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Isolation of novel gibberellin signalling factors from barley aleurone

by

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CANDIDATE'S STATEMENT

The research described in this dissertation is original and has not been submitted for any other degree at this or any other university. The work contained herein was entirely my own with the proviso that technical assistance was supplied by Margaret Keys for the execution of the aleurone co-bombardment experiments and for aleurone protoplast transfections.

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ABSTRACT

The delineation of plant hormone signalling pathways provides a mechanistic insight into the powerful effects hormones exert on plant growth and development. The investigation of the control of hydrolase synthesis and secretion in the cereal aleurone layer by gibberellin (GA) and abscisic acid (ABA) is currently a dynamic area within this field. The experimental work described in this dissertation was directed toward the identification of novel components of the signalling pathway in barley aleurone leading to GA-regulated expression of the gene encoding the key hydrolytic enzyme, α-amylase. Recent work has identified GAMYB, a GA-regulated transcriptional activator of α-amylase gene expression in barley aleurone cells. Accordingly, a proteomics strategy was adopted to identify proteins which regulate GAMYB action in aleurone.

A yeast two-hybrid screen of barley aleurone cDNA-expression libraries for proteins which bind to GAMYB was conducted. Four cDNAs encoding putative GAMYB-binding proteins containing features consistent with a function in signal-regulated gene expression were isolated. Two of these factors, a MAP kinase-like gene termed KGM (for *kinase associated with GAMYB*) and GMPOZ (for *GAMYB associated POZ-domain factor*) were selected for further functional characterisation. Transient expression assays indicate that KGM negatively regulates α-amylase promoter activity in barley aleurone at the level of GAMYB function. Indirect evidence suggests that this repression may be the result of the phosphorylation of GAMYB by KGM. By contrast GMPOZ appears to function as an activator of this pathway, but upstream of GAMYB-mediated transactivation of the α-amylase promoter. The possibility that GAMYB autoactivates GAMYB gene expression in conjunction with GMPOZ is discussed. An augmented model of the GA-response pathway in aleurone, which incorporates these findings, is proposed.

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ABBREVIATIONS

aa	amino acid
ABA	abscisic acid
acc. no.	accession number
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
CaM	calmodulin
cAMP	cyclic adenosine 3':5'-monophosphate
cdc2	cell division cycle 2
cDNA	complimentary cDNA
d	day
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
cGMP	cyclic guanosine 3':5'-monophosphate
dicot	dicotyledonous species
dNTP	deoxynucleoside triphosphate
dsRNA	double stranded RNA
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

DTT	dithiothreitol
EDTA	diaminoethanetetra-acetic acid.disodium salt
ER	endoplasmic reticulum
EST	expressed sequence tag
g	gravity
GA	gibberellin
GDP	guanosine 5'-diphosphate
GFP	green fluorescent protein
GTP	guanosine 5'-triphosphate
GUS	β-glucuronidase
h	hour
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
ir	inverted repeat
LB	Luria-Bertani medium
kDa	kilodalton
kPa	kilopascal
MES	(2-(*N*-morpholino)ethanesulphonic acid
min	minute
MOPS	morpholinopropanesulphonic acid
mRNA	messenger RNA

monocot	monocotyledonous species
MUG	methylumbelliferyl β-D-glucuronide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
poly(A^+)	poly-adenylate
RNA	ribonucleic acid
RNase	ribonuclease
RRS	RAS-recruitment system
s	second
SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)aminoethane
Tween-20	polyoxyethylenesorbitan monolaurate
U	unit
UTP	uridine 5'-triphosphate
UTR	untranslated region
UV	ultra-violet

Chapter 1

Introduction

(1.1) Plant hormone signal transduction – general features

In higher plants and animals a complex network of stimulus-response pathways mediates adaptation to environmental change and facilitates the co-ordinated execution of developmental programmes. Signal transduction is an important component of this system - it is the process by which a molecular "messenger", such as a hormone, evokes a response at the cellular level (for a review see Alberts et al., 1994). In general terms, signal transduction proceeds via a sequence of molecular events which commence with the stimulation of a high-affinity cellular receptor. The primed receptor-complex then activates signalling intermediates, which in turn modulate the activity of various cellular effectors such as transcription factors. It is the activated effector molecules in a signalling pathway which generate a specific cellular response, such as a modulation in gene expