		603	13		
FOXG_17700		624	13		
FOXG_17723	Cyanovirin/LysM	1221	91	n	
FOXG_17733	Methyltransferase	852	16	n	
Chromosome 3					
FOXG_16124T0	Hypothetical protein	378	0		n
FOXG_17327T0		1176	29		
FOXG_06940T0		2031	20		
FOXG_12447T0	Hypothetical protein	333	49		n
FOXG_14811T0		747	135		
FOXG_14848T0		2406	11		
FOXG_06920T0	Hypothetical protein	177	13		n
FOXG_14947T0		1488	31		
FOXG_14931T0		2226	27		
FOXG_14902T0		747	29		
FOXG_14884T0		999	164		
Chromosome 6					
FOXG_07063T0		813	850		
FOXG_07105T0	hypothetical protein	288	71	n	
FOXG_07181T0	Ribonuclease	348	16	n	
FOXG_06948T0	hypothetical protein	258	112	n	
FOXG_07055T0		903	116		
FOXG_17124T0		1146	11		
FOXG_16255T0	hypothetical protein	378	32	n	
FOXG_07113T0		1776	21		
FOXG_07265T0	hypothetical protein	921	1363	n	
FOXG_06955T0		2286	273		
FOXG_14035T0		1704	85		
FOXG_17106T0	catalase/peroxydase	1554	55	n	
FOXG_07180T0	hypothetical protein	504	35	n	
FOXG_17123T0	DNA-binding	2325	25	n	
Chromosome 14					
FOXG_14152T0		669	40		
FOXG_16447T0		2379	17		

FOXG_17457T0	SIX10	447	15	
FOXG_16418T0	SIX1	399	49	
FOXG_14278T0	hystidine kinase	303	24	n
FOXG_14297T0		861	309	
FOXG_14290T0		675	11	
FOXG_16448T0		1881	80	
FOXG_17458T0	Zn-DNA bindig	2829	22	n
FOXG_14233T0		2538	8	
FOXG_14156T0		1176	9	
FOXG_14266T0	hypothetical protein	297	51	n
FOXG_14234T0	peroxydase/catalase	2343	28	y
FOXG_14144T0		1992	20	
FOXG_14258T0	putative oxydoreductase	1830	20	y

F. oxysporum candidate effectors (vanDam et al., 2016, Taylor et al., 2016 and Williams et al., 2016) identified in _Foph_

ID	f. sp.	Annotation	Gene length	Unique gene reads	_Fol_ homologue	_Fol_ chromosome
SIX1	lycopersici 4287		855	107		
SIX7			663	19		
SIX10			450	15		
SIX12			384	17		
FOTG_18786	vasinfectum		1362	21		
FOMG_18850	melonis	minor extracellular protease	2235	22	FOXG_14564	chr12
FOMG_11638	melonis		2544	16		
FOYG_00459	FOSC-3a human pathogenic strain	tyrosinase	1212	50	FOXG_04315	chr4
FOVG_19731	pisi	catalase-peroxidase 2	2367	266	FOXG_17106	chr6
FOPG_13541	conglutinans	pectinesterase precursor	450	15	FOXG_12330	chr13
FOCG_01737	radicis-lycopersici	metallo-beta-lactamase family protein	975	109	104737-105311	chr5
FOXG_13844	lycopersici 4287		957	7		
FOWG_17947	lycopersici MN25	Ave1	375	9		
FOXB_19077	conglutinans	Hypotetical protein	306	454	FOXG_15064	chr1
FOXG_22828	lycopersici 4287	Hypotetical protein (SIX15)	339	14	1160235-1160458	chr14
FOQG_14751	raphani	Hypotetical protein	660	7	FOXG_04853	chr7
FOXM_16306	medicaginis	Candidate effector		8		
FOXG_17088		Endopolyglacturonase	1140	14		

Transcripts discarded due to low coverage (< 80%) by *Foph* RNAseq reads

Homologues identified in the *Fol* mapping analysis

Appendix 1.2

DNA and protein sequences of *Foph* homologous effectors

>*Foph*_SIX1a coding sequence

ATGGCGCCCTATAGCATGGTACTTCTTGGCGCTCTCTCGATCCTTGGGTTTGGGGCTTATG
TCAAGAGGCTGCGGTTCGGGAACCCCAGATATTTTTCAACTTGACCTTTACGGAATATCTG
GATAAGGTGGCAGAATCCCACGGGAGCCCCCCAGACAAGAGTGATTTGCCCTGGAATGAC
ACTATGAGCAGCTTTCCTGGAAACGAGACGGACGATGGTGTTCAGACCGAGACGGGCAGC
AGCCTATCGAGGCGTGGTCACATTGTCAACCTTGGAAAGCGTGAGATTGTCCGCGGGGAG
TCTCGCAACGATGCTGTCACCAATGACATGCTCCAGGTGCTTCATAATATTTGCGTTGAAA
GATTTGGAACTGGCTGGCGAGCGGTTGGCGGGTATTGTGACCGCCGGTCCAGGAGAGTA
AAATGTAGGAGGCCGGATGACACTGGGCTAGAGCGGCGTGCGAACAAAGCTTGCCCACC
GCGCGAGGAGTGCTCCACTTTCCAAGCGTACAACTTTATTACTACCGGTCAATATGGCCGT
ACGCCGCACAATTTTCCTGTCTGTGGGCACAAAATTGAGGTGAATGACAGACAGGATCAAG
GGAGTCACACGGAGTGGGATGGAACCTGGTACCCCGAAGAGCCTAAATCGCCTGGGACC
TACGATTCTTTCGTCCAGATGGCGGGCAGTCTCAATGCGTACTTCAACTTTGATGGTGTTTA
TTCTGATGGAGAGACGATGAACTCTCGGGGACATGCAGTCTCATGGTCATGCCTTAATTGC
CCGGGAGGCAAGCTGACTATCACTAACACGTATCGCCCAACTTGGGCGGTTGGATATTCC
CACACCGTCTAAGTTGCGATCCAATCTA

>*Foph*_SIX1a protein sequence

MAPYSMVLLGALSILGFGAYAQEAAVREPQIFFNLTFTEYLDKVAESHGSPPDKSDLPWNDTM
SSFPGNETDDGVQTETGSSLSRRGHIVNLGKREIVRGESRNDAVTNDMLQVLHNICVERFGTG
WRAVGGYCDRRSRRVKCRRPDDTGLERRANKACPPREECSTFQAYNFITTGQYGRTPHNFP
VCGHKIEVNDRQDQGSHTEWDGTWYPEEPKSPGTYDSFVQMAGSLNAYFNFDGVYSDGETM
NSRGHAVSWSCLNCPGGKLTITNTYRPTWAVGYSHT

>*Foph*_SIX1b coding sequence

ATGGCGCCCTATAGCATGGTACTCCTTGGCGCCCTCTCGATCCTTGGGTTTGGGGCTTAT
GCTCAAGAGGCTGCGGTTCGGGAGCCCCAGATATTTTTCAACTTGACCTACACGGAATATC
TGGATAAGGTGGCAGCATCCCACGGGAGTCCCTCAGACAAGAGTGATTTGCCCCGGAATG
ACACTATGAGCAGCTTTCCTGGAAACGAGACGGACGATGGTGTCCAGACCGAGACGGGCA
GCAGCTTATCGAGGCGTGGTCACATTGTCAACCTTAGAAAGCGTGAGCCTGTCGGCGAGG
AGTCTCGCAACGATCGTGTCACACAAGACATGCTCCAGGCGCTTCATGATCTGTGCGTTGA
AAATTTTGGCACAGGCTATCGAGCGGTTAGTGGGATGTGTGTACCTGGACGTCGGGCGAC
TAGGAGCTTCGAATGTGGGACACCGGGTGTCAGGGGGGGAGACAGAGCAATGACCAGAG
CTTGCCCAAAGGACCAGGAGTGCACCACTTTCCAGGCGTACAACTATCGTAACGGTAAGC
GTCAGGTTACTTTTCCTGTCTGTGGGCACAAAATTGAGGTGAAGGAAAGACATGATATAGG
GATAGACACGGAGTGGGATGGATCCTGGTTCCCCGAAAAGCCTGAATCGCCTGGGACTTA
CGATTCTTTCGTCCAGATGGCTGGCAGTCTCGGTGCGCACTTCGACTTTCATGGTGTTTAT
TCGGATGGATATGAGATGAACTCCCGGGGAAATGGACACTCATGGGCATGCGTTAATTGC
CCGGGAGGCGAGCTGACTATCACAAGCACATATCGCCCAACTTGGGCCATTGGATATACC
ACCCCCAACTAAGTTGCGATCCAAAGCTCTA

>*Foph*_SIX1b protein sequence

MAPYSMVLLGALSILGFGAYAQEAAVREPQIFFNLTYTEYLDKVAASHGSPSDKSDLPRNDTMS
SFPGNETDDGVQTETGSSLSRRGHIVNLRKREPVGEESRNDRVTQDMLQALHDLCVENFGTG
YRAVSGMCVPGRRATRSFECGTPGVRGGDRAMTRACPKDQECTTFQAYNYRNGKRQVTFP
VCGHKIEVKERHDIGIDTEWDGSWFPEKPESPGTYDSFVQMAGSLGAHFDFHGVYSDGYEMN
SRGNGHSWACVNCPGGELTITSTYRPTWAIGYTTP

>*Foph*_SIX7 coding sequence

ATGAAGTACCTTTACCTCCTTTTCCATTTCGCCCTGTTTGCGAGTGCTATACCTATGCTCGA
CCTATTTCCAAGACAAGGGCAATGCTTTAGCACCACCGGCTCAACACCACCACGACCACCA
CCCGCGGCTAGAGAGGTGACATTTGACATCACCCAAAATGTCAACACGTTCACTAGCGCC
GCGTCCACACCATGGACTGAGGGTGTAGGACTCTCGAATATTAGATACCAATGGCGCGCA
TACTACAGTACCAGGCAACAAACTACATTCGTTGAGGTTCGGGTTTTCGGTACTGCCGAGG
CGCAAGTAGTCTTGCTTCCGGATGCTCCCGGAACAAGTCGATATCGCGCTATCGATAGCAA
CGTCTTTAGGCCCAACGAAGCGGTCACAGGCGGTGGTTTAGCGGGCTGGGGTCAGGTGA
CTACGGTCTGCCTTCAAACTTGGGGTCGGCGAGGAGATATTACCTATCGGCTCCGGATTC
AAAGTAAGCCCAAGCACTCCTACCCCCTTGAAGTCATTCTTGAGAGGCAATACGCCAACCG
CTTAATGAATGCCCGCTGCTACGCCAAAACCCATCAGATGATTTATAAGCCCAGGGGCCTT
GCTGACTTTCGTTTTCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGGGATC

>Foph_SIX7 protein sequence

MKYLYLLFHFALFASAIPMLDLFPRQGQCFSTTGSTPPRPPPAAREVTFDITQNVNTFTSAASTP
WTEGVGLSNIRYQWRAYYSTRQQTTFVEVRVFGTAEAQVVLLPDAPGTSRYRAIDSNVFRPNE
AVTGGGLAGWGQVTTVCLQTWGRRGDITYRLRIQSKPKHSYPLEVILERQYANRLMNARCYA
KTHQMIYKPRGLADFRFQGRIPAHWRPLLGI

>*Foph*_SIX10 coding sequence

ATGAAGCTCTTGTGGTTGATTCCTGTTGTCGCGTCTACCGCAATTCCAGACTCTGGTGTAT
CCACTGGCACCAAAGACCTCTCTAAACGAGACGATGCATACGTATTTGATGTCACGTTTCG
AGTTGGTCCAGCCGGGGCTAACGTCGCGCCATTCTCTGGATCTGTGTACGTCCAGGATGG
TCTTACCCCACTTGTTCGTTCAGGTTCAGGATCTTCGATCTCGGATCACGGTTACAACGCTT
TCAGAGGAATAGTGTACTTCACATTTACTCACGGCTACAACCAGTACTCCGCATCGACTCG
ATTTGGTGTCTACGTTGATACTGGATTAATTGTCGACTCGAACGGCAGACCAATCTACGGG
ACCGCGCCCCGCAAAGCCTGCATCGACTATTCACCTCATGGTCCCACCGACGTTTGTTCT
GTAACAATTACCAGGTCTAAGTAG

>Foph_SIX10 protein sequence

MKLLWLIPVVASTAIPDSGVSTGTKDLSKRDDAYVFDVTFRVGPAGANVAPFSGSVYVQDGLT
PLVRSGSGSSISDHGYNAFRGIVYFTFTHGYNQYSASTRFGVYVDTGLIVDSNGRPIYGTAPRK
ACIDYSPHGPTDVCSVTITRSK

>*Foph*_SIX12 coding sequence

ATGGGCTTTTACTCCGTTATAAAAGTCCTCTCTATTTATGGCATGCTGCTCCAAGTACAACC
TACCTTGGCCCAAGCGTCCAGTTGTCTCAGCGTTGGACCCAAAGGTATCAGCAACCAGAAT
GCTTGTGTGTGTGGAGGTCAATGCGTCATGAAAGACTTGGTCGTTGCTCGCAGAAAGGTTT
GCTGTGAATACACAGTTCAGATACAGGGTGGTTGGCCTGTTCTTGCCCAAAGCCGTTGTGT
TTACGGCTCTACTGGTGCCAACGGGGGTTCATGCTCTGGTGATAATGTCTCCCTTGCTTGG
TGGCTCAACTATGAGCCGGAGGTGAAGAGCACAGATCCCACATGCATTTTTGCGAAGCCC
AAGCTATGCCACTCCTGA

>Foph_SIX12 protein sequence

MGFYSVIKVLSIYGMLLQVQPTLAQASSCLSVGPKGISNQNACVCGGQCVMKDLVVARRKVCC
EYTVQIQGGWPVLAQSRCVYGSTGANGGSCSGDNVSLAWWLNYEPEVKSTDPTCIFAKPKLC
HS

>*Foph*_SIX15 coding sequence

ATGAAGCTTTCCGCGCTACTCGGCTTTGCAGTTTGTGTGCCTAGCACTATGGCCACCATTT
ACTGCAGGGACGTCAGCCCCCCTGGTGACACACGAAGCTGGTGTAGAACCAACACGCCA
GCGTGGCAAGGATGTCAACGTTTCTGTTCCGAGCACTGTAGAAGTACCCCAAGGGATTAC
CCAGGTGGTTGCATGTACCATTTACAAGTCGGTGGAGACTATGACTGCTTTTGTAAATAG

> *Foph*_SIX15 protein sequence

MKLSALLGFAVCVPSTMATIYCRDVSPPVSIIDALKRINIDLYSVTHEAGRKPTRQRGKDVNVSV
PSTVEVPQGITQVVACTIYKSVETMTAFVNSRPSYELSPVRKSASIT

>*Foph*_Ave1 coding sequence

ATGAAACTACTCGCACTATTGGCTCTCGCGTCACCATTAGTCAGCGCCGATATCGGAACTG
CCAATATTCTCAACGGGCCGCCCTTTACCCCTACGGCTTGTTATGGTAATGACTTAAGCCA
GTTCCCTGCTGGTAACTTGTTTGCGGCTGCTGGAGAGGGAATATGGGACAACGGGGCCGC
TTGTGGCAGACGGTATAGGGTGAAATGTATAAGCCCAAGACCGTGTAAGAGTGATATGGT
GGATGTGAGAATAGTTGATCGAGCGAAAAATCATCGAGAGTATGCAGGTGAATTCCTTTTA
CTACACGCTGCAGCATACAATACTATTGCGGCCGGGGGACGACGACGCTTAAATATCAATT
TTGTACAAAGATGA

>*Foph*_Ave1 protein sequence

MKLLALLALASPLVSADIGTANILNGPPFTPTACYGNDLSQFPAGNLFAAAGEGIWDNGAACGR
RYRVKCISPRPCKSDMVDVRIVDRAKNHREYAGEFLLLHAAAYNTIAAGGRRRLNINFVQR

>*Foph*_M16306 coding sequence

ATGCGTCTCAAAGCAACCATCGCCGCCGCCCTCTGCGCGAACATATCCCTCATCCAAGCTT
GCTCCCCAGGCTTTTCCTCGAACCGCAACCTGTCCCCCGGCCAATTCTCCTGGCACTTGG
CGGACAAAAGCGTCGACGACGTGTCCGGCTGCAGCAGCCGCTGCGCGCGCCAGTGCACC
GCACCGGGTAAAAAGTACGGCGCCTGCATGGGCTGCGCCAGGTGGAAGGGTAATGCCCA
GGGTCGGATGGACCTCCCGCCTTGCAGCAAGCCTATCACGACTGCAAATGACGATGGCGA
GTACGAATGCCACGGGTACGGAGACGGGTTTTGGAACTGTATCACGCGGTGCCCTCCTGG
TTACGTGTCTAAGAATCTGGGCCGTATTAAATAA

>*Foph*_M16306 protein sequence
MRLKATIAAALCANISLIQACSPGFSSNRNLSPGQFSWHLADKSVDDVSGCSSRCARQCTAPG
KKYGACMGCARWKGNAQGRMDLPPCSKPITTANDDGEYECHGYGDGFWNCITRCPPG
YVSKNLGRIK

Appendix 1.3

Clustal-Omega alignment of homologue effectors between *Fol* and *Foph*

SIX7 alignment

```
Fol_SIX7        MQVMKYLYLLFHFALFASAIPMLDLFPRQGQCFSTTGSTPPRPPPAAREVTFDITQNVNT
Foph_SIX7       MRVMKYLYLLFHFALFASAIPMLDLFPRQGQCFSTTGSTPPRPPPAAREVTFDITQNVNT
                *:************************************************************

Fol_SIX7        FTSAASTPWTEGVGLSNIRYQWRAYYSTRQRTTFVEVRVFGTAEAQVVLLPDAPGTSRYR
Foph_SIX7       FTSAASTPWTEGVGLSNIRYQWRAYYSTRQQTTFVEVRVFGTAEAQVVLLPDAPGTSRYR
                ********************************:***************************

Fol_SIX7        AIDSNVFRPNEEVTGGGLAGWGQVTTVCLQTWGRRGDITYRLRIQSKPKHSYPLEVIPER
Foph_SIX7       AIDSNVFRPNEAVTGGGLAGWGQVTTVCLQTWGRRGDITYRLRIQSKPKHSYPLEVILER
                *********** ********************************************** **

Fol_SIX7        QYANRLMNARCYAKTHQMIYKPRGLADFRFHRLTQSVVNL----
Foph_SIX7       QYANRLMNARCYAKTHQMIYKPRGLADFRFQGRIPAHWRPLLGI
                *********************************:    :  .
```

SIX10 alignment

```
Fol_SIX10       MKLLWLIPVVASTAIPDSGVSTGTKDLSKRDDAYIFDVTFRVGPAGANVAPFSGSVYVQD
Foph_SIX10      MKLLWLIPVVASTAIPDSGVSTGTKDLSKRDDAYVFDVTFRVGPAGANVAPFSGSVYVQD
                ************************************:***********************

Fol_SIX10       GLTPLVRSGSGSSISDRGYNAFRGIVYFTFTHGYNQYSASTRFGVYVDTGLIVDSNGRPI
Foph_SIX10      GLTPLVRSGSGSSISDHGYNAFRGIVYFTFTHGYNQYSASTRFGVYVDTGLIVDSNGRPI
                ****************:************************************************

Fol_SIX10       YGTAPRKACIDYSPHGPTDVCSVTITRSK
Foph_SIX10      YGTAPRKACIDYSPHGPTDVCSVTITRSK
                ******************************
```

SIX12 alignment

```
Fol_SIX12       MGFYSVIKVLSIYGMLLQVQPTLAQASSCLSVGPKGISNQNACVCGGQCVMKDLVVARRK
Foph_SIX12      MGFYSVIKVLSIYGMLLQVQPTLAQASSCLSVGPKGISNQNACVCGGQCVMKDLVVARRK
                ************************************************************

Fol_SIX12       VCCEYTVQIQGGWPVLAQSRCVYGSTGANGGSCSGDNVSLAWWLNYEPEVKSTDPTCIFA
Foph_SIX12      VCCEYTVQIQGGWPVLAQSRCVYGSTGANGGSCSGDNVSLAWWLNYEPEVKSTDPTCIFA
                ************************************************************

Fol_SIX12       KPKLCHS
Foph_SIX12      KPKLCHS
                *******
```

Ave1 alignment

```
Foph_Ave1       MKVLALLALASPLVSADIGTANILNGPPFTPTACYGNDLSQFPAGNLFAAAGEGIWDNGA
Fol_Ave1        MKLLALLALASPLVSADIGTANILNG-PFTPTACYGNGVSHFPSGNLFAAAGEGMWDNGA
                **:************************ **********.:*:**:**********:*****

Foph_Ave1       ACGRRYRVKCISPRPCKSDMVDVRIVDRAKNHREYAGEFLLLHAAAYNTIAAGGRRRLNI
Fol_Ave1        ACGRLYTMKCISPGPCKSDTVDVRIVDRAKNHRGYAGEFFLLHAAAYNTIAAGGRRRLNI
                **** * :***** ***** ************* *****:********************

Foph_Ave1       NFVQR
Fol_Ave1        DFVQR
                :****
```

SIX15 (FOXG_22828) Alignment

```
Fol_FOXG_22828      MKLSALLGFAVCVPSTMATIYCRDVSPPVSIIDALKRINIDLYSVTHEAGVKPTRQRGKD
Foph_FOXG_22828     MKLSALLGFAVCVPSTMATIYCRDVSPPVSIIDALKRINIDLYSVTHEAGRKPTRQRGKD
                    ********************************************** *********

Fol_FOXG_22828      VNVSVPSTVEVPQGITQMVACTIYKSVETMTAFVNSRPSHELSPVRKSASLT
Foph_FOXG_22828     VNVSVPSTVEVPQGITQVVACTIYKSVETMTAFVNSRPSYELSPVRKSASIT
                    ****************:*********************:**********:*
```

*Foph*_M16306 Alignment

```
Foph_M16306     MRLKATIAAALCANISLIQACSPGFSSNRNLSPGQFSWHLADKSVDDVSGCSSRCARQCT
FOXM_16306      MRLKATIAAALCANISLIQACSPGFSSNRNLSPGQFSWLFADYSVDDVSGCSSRCARQCT
                ************************************** :** ****************

Foph_M16306     APGKKYGACMGCARWKGNAQGRMDLPPCSKPITTANDDGEYECHGYGDGFWNCITRCPPG
FOXM_16306      APGKRYGACMGCARWKGNAQGRMDLPPCSKPITTANDDGEYVCHGYGDGFWNCITRCPPG
                ****:*************************************** ****************

Foph_M16306     YVSKNLGRIK
FOXM_16306      YVSKDLGRIK
                ****:*****
```

Appendix 1.4

Alignment of SIX gene cluster homologous intergenic regions (DNA) with CLUSTAL O (1.2.3)

Intergenic region 1 (SIX10-SIX12)

```
Fol_int1       ------------------------------------------------------------ 0
Foph_int1      AAGCAAAACTAGATGGTTCTAAGGGAAGTTCGGGTCGTCCGAAAAAGAAGTATCTTACAG 60

Fol_int1       ------------------------------------------------------------ 0
Foph_int1      GTCTTTGGTGCCAGTGGATACACCAGAGTCTGGAATTGCGGTAGACGCGACAACAGGAAT 120

Fol_int1       ------------------AGTTGAGGTTTTCTAGTGTCTCGCAGTTGCTAACACTCTTAT 42
Foph_int1      CAACCACAAGAGCTTCATAGTTGAGGTTTTCTAGTGTCTCGCAGTTGCTAACACTCTTAT 180
                                 ****************************************

Fol_int1       AATATGAATAAGACACGAATTTAATAAAGCTCAGAAGGGTTATCGCTTGGTATAGGGTCT 102
Foph_int1      AATATGAATAAGACACGAATTTAATAAAGCTCAGAAGGGTTATCGCTTGGTATAGGGTCT 240
               ************************************************************

Fol_int1       AGGGAGTAAGGAAAGTTGGCTTTAGAGTGCCAACTATTGCTTTTATACCTGTATCCCTAA 162
Foph_int1      AGGGAGTAAGGAAAGTTGGCTTTAGAGTGCCAACTATTGCTTTTATACCTGTATCCCTAA 300
               ************************************************************

Fol_int1       TGATGACGTATTCAAGAGGATGCTCCCGTCCATAGAAAGCATCTCAGCTTAGCAAAGGGG 222
Foph_int1      TGATGACGTATTCAAGAGGATGCTCCCGTCCATAGAAAGCATCTCAGCTTAGCAAAGGGG 360
               ************************************************************

Fol_int1       GCTTCGGCTCTAATAATGCAGTTTATAATGCAACCTTGGATTCGTAAGGTTTGCGACCCA 282
Foph_int1      GCTTCGGCTCTAATAATGCAGTTTATAATGCAACCTTGGATTCGTAAGGTTTGCGACCCA 420
               ************************************************************

Fol_int1       TGGTAGTACAGTCCAGAGAGGGGCGAGGAGGGGATAGTGGCTAAAAAAAAATAAAAAAAA 342
Foph_int1      TGGTAGTACAGTCCAGAGAGGGGCGAGGAGGGGATAGTGGCTAAAAAAAAATAAAAAAAA 480
               ************************************************************

Fol_int1       AGAATACTAGATGAATTTTTAGGAAGGTTTTCCTAGAGTATACAGTGGGGTGCAATAAGT 402
Foph_int1      AGAATACTAGATGAATTTTTAGGAAGGTTTTCCTAGAGTATACAGTGGGGTGCAATAAGT 540
               ************************************************************

Fol_int1       TTGAATGCACCCAGAGGTACCGATTATTGAGGCTACCCGTAACAGGCGTCGAAAACGCGG 462
Foph_int1      TTGAATGCACCCAGAGGTACCGATTATTGAGGCTACCCGTAACAGGCGTCGAAAACGCGG 600
               ************************************************************

Fol_int1       TCCCGATCGGTAGCCCGGGTTGAGATAC------------------------------- 490
Foph_int1      TCCCGATCGGTAGCCCGGGTTGAGATACAGTGGGGGGCAATAAGTTTGAATCCAAACTGA 660
               ***************************

Fol_int1       ------------------------------------------------------------ 490
Foph_int1      AAGTACATATTCGCAGATGAACCAAGGTGAGACCAGTTTCCCACGCAGTAATATCTCAAT 720

Fol_int1       ------------------------------------------------------------ 490
Foph_int1      TAAGATAGCATTTTGCCTAATATAGCTTAGTTTTTACTTTATTAAGCTAAAGCTTTATAA 780

Fol_int1       ------------------------------------------------------------ 490
Foph_int1      ACAGGAGCTAGATTACAACAGCTGGTACTTCAGCCTGGATTCAAACTTATTGCATCCCAC 840

Fol_int1       -----CTACAGACGTATTCAAACCTATTGCACCCCACTGTACAAAAGTCCCGTGCTATTG 545
Foph_int1      TGTACCTACAGACGTATTCAAACCTATTGCACCCCACTGTACAAAAGTCCCGTGCTATTG 900
                    ****************************************************

Fol_int1       ATTAATTGATATACTTTATTCACGGAACTTTTGTATACTCTAGGAAAACCTTCCTAAACA 605
Foph_int1      ATTAATTGATATACTTTATTCACGGAACTTTTGTATACTCTAGGAAAACCTTCCTAAACA 960
               ************************************************************

Fol_int1       AACTTCCCTAGTCTCAGGTAAACTCAAAGGTACCGCTAAAACATCTACTAAACTTTCCCT 665
Foph_int1      AACTTCCCTAGTCTCAGGTAAACTCAAAGGTACCGCTAAAACATCTACTAAACTTTCCCT 1020
```

```
                ************************************************************

Fol_int1        AATCTTGTAATATACTTTCTCTAACCATGGATAAGTTCAATAACACGAGGGAATCTAATA 725
Foph_int1       AATCTTGTAATATACTTTCTCTAACCATGGATAAGTTCAATAACACGAGGGAATCTAATA 1080
                ************************************************************

Fol_int1        GACTTTATCAACCCTTTTAAACTTCCTCTAGTATATAGTAAGTTTAATAATACGAACAAA 785
Foph_int1       GACTTTATCAACCCTTTTAAACTTCCTCTAGTATATAGTAAGTTTAATAATACGAACAAA 1140
                ************************************************************

Fol_int1        TCTAATGCATATTCCTTCCTGGGTAACGTAGGTAACTCCACTCATCTAGACATCTATACT 845
Foph_int1       TCTAATGCATATTCCTTCCTGGGTAACGTAGGTAACTCCACTCATCTAGACATCTATACT 1200
                ************************************************************

Fol_int1        CGTAGTGTTTTAGCAGACCATTATTTGATTACCAAGAGTTCCGCTCATGAATAAGTGGTC 905
Foph_int1       CGTAGTGTTTTAGCAGACCATTATTTGATTACCAAGAGTTCCTCTCATGAATAAGTGGTC 1260
                ****************************************** ******************

Fol_int1        TGAGACACAAGCGGTAAAAGGACATCGTAGCTGACCTTGGGAGTCCCGCCTTTTGGTGAG 965
Foph_int1       TGAGACACAAGCGGTAAAAGGACATCGTAGCTGACCTTGGGAGTCCCGCCTTTTGGTGAG 1320
                ************************************************************

Fol_int1        TACCGGTACTAATTATGAGTTGTTGCTTATAGACTGCATTAAACACATGATTTATGAGAA 1025
Foph_int1       TACCGGTACTAATTATGAGTTGTTGCTTATAGACTGCATTAAACACATGATTTATGAGAA 1380
                ************************************************************

Fol_int1        TTATATCTCTCTACGAATGAAGGGCCTTTGATACAGTATCTCAAATTCGATTTAAGAACA 1085
Foph_int1       TTATATCTGTCTACGAATGAAGGGCCTTTGATACAGTATCTCAAATTCGATTTAAGAACA 1440
                ******** ****************************************************

Fol_int1        ATCCGAAGTAGGGTCAAGATCTGTGATGAGGAAGTTATTGTGCATCTCTTCCTTCGCGTA 1145
Foph_int1       ATCCGAAGTAGGGTCAAGATCTGTGATGAGGAAGTTATTGTGCATCTCTTCCTTCGCGTA 1500
                ************************************************************

Fol_int1        CTTGCTAGCCTCGCTGGCAAGTATTTGTTCCTTCTCATCCACTATACAGCTACGCTGTTA 1205
Foph_int1       CTTGCTAGCCTCGCTGGCAAGTATTTG---------------------------------- 1527
                ***************************

Fol_int1        ACTAAGTTGGAGATTATC 1223
Foph_int1       ------------------ 1527
```

Intergenic region 2 (SIX12-SIX7)

```
Fol_int2        ------------------------------------------------------------ 0
Foph_int2       GGGAAAACGAAATACGATCGTGCTCGAGACCACACGCCATACATCGAGAGGACTTCTCTT 60

Fol_int2        ------------------------------------------------------------ 0
Foph_int2       CACTTCTCTAGTTGTGAACATGATAAATAAAACGACGTCATACTTATCTACGGAGTAAAA 120

Fol_int2        ------GGTGCTTGATTTGTAAGTGTTGAGGAAAAATATGATCAATATGTATCGACTGTC 54
Foph_int2       GCCCATGGTGCTTGATTTGTCAGTGTTGAGGAAAAATATGATCAATATGTATCGACTGTC 180
                      ************** ****************************************

Fol_int2        TTGGTTAGGGAATGACTAGGAGTGCGAACGGCTACCACATGTTGGGGAGAGCATGCTGCT 114
Foph_int2       TTGGTTAGGGAATGACTAGGAGTGCGAACGGCTACCACATGTTGGGGAGAGCATGCTGCT 240
                ************************************************************

Fol_int2        ATATAGTGTCACATACTCTCCGTATTGCCGGCCTTAGTTCAGAGCATGAGGTGAAATGGG 174
Foph_int2       ATATAGTGTCACATACTCTCCGTATTGCCGGCCTTAGTTCAGAGCATGAGGTGAAATGGG 300
                ************************************************************

Fol_int2        TCGCAATGCCAAGGGGTCTTTAGTATCTCCACAAGGGCAAAGATACCCGAGCGCGCCAGC 234
Foph_int2       TCGCAATGCCCAGGGGTCTTTAGTATCTCCACAAGGGCAAAGATACCCGAGCGCGCCAGC 360
                ********** ***************************************************

Fol_int2        AACCATTGCAATGTTACAGTATGCAGAGACCTCAAGTCCCGGCTATAATAAACCTAAAGG 294
Foph_int2       AACCATTGCAATGTTACAGTATGCAGAGACCTCAAGTCCCGGCTATAATAAACCTAAAGG 420
                ************************************************************

Fol_int2        CGTGGCATTCAGAGGGCCCAGTAGCCATATGGCCCGAGCTGTTAAACGCATAGCTATACC 354
Foph_int2       CGTGGCATTCAGAGGGCCCAGTAGCCATATGGCCCGAGCTGTTAAACGCATAGCTATACC 480
                ************************************************************

Fol_int2        TATAATAAAGTGAATTTGATTGTAATCTCACTAGGTTTACGAGAGAAGAGATTATAAAAC 414
Foph_int2       TATAATAAAGTGAATTTGATTGTAATCTCACTAGGTTTACGAGAGAAGAGATTATAAAAC 540
                ************************************************************
```

```
Fol_int2       CTTCCTCTAGCCTATTTATTAAGTTATTAATATTACTAATTAAAGGAAGGTTTATTTAAA 474
Foph_int2      CTTCCTCTAGCCTATTTATTAAGTTATTAATATTACTAATTAAAGGAAGGTTTATTTAAA 600
               ************************************************************

Fol_int2       TTAGTTAAAAATTTAAGGAAAACTTAAAATTATAAATACTTTTAGGGAATTAATTTTACT 534
Foph_int2      TTAGTTAAAAATTTAAGGAAAACTTAAAATTATAAATACTTTTAGGGAATTAATTTTACT 660
               ************************************************************

Fol_int2       AAGTTTATAAAAGAAGAAACTATAAACCCTTTCTCTAATTTACGGTGTACAGTGGGGTGC 594
Foph_int2      AAGTTTATAAAAGAAGAAACTATAAACCCTTTCTCTAATTTACGGTGTACAGTGGGGTGC 720
               ************************************************************

Fol_int2       AATAAGTTTGAGGATCTTTGAAGGTATCGAAGCAGCCGATCTAGCCGGATTAGATCCTTG 654
Foph_int2      AATAAGTTTGAGGATCTTTGAAGGTATCGAAGCAGCCGATCTAGCCGGATTAGATCCTTG 780
               ************************************************************

Fol_int2       TTACATATTTGTCGGTCGTGTTCATCTGCCGATTTTGTTCATCTGCCGATTTTATTTATC 714
Foph_int2      TTACATATTTGTCGGTCGTGTTCATCTGCCGATTTTGTTCATCTGCCGAATTTATTTATC 840
               ********************************************** **********

Fol_int2       TGCCGGTCTTATGTAATATTAAGACCGAGAAGGTAACTTTAAGTCTCTTACAGTGGGATG 774
Foph_int2      TGCCGGTCTTATGTAATATTAAGACCGAGAAGGTAACTTTAAGTCTCCTACAGTGGGATG 900
               ********************************************* ************

Fol_int2       CAATAAGTTTGAATACGTTTTCAGGTACTGTTACCCCCTGTCTAGCCTATTTGGAGTTCA 834
Foph_int2      CAATAAGTTTGAATACGTTTTCAGGTTACTGTTACCCCTGTCTAGCCTATTTGGAGTTCA 960
               *************************     *  *************************

Fol_int2       GCCTGCTTCTAATCCGCACAGCGGATCCGTGCCAATCTTAGCGCACTTCCACAAACCTTT 894
Foph_int2      GCCTGCTTCTAATCCGAGGAAGAAGCTCGATCTAGCCAAATCATATATGCTTACTCGCCA 1020
               ***************   *       **  * *  *  * *  *  * *  *  *

Fol_int2       CATCGTA--TATTTTGTAACACATATACAATTTCCTTCGCAGAAAATAGTCAACATACCT 952
Foph_int2      TGCAAAAGCTGATATGCACGTGGTAGCTGCGCTCAAGAGCTAGCGGCAAAGAAGAACCCG 1080
                     *  *  * ** *     **       **    **       *   ** *  **

Fol_int2       CTCTCTTCCTCCCTTACAATCTCCTTCACTAAGATTCCATCTTAGAAAAAATAGAAGACG 1012
Foph_int2      CCCGGATTCAACTTATGCATCC-----ACTTGTAGACGATACGAGAAGGAATTGTTGCCG 1135
               * *   * *  * *    ***      ***   *  * **   ****  *** *  * **

Fol_int2       G----GACACCTATGGTGAAGTATTTGCTATTTTTCTTATTTACTGTAAGAATATCTCCC 1068
Foph_int2      GCCTCGATACCTAGTGAAAATAAT-TCTTAATGAAC----TTAATGTACCGGATACAAGA 1190
               *    ** *****  *  **  ** *  ** *   *    *** ****       *

Fol_int2       ATACCTATGACGATTTAGAAGATATAGAAGAACAGCGCACTAACCACAAGGGGACGGGTG 1128
Foph_int2      TACCGTATGAAAGTTTGGGTTTTAGGGAGGAGAATTTTATTTAAATAATAAATATAGAGG 1250
                  * *****   *** *    **  ** **  *    * * *    *     *  *  *

Fol_int2       TCCGACTACCCCCCGAGGAACGCCCACCCCTCTCTAACTACCCCCGCCTATTATTAAATA 1188
Foph_int2      TAATAG---AAAGGTAGATA--------------TA---------------------- 1269
               *   *          **  *              **
```

Alignment of homologous transcripts of αTF1 (FOXG_17458), between *Fol* and *Foph*

```
FOXG_17458      ATGTCCGGCCGTGCCGTTTTGAGCCCTCAACATGCCCAAGCGTCCTTTGATTCTGGTCTT 60
Foph_17458      ATGTCCGGCCGTGCCGTTTTGAGCCCTCAACATGCCCAAGCGTCCTTTGATTCTGGTCTT 60
                ************************************************************

FOXG_17458      CAACTCTATGGCGACGTGCCTGAATTGAACGCTGTGCCTCCTCCCTCTCACAGCGCCTTA 120
Foph_17458      CAACTCTATGGCGACGTGCCTGAATTGAACGCTGTGCCTCCTCCCTCTCACAGCGCCTTA 120
                ************************************************************

FOXG_17458      ATGGATTTCACACGCTTCGACGATTTTGCGTTTGCTTACTATGGTCTTCCTGACCAGTCT 180
Foph_17458      ATGGATTTCACACGCTTCGACGATTTTGCGTTTGCTTACTATGGTCTTCCTGACCAGTCT 180
                ************************************************************

FOXG_17458      TCTCTCGTTTCCCTAGTAGATCACACCCACACGTTCCAATCTCTCACTACGTTTCCCCAG 240
Foph_17458      TCTCTCGTTTCCCTAGTAGATCACACCCACACGTTCCAATCTCTCACTACGTTTCCCCAG 240
                ************************************************************

FOXG_17458      CACCAGGCCATATCTGGCCTTGCACATAGCGGTCTGCCGTTCGGCACCTTGCCTACGGAC 300
Foph_17458      CACCAGGCCATATCTGGCCTTGCACATAGCGGTCTGCCGCTCGGCACCTTGCCTACGGGC 300
                ************************************ ***************** *

FOXG_17458      AACTACAACCAGAGCATGGAAGACTCCAAAGCTCCCCCAGATCGGACATCTCCCGCATCC 360
Foph_17458      AACCGAAGCCAGAGCATGGAAGACTCCAAAGCCCCCCCAGATCGGACATCTCCCGCATCC 360
```

```
                ***   * ********************** ************************** 

FOXG_17458      AACGCCCTCGAAGACCCGACTACCGATGAGTTTGGTTTGGCTTCCCCTAACCGTGCGGGT 420
Foph_17458      AACGCCCTCGAAGACCCGACTACCGATGAGTTTGGTTTGGCTTCCCCTAACCGTGCGGGT 420
                ************************************************************ 

FOXG_17458      GGCACTGATCTAGGCGGTAAACCTAAGGAGGATAAAGCCGATGCCACACCTGCGTGGAGT 480
Foph_17458      GGCACTGATCTAGGCGGTAAACCTAAGGAGGATAAAGCCGATGCCACACCTGCGTGGAGT 480
                ************************************************************ 

FOXG_17458      GAACTTAAAACAAAGGCTGGCAAGGAAAGAAAACGCCTCCCGCTTGCTTGCATTGCTTGT 540
Foph_17458      GAACTTAAAACAAAGGCTGGCAAGGAAAGAAAACGCCTCCCGCTTGCTTGCATTGCTTGT 540
                ************************************************************ 

FOXG_17458      CGCCGAAAGAAGATCCGTTGTTCTGGCGAGAAACCCGCCTGCAAGCAGTGCCTACACTCA 600
Foph_17458      CGCCGAAAGAAGATCCGTTGTTCTGGCGAGAAACCCGCCTGCAAGCAGTGCCTACACTCA 600
                ************************************************************ 

FOXG_17458      TGTATCCCATGTGTCTACAAGGTTGCGACTCGGAAGGCTGCGCCTCGGACAAATTGCATG 660
Foph_17458      TGTATCCCATGTGTCTACAAGGTTGCGACTCGGAAGGCTGCGCCTCGGACAAATTGCATG 660
                ************************************************************ 

FOXG_17458      GCTATGCTCGATAAGCGACCGAAGCGCATGGAAGAACGCGCCATCAAGGCCATATCCAAG 720
Foph_17458      GCTATGCTCGATAAGCGACCGAAGCGCATGGAAGAACGCGCCATCAAGGCCATATCCAAG 720
                ************************************************************ 

FOXG_17458      TCGGATCAGGAAGTCGCCTCATCTGTGACTCACCCCGTGGTCAAACAGGCGATACCAGGA 780
Foph_17458      TCGGATCAGGAAGTCGCCTCATCTGTGACTCACCCCGTGGTCAAACAGGCGATACCAGGA 780
                ************************************************************ 

FOXG_17458      ACCGTAACTTCCAGTAGGCCAACCAAGAAGCGCGGCGCCGAAGAAGCATTTGAGCCTGAT 840
Foph_17458      ACCGTACCTTCCAGTAGGCCAACCAAGAAGCGCGGCGCCGAAGAAGCATTTGAGCCTGAT 840
                ****** ****************************************************** 

FOXG_17458      CTGGAAGCTTGGGCGAAGGCATCTTCGGAGCCAAAGATTGAGGGCGATGATGGATCCAGC 900
Foph_17458      CTGGAAGCTTGGGCGAAGGCACCTTCGGAGCCAAAGATTGAGGGCGATGATGGATCCAGC 900
                ********************* ************************************** 

FOXG_17458      AGCTTACAAGTCCAGGAAGGGGAGGAGAATAAGCTGCAACATGAAGGCACCGAAGCACTC 960
Foph_17458      AGCTTACAAGTCCAGGAAGGGGAGGAGAATAAGCTGCAACATGAAGGCACCGAAGCACTC 960
                ************************************************************ 

FOXG_17458      CCCTCCAGGGAGATACAGGAGCATCTGGCAGAGGTGTTTTTCGAAAACATCTACGGTCAA 1020
Foph_17458      CCCTCCAGGGAGATACAGGACGCT---------------------------------- 984
                ********************   * 

FOXG_17458      TCCTACCATCTTCTACACAAGCCAAGCTATATGCGAAAGCTAAATGTGCGCTGTAGCTGC 1080
Foph_17458      ----------------------------------------------------GTAGCTGC 992
                                                                    ******** 

FOXG_17458      TCGTTTTACCTCAAACCCTCTAACCGACCCATGTTCATCAGGGAGGATACAATTCAGATT 1140
Foph_17458      TCGTTTTACCTCAAACCCTCTAACCGACCCATGTTCATCAGGGAGGATACAATTCAGATC 1052
                ************************************************************ 

FOXG_17458      CCTCTGC—CGGTAAAGGAAAAGTACTTCCAGTTCGACATGCCTGCGCCCACCGAGATGTT 1198
Foph_17458      CCCCCCGCGGGAAAAAGGAAACGTACTTCCAGTTCGACATGCCTGCGCCCACCGAGATGT 1112
                ** *      *  ******** ************************************* 

FOXG_17458      TGGACGGTCGAGTACCTCATCCGCCGTCGCCCAACGACGGACAAATCGCGAATTCACGAG 1258
Foph_17458      TGGACGGTCGAGTACCTCATCCGCCGTCGCCCAACGACGGACAAATCGCGAATTCACGAG 1172
                ************************************************************ 

FOXG_17458      AGAACATGGGAGTTGCGGCCTTCCTGATTCGAGCCATTGCCTTATGGGGACGAATCATCA 1318
Foph_17458      ATAACATGTGAGTTGCGTCTTTCCTGATTCGAGCCATTGCCTTATGGGGACGGATCACCA 1232
                * ****** ******** * ********************************** **** ** 

FOXG_17458      CCTACCTGAGCCAAGGGTGTAAGGATCTAGACCCCAATCCATTGTGGGAAGACGAGTCTC 1378
Foph_17458      CCTACCTGAGCCAAGGGTGTACGGATCTAGACTCCAATCCATTGTGGGAAGACGGGTCTC 1292
                ********************* ********** ********************* ***** 

FOXG_17458      ACTACATGAAGCACCTCGATGATGTTGTAAACCTTGAAGCTAGTCTGCCCTTGTCACTCA 1438
Foph_17458      ACTACATGAAGCATCTCAATGATGTTGTAGACCTTGAAGCTAGTCTGCCCTTGTCACTCA 1352
                ************* *** *********** ****************************** 

FOXG_17458      AGCACTCTGCAGAGAACCTGGAGGTCCACAAGACAGAGAACACGGCAAGCCAGTTTCTTT 1498
Foph_17458      AGTACTCTGCAGAGAACCTCGAGGTCCACAAAGCAGAGAACACGCCAAGCCAGTTTCTTT 1412
                ** ***************** ***********  ************ *************** 

FOXG_17458      TCATGCATATCTGCCTGCAGCATAACATTCTCTTTGTCAACCGAGCTGCTATGTCAGCAC 1558
Foph_17458      TCATGCATATCTGCCTGCAGCATAACATTCTCTTTGTCAGTCGAGCTGCTATGGCAGCAC 1472
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                ************************************  ************ ******

FOXG_17458      GAAAGCAACATGGTGTACATGATGATTTCGTCTCTGAAGCAAGCAAGAGGGCCTTCAACG 1618
Foph_17458      GAAAGCAACATGGTGTACATGATGATTTCTTCTCTGAAGCAAGCAAGAGGGCCTTCAACG 1532
                **************************** ****************************

FOXG_17458      CTGCGAACCGAATATCCGAGCTTCTTCGTGAGGCTGAACAGTCGGGATGCTTTGTTTCGG 1678
Foph_17458      CTGCGAACCGAATATCCGAGCTTCTTCGTGAGGCTGAACAGTCGGGATGCTTTGTTTCGG 1592
                ************************************************************

FOXG_17458      CCCCGTTTGCTGGATACTGCGCCTTTTCCTCGACAACAGTTCATATCTTGGGTATTATCT 1738
Foph_17458      CCCCTTTTGCTGGATACTGCGCCTTTTCCTCGACAACAGTCCACATATTGGGCGTCATAT 1652
                **** ********************************** ** ** *****  * ** *

FOXG_17458      CTCGCAATCCCTTCACGAAGCTAGCAGCCCAGGCCAATTTGACCACCAATATAAAGTATC 1798
Foph_17458      CTCGAAACCCCAACATGAAGCTAACAGCCGAGGCCAATTTGACCACCAATGTCAAGTATC 1712
                **** ** ***  ** ******* ***** ******************** * *******

FOXG_17458      TTCACAGAATGAAGAAGTATTGGGGCATGTTCCACTGGATGGTGGAGAACGTTCGCACTC 1858
Foph_17458      TTCACAAAATGAAGAAGTATTGGGCCATGTTTCACTGGATGGTGGAGAACGTTCGCACTC 1772
                ****** ***************** ****** ****************************

FOXG_17458      AGTATCGAAATGTCTTGGACGCTATGAGAGCTGGTGCGAATGTCGAAGAACGAGCCACAC 1918
Foph_17458      AGTATCGAAATGTCTTGGACGCTATGAGAGCTGGTGCGAATCTCGAAGAACGAGCCACAC 1832
                ****************************************** ******************

FOXG_17458      AGCCGTCTTTCCTGCAATACGGAGACTGGTTTAACCGTTACCCTCGCGGTCTTTCTGACG 1978
Foph_17458      AGTCGTCTTTCCTTCAATACGGAGACTGGTTTAACCGTTACCCTCGCGGTCTTTCTGACG 1892
                ** ********** ************************************************

FOXG_17458      CTGAGTTCATGGATCCTGCCACTCACAAACGAAAGGATTCAGGAGCAGACGGCGTCCTCG 2038
Foph_17458      CTGAGTTCATGGATCCTGCCACTCACAAACGAAAGGATTCAGGAGCAGACGGCGTCCTCG 1952
                ************************************************************

FOXG_17458      AAGCGAAGCGCGAACTGCGATCAGTGGAGGAATACTTCACGCTTCCAACACCACGAAGGG 2098
Foph_17458      AAGCGAAGCCCGAACTGCGATCAGTGGAGGCATACTTCACCCTTCCAACACCCCAGAGTG 2012
                ********* ******************** ********* *********** *  ** *

FOXG_17458      TTGAGAATAAGGATACTATCCGCGCAAC-AGCGCCGAAACGAAAGCAAAGCGCCAAGAAA 2157
Foph_17458      TCGAACATAAGGATACCACCCACACAAACAGCAACAAAAAAAAAAAAAAAGCCCCAAGAAA 2072
                * **  ********** * ** * ***  ***  * ***  ***  ***** ********

FOXG_17458      CAGGCTGGCATGCCAGCACAACCTGGCCAGCATCTCGATTCGTTGCAGAGTATAGACGCA 2217
Foph_17458      CAGGCTGCCACCCCACCACAACCTGGCCAGCATCTCGATTCGTTGCAGAGTATAGACGGA 2132
                ******* **  *** ****************************************** *

FOXG_17458      GACGCAGTCTCCCAGGAACGCAAGTTCTCGGGTGGTTTGGGATTACAAATCACAGGTGCA 2277
Foph_17458      GACGCAGTCTCCCAGGAACGCAAGTTCTCGGGTCATTACGGACTACAAACCACAGGTGCA 2192
                ********************************  **  *** ****** **********

FOXG_17458      GCAGGCTTCAACCCTCTGGCAGCATCAAACCAGCAGAGCCCGGATTTCAGCACCACCATA 2337
Foph_17458      GCAGGCTTCAACCCTCTGGCAGCATCAAACCAGCAGAGCCCGGATTTCAGCACCACCATA 2252
                ************************************************************

FOXG_17458      TCACCTACGAGACCGGCCAACATGACTCCATTTGCTCACCACGCACACACGCCCACCTTT 2397
Foph_17458      TCACCTACGAGACCGGCCAACATGACTCCATTTGCTCACCACGCACACACGCCCACCTTT 2312
                ************************************************************

FOXG_17458      TTCCCACCCGAGCTGCTCGCAATGAACTTTGGGCAGGGTTCGAATGGAAACATCGACCCG 2457
Foph_17458      TTCCCACCCGAGCTGCTCGCAATGAACTTTGGGCAGGGCTCGAATGGAAACATCGACCCG 2372
                ************************************ ********************

FOXG_17458      CTTGATCGTCAGCTTATCTATGGCGGATACTCAATGGATGCTAGTACCGGCCTGGGTGGT 2517
Foph_17458      CCTGATCGTCAGCTTATCTATGGCGGATACTCAATGGATGCTAGTACCGGCCTGGGTGAT 2432
                * ********************************************************** *

FOXG_17458      GGCCAGGATATGATGAGCGGCCTTGGTTGGGATACCGTCGCTTTAGGCGCTCAGCCAGAT 2577
Foph_17458      GGCCATAATAGCGCATGGGC—ATTGTAGAGGAAACATTACCCACTCCGCCCAGCCAGATT 2490
                *****  ***      * *    * ** * *  * * *  *      *** *********

FOXG_17458      GGACGCTTGCAAAGTCGGCCGTCAAATGCCAAGGCAGGCATGCATGGACAAAGTGCAGGC 2637
Foph_17458      GGACGCTTGCAAAGTCGGCCGTCAAATGCCAAGGCAGGCATGCATGGACAAAGTGCAGGC 2550
                ************************************************************

FOXG_17458      ATGGCTGATGGGGCAGGATTGAGCAGACCGGAAGCATCATCCGCATGGTTCATGCCATTC 2697
Foph_17458      ATGGCTGATGGGGCAGGATTGAGCGGACCGGAAGCATCGTCCGCATGGTTCATGCCATTC 2610
                ************************ ************** ********************

FOXG_17458      AACATGGAGCCTCCAGATATGGATCAAGATGCCGGCTTCAACATGGGCGGGATTGATCCG 2757
Foph_17458      AACATGGAGCCTCCAGAGATGGGCCAAGATTCCGGCTTCAACATGGGTGGAATTGACCCA 2670
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                ***************** ****  ****** *************** ** ***** **

FOXG_17458      TTCACGGGAGTGTTTGGCGGAGGAGGCAGTGGTTTGGCAACGCCGAATGCGCTAGCATAC 2817
Foph_17458      TTTACGGGAGTGTTTGGAGGAGGGGGTAGTGGTTTGGCAACGCCGAATGC---------- 2720
                ** ************** ***** ** ***********************

FOXG_17458      TTGGCACTACGT 2829
Foph_17458      ------------ 2720
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Appendix 1.5

Alignment of SIX1 homologous effectors (DNA) with CLUSTAL O (1.2.3)

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Foph_SIX1a      ATGGCGCCCTATAGCATGGTACTTCTTGGCGCTCTCTCGATCCTTGGGTTTGGGGCTTAT      60
Foph_SIX1b      ATGGCGCCCTATAGCATGGTACTCCTTGGCGCCCTCTCGATCCTTGGGTTTGGGGCTTAT      60
Fol_SIX1        ATGGCGCCCTATAGCATGGTACTCCTTGGCGCCCTCTCAATCCTTGGGTTTGGGGCTTAT      60
                ********************** ******** ***** *********************

Foph_SIX1a      -GTCAAGAGGCTGCGGTTCGGGAACCCCAGATATTTTTCAACTTGACCTTTACGGAATAT      119
Foph_SIX1b      GCTCAAGAGGCTGCGGTTCGGGAGCCCCAGATATTTTTCAACTTGACCTACACGGAATAT      120
Fol_SIX1        GCTCAAGAGGCTGCGGTTCGGGAGCCCCAGATATTTTTCAACTTGACCTACACGGAATAT      120
                  ******************** ************************  *********

Foph_SIX1a      CTGGATAAGGTGGCAGAATCCCACGGGAGCCCCCCAGACAAGAGTGATTTGCCCTGGAAT      179
Foph_SIX1b      CTGGATAAGGTGGCAGCATCCCACGGGAGTCCCTCAGACAAGAGTGATTTGCCCCGGAAT      180
Fol_SIX1        CTGGATAAGGTGGCAGCATCCCACGGGAGTCCCCCAGACAAGAGTGATTTGCCCTGGAAT      180
                **************** ************ *** ******************** *****

Foph_SIX1a      GACACTATGAGCAGCTTTCCTGGAAACGAGACGGACGATGGTGTTCAGACCGAGACGGGC      239
Foph_SIX1b      GACACTATGAGCAGCTTTCCTGGAAACGAGACGGACGATGGTGTCCAGACCGAGACGGGC      240
Fol_SIX1        GACACTATGGGCAGCTTTCCTGGAAACGAGACGGACGATGGTGTCCAGACCGAGACGGGC      240
                ********* ********************************** ***************

Foph_SIX1a      AGCAGCCTATCGAGGCGTGGTCACATTGTCAACCTTGGAAAGCGTGAGATTGTCCGCGGG      299
Foph_SIX1b      AGCAGCTTATCGAGGCGTGGTCACATTGTCAACCTTAGAAAGCGTGAGCCTGTCGGCGAG      300
Fol_SIX1        AGCAGCCTATCGAGGCGTGGTCACATTGTCAACCTTAGAAAGCGTGAGCCTTTCGGGGAG      300
                ****** ***************************** ***********  * ** * * *

Foph_SIX1a      GAGTCTCGCAACGATGCTGTCACCAATGACATGCTCCAGGTGCTTCATAATATTTGCGTT      359
Foph_SIX1b      GAGTCTCGCAACGATCGTGTCACACAAGACATGCTCCAGGCGCTTCATGATCTGTGCGTT      360
Fol_SIX1        GAGTCTCGCAACGATCGTGTCACACAAGACATGCTCCAGGCGCTTCATGATCTGTGCGTT      360
                ***************  ******  * ************* ******* ** * ******

Foph_SIX1a      GAAAGATTTGGAACTGGCTGGCGAGCGGTTGGCGGGTATTGTGACCGCCGGT--------      411
Foph_SIX1b      GAAAATTTTGGCACAGGCTATCGAGCGGTTAGTGGGATGTGTGTACCTGGACGTCGGGCG      420
Fol_SIX1        GAAAGATTTGGCACAGGCTATCGAGCGGTTAGTGGGTTATGTTACACTGATCGTCGGGCG      420
                ****  ***** ** ****  ********* * ***   ***

Foph_SIX1a      -CCAGGAGAGTAAAATGTAGGAGGCCGGATGACACTGGGCTAGAGCGGCGTGCGAACAAA      470
Foph_SIX1b      ACTAGGAGCTTCGAATGTGGGACACCGGGTGTCAGGGGGGGAGACAGAGCAATGACCAGA      480
Fol_SIX1        ACTAGGAAAATCGAATGTAATAAACCGTCTGTCAGGGAGAGAGACAGATCAGTGACCAGA      480
                 * ****   *  *****   *  ***  ** **  * *  ***  *      ** ** *

Foph_SIX1a      GCTTGCCCACCGCGCGAGGAGTGCTCCACTTTCCAAGCGTACAACTTTATTACTACCGGT      530
Foph_SIX1b      GCTTGCCCAAAGGACCAGGAGTGCACCACTTTCCAGGCGTACAACTATCGTAA-------      533
Fol_SIX1        GCTTGCCCAGAGGGCCAGGAGTGCACCACTTTCAATGCGTACAACTTTCGTAA-------      533
                *********  *  * ******** ******** * ********** *  **

Foph_SIX1a      CAATATGGCCGTACGCCGCACAATTTTCCTGTCTGTGGGCACAAAATTGAGGTGAATGAC      590
Foph_SIX1b      -----CGGTAAGCGTCAGGTTACTTTTCCTGTCTGTGGGCACAAAATTGAGGTGAAGGAA      588
Fol_SIX1        -----CCGTCACCATCAGGTTACTTTTCCTGTCTGTGGGCCCCGAATTGAGGTGAAGGAC      588
                       *       * *   * ***************** *  ************ **

Foph_SIX1a      AGACAGGATCAAGGGAGTCACACGGAGTGGGATGGAACCTGGTACCCCGAAGAGCCTAAA      650
Foph_SIX1b      AGACATGATATAGGGATAGACACGGAGTGGGATGGATCCTGGTTCCCCGAAAAGCCTGAA      648
Fol_SIX1        AGACATGATATAGGGATACACACGGAGTGGCAGGGAACCTGGTACCCCGAATCGCCTAAA      648
                ***** ***  *****   *********** * *** ****** *******  **** **

Foph_SIX1a      TCGCCTGGGACCTACGATTCTTTCGTCCAGATGGCGGGCAGTCTCAATGCGTACTTCAAC      710
Foph_SIX1b      TCGCCTGGGACTTACGATTCTTTCGTCCAGATGGCTGGCAGTCTCGGTGCGCACTTCGAC      708
Fol_SIX1        TCGCCTGGGACCTACGATTATTTCGCCCAGATGGCGGGCACTCTCAATGGGTACTTCGGG      708
                *********** ******* ***** ********* **** ****  ** * *****

Foph_SIX1a      TTTGATGGTGTTTATTCTGATGGAGAGACGATGAACTCTCGGGGACATGCAGTCTCATGG      770
Foph_SIX1b      TTTCATGGTGTTTATTCGGATGGATATGAGATGAACTCCCGGGGAAATGGACACTCATGG      768
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Fol_SIX1        TATGACGGTGTTTATTCGGATGGATATAAGACGAGCTCTCACGGATATGGACATTCATGG      768
                * * * *********** ****** *   ** ** *** *  *** *** *   ******

Foph_SIX1a      TCATGCCTTAATTGCCCGGGAGGCAAGCTGACTATCACTAACACGTATCGCCCAACTTGG      830
Foph_SIX1b      GCATGCGTTAATTGCCCGGGAGGCGAGCTGACTATCACAAGCACATATCGCCCAACTTGG      828
Fol_SIX1        TCATGCATTAATTGCCCGAGAGGCAAGGTGACTATCACAAACACATATCGCGCAACTTGG      828
                 ***** *********** ***** ** ********** * *** ****** ********

Foph_SIX1a      GCGGTTGGATATTCCCACACCGTCTAAGTTGCGATCCAATCTA---     873
Foph_SIX1b      GCCATTGGATATACCACCCCCAACTAAGTTGCGATCCAAAGCTCTA     874
Fol_SIX1        GCGTTTGGATATACCAGCCCACACTAA-------------------     855
                **  ******** **  * *   ****
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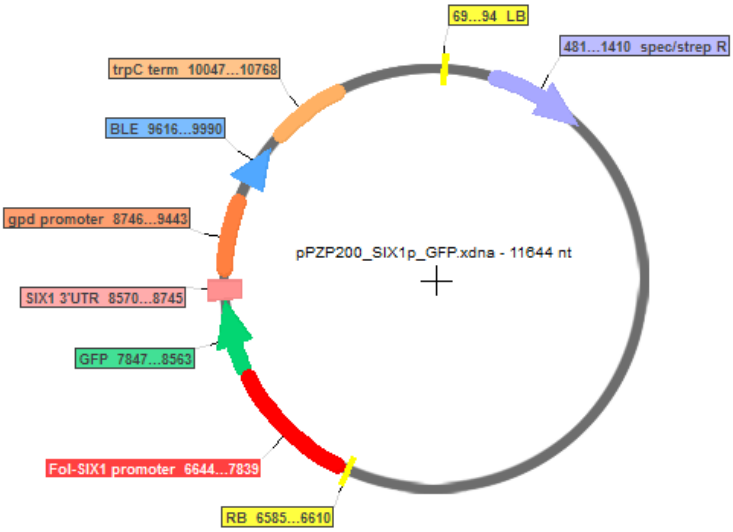
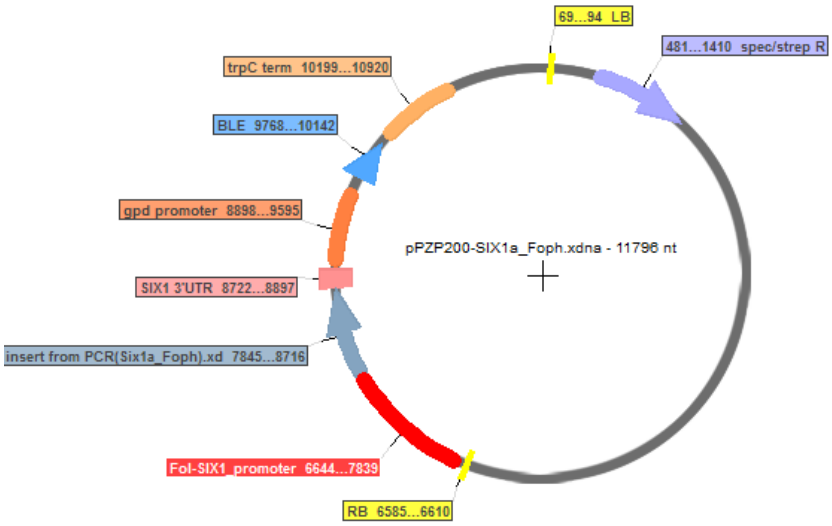
Appendix 2

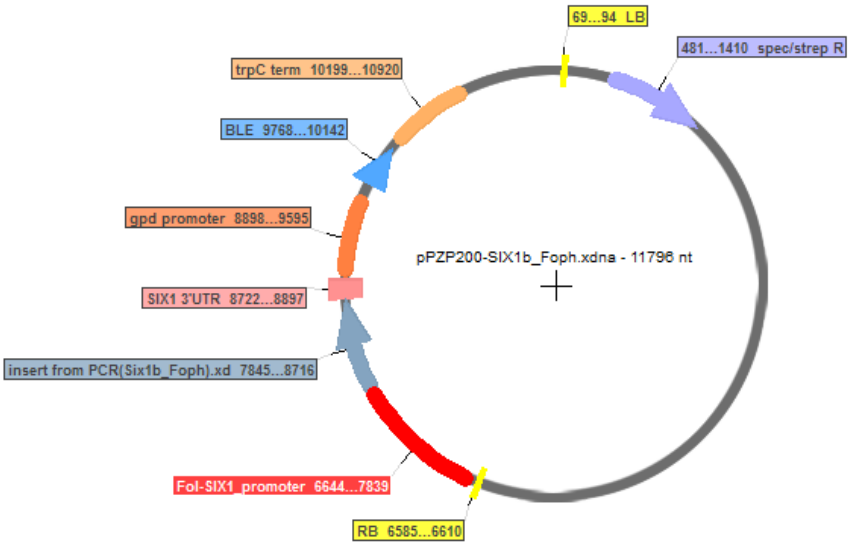
2.1 Map of the pPZP200-pSIX1GFP vector



2.2a Map of pPZP200-pSIX1:SIX1a vector



2.2b Map of pPZP200-pSIX1:SIX1b vector



2.3 Media used for *Agrobacterium tumefaciens*-mediated transformation

Induction medium 1 litre

K_2HPO_4 x $3H_2O$	3.44 g
KH_2PO_4	1.45 g

Dissolve in 900 mL and autoclave (**for IM plates add 15 g/L of Bacto agar)**

NaCl	0.15 g
MgSO4 x $7H_2O$	0.5 g
$(NH_4)_2SO_4$	0.5 g
$CaCl_2$ x $6H_2O$	0.1 g
$FeSO_4$ x $7H_2O$	0.0025 g
MES (pH 5.3)	40 mM
Glucose	10 mM (1.8 g),
Glycerol	0.5 % (w/v) (5 ml)

Dissolve in 100 ml dH_2O, filter-sterilize (0.2 µm) and combine with the 900 ml

Co-cultivation Medium (CM)

Same as IM, except:

5 mM glucose

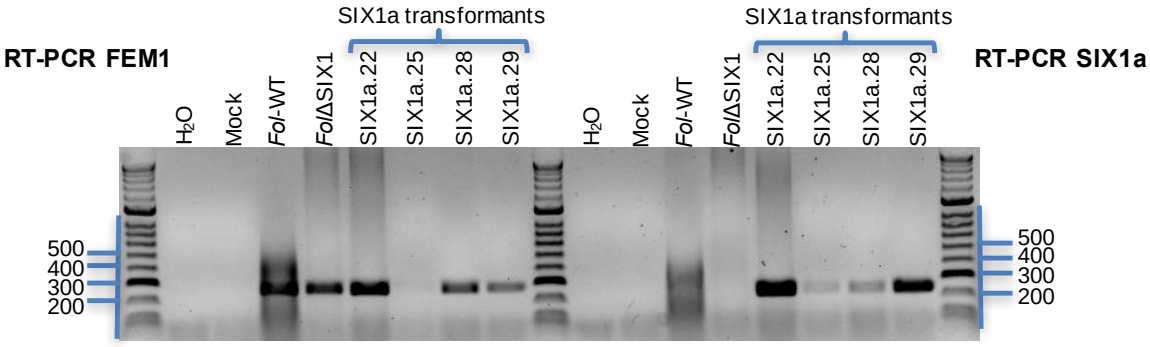
1.5% (w/v) Bacto agar

Add into final concentration 200 µM acetosyringone

2.4 *Fol* genomic DNA extraction protocol

Fungal biomass was produced in PDB. Mycelia were recovered by filtration through four layers of miracloth and air-dried. Dried mycelia (200 mg approximately) was frozen using liquid nitrogen and immediately ground with a pestle and mortar. 1 mL extraction buffer (200 mM Tris-Cl pH 8.0; 50 mM EDTA pH 8.0; 200 mM NaCl; 2% (w/v) sodium N-laurylsarcosinate; 0.1% (v/v) 2-(β) mercaptoethanol) was added to the powder and mixed gently. The mycelial mush was transferred to 1.5 mL tubes and incubated at 65 °C for 30 min. The tubes were cooled down to room temperature and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) solution was added. The suspension was mixed gently until an emulsion formed and was then centrifuged at 6,000 g for 10 min. Supernatants were transferred to new tubes and an equal volume of chloroform:isoamylalcohol (24:1) solution was added. The solution was mixed gently until an emulsion formed and centrifuged again at 6,000 g for 10 min. Supernatants were transferred to new tubes and an equal volume of isopropanol was added and mixed. To enhance precipitation, DNA solutions were stored at -20 °C for 1 hour. High molecular weight DNA appeared as an aggregate, and was centrifuged again at 6,000 g for 10 min. The supernatant was discarded and the precipitated DNA was washed several times with 70% (v/v) ethanol. DNA was air-dried and redissolved in distilled and deionised H_2O.

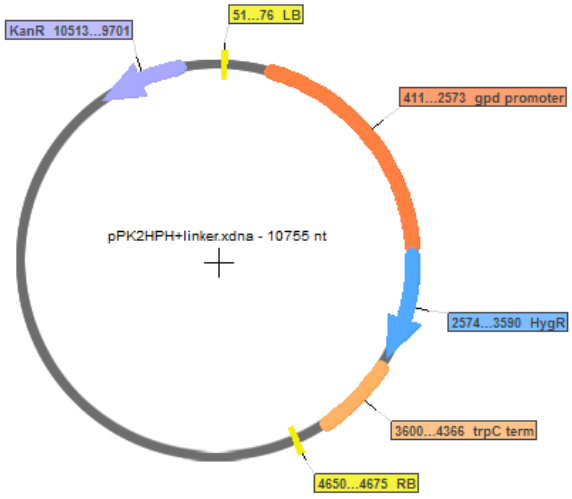
2.5 RT-PCR analysis of four additional *Fol-ΔSIX1* transformants expressing *Foph SIX1a*



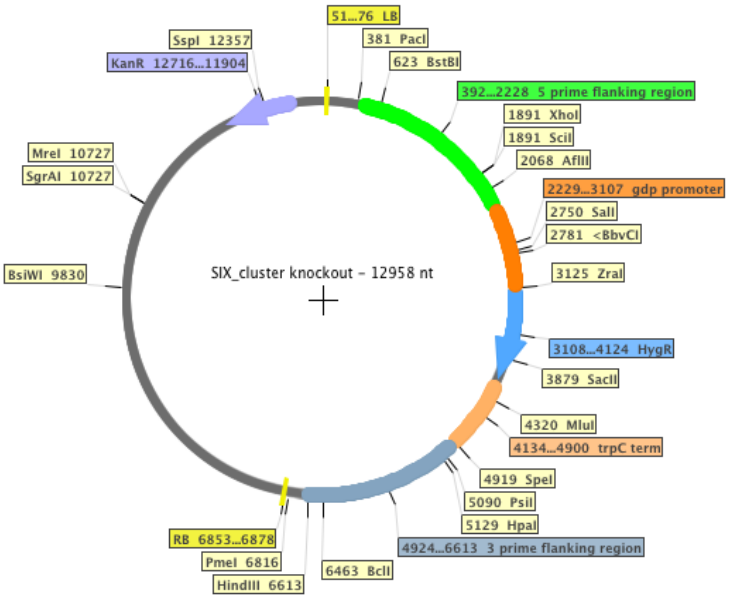
Reverse transcriptase PCR *Fol-ΔSIX1* transformants expressing *Foph-SIX1a* during infection of tomato roots. Left gel image shows bands (expected size 250 with q_Fem1 primers) consistent with *FEM1* expression in *Fol*-infected roots at 5 dpi. Right gel image shows bands (expected size 250 bp) consistent with *SIX1a* expression in *Fol-ΔSIX1:SIX1a*-infected roots at 5dpi, compared with mock, *Fol*-WT or *Fol-ΔSIX1*-inoculated controls where no PCR product was observed.

Appendix 3

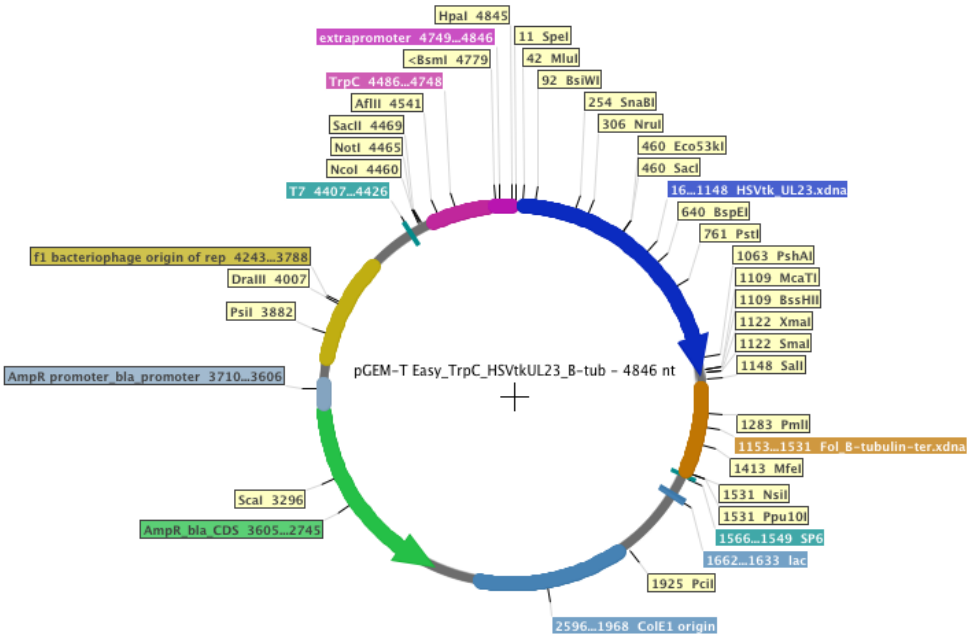
3.1 Map of pPK2*HPH* construct



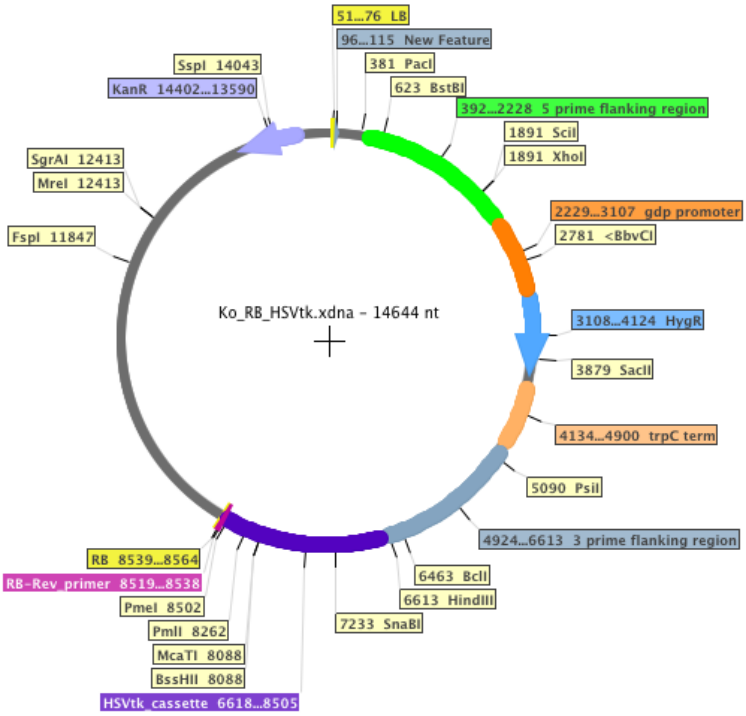
3.2 Map of ΔSIXcl construct



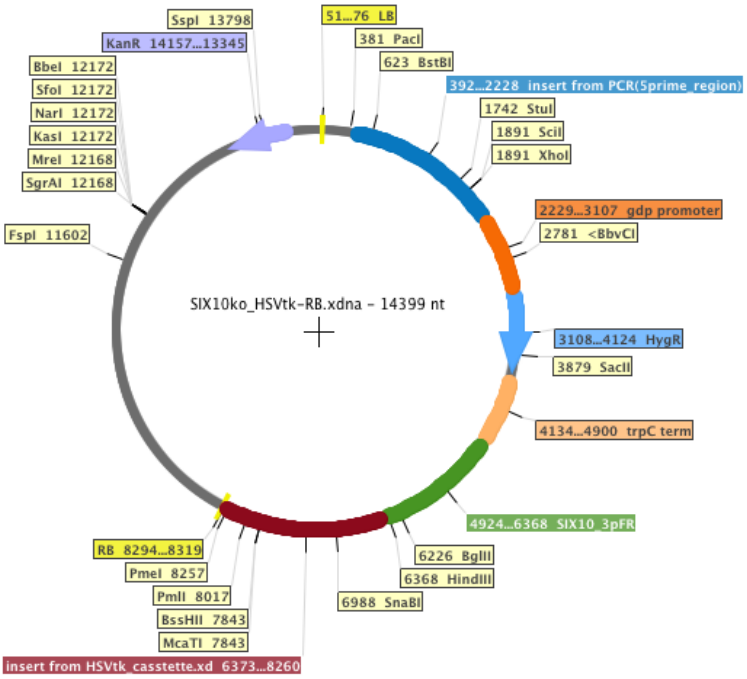
3.3 Map of HSVtk cassette in pGEM-T easy vector



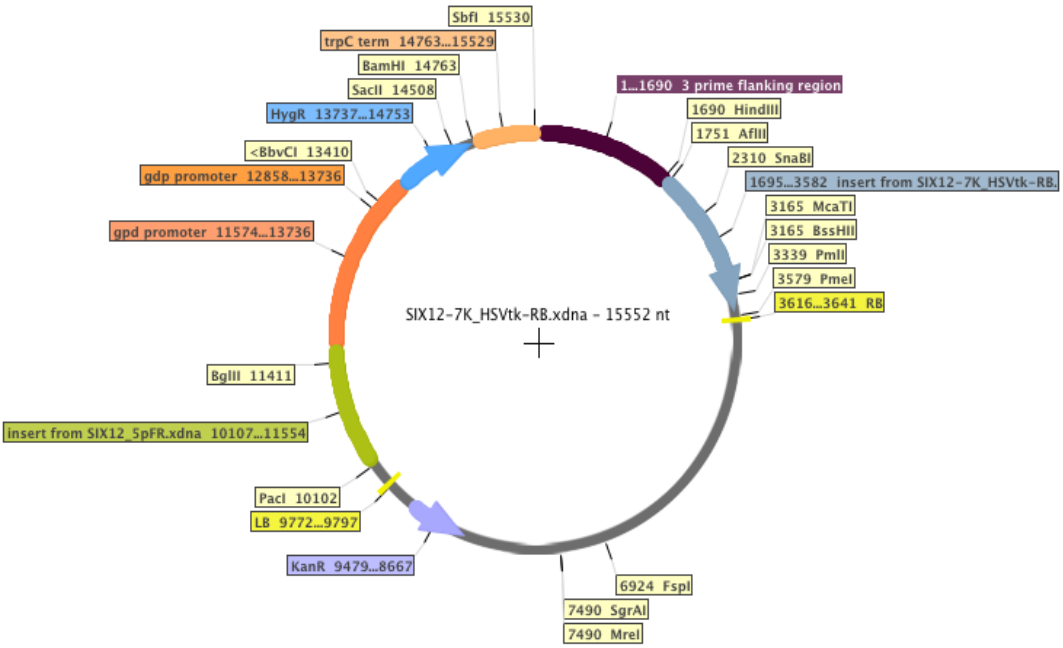
3.4 Map of ΔSIXcI:HSVtk-RB construct



3.5 Map of ΔSIX10::HSVtk-RB construct

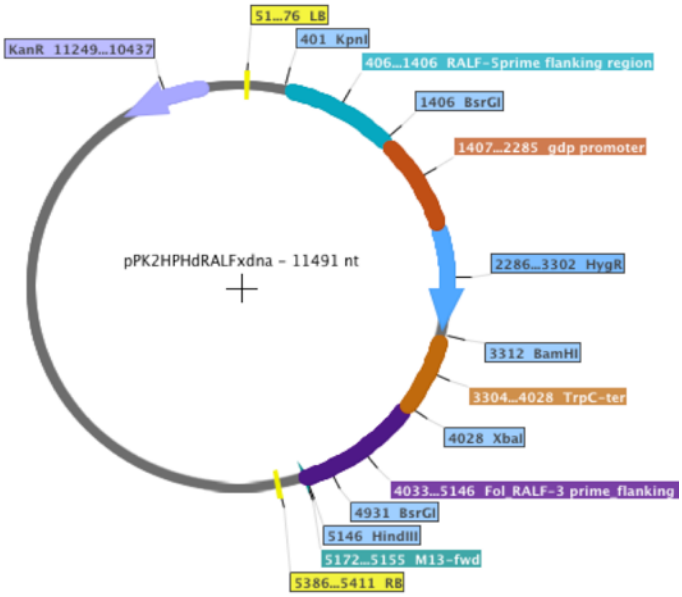


3.6 Map of ΔSIX12/7::HSVtk-RB construct

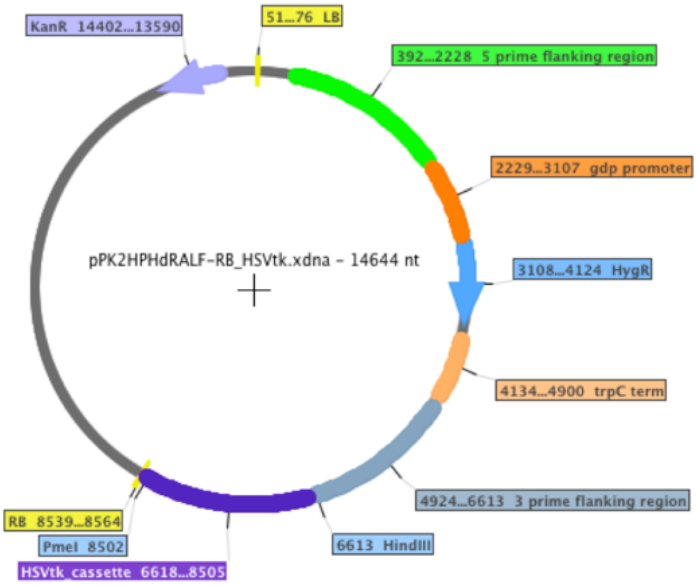


Appendix 4.

4.1 Map of ΔRALF construct

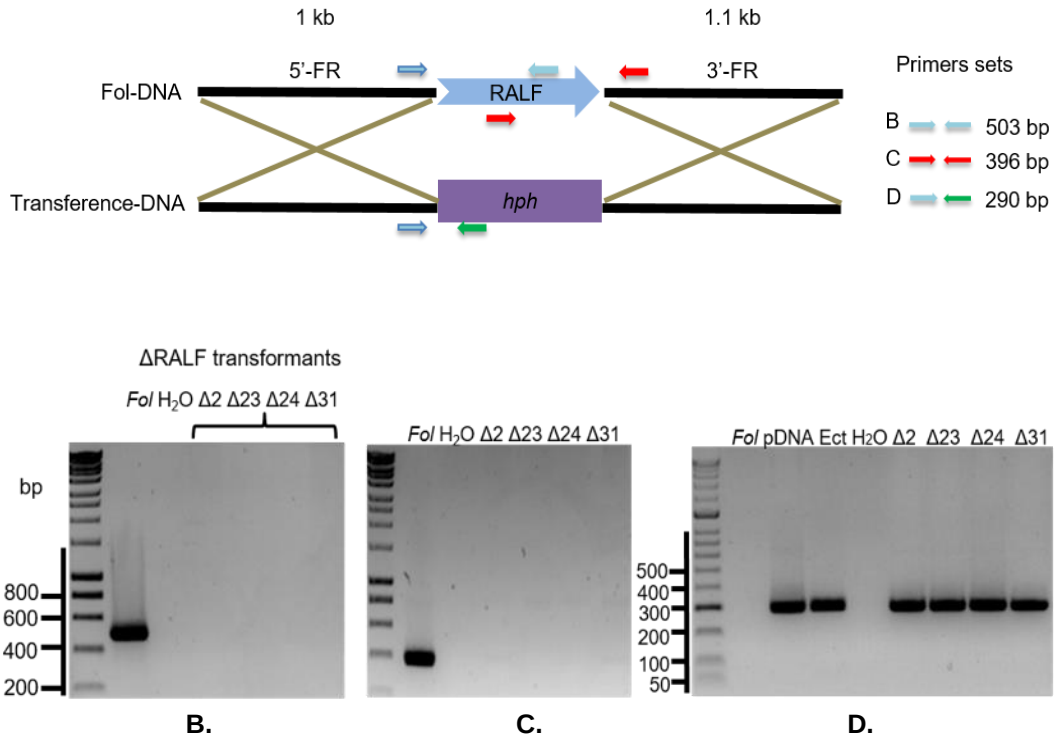


4.2 Map of ΔRALF+HSVtk cassette construct



4.3 Screening of ΔRALF transformants

A.



PCR screening of ΔRALF transformants. **A.** schematic representation of the *RALF* gene replacement by the *HPH* cassette. Arrows indicate primers pairs used for the screening. **B.** PCR with scRALF 5'-F/RALF_int-R primers **C**. PCR with RALF_int-F/ scRALF 3'-R, both images indicate no presence of the *RALF* coding sequence in the four ΔRALF transformants. **D**. PCR with scRALF 5'-F/sc_gdp-R indicate the presence of the *HPH* cassette in the ΔRALF transformants. *Fol* = *F. oxysporum* f.sp. *lycopersici*, Ect 50= transformant with an ectopic insertion of the *HPH* cassette in *Fol*

4.4 *Foph-RALF* sequence

>Foph_RALF

ATGAAGTTCTCTATCATTACATTATCCCTCATCACTCTCGCTTCTGCAGCTCCTGCAGCCAA
GCCTCAGAGCGGTGAGATCTCATATGGAGCCCTCAACCGCGACCATATTCCTTGCTCAGT
CAAGGGCGCGTCGGCCGCAAACTGCCGCCCTGGTGCTGAAGCCAACCCCTATAACCGGG
GCTGCAACGCCATTGAAAAATGCCGTGGTGGTGTTGGTGACAACGTAAGTTCAACAGACG
AGTTTCCTCGTGTGCAACATCAATTAACTGTTTATTCAGTAAGAGAGGACCCATCAAGTTCG
GTTATTGGAATGGCTTGGTAACCGGATCGTAA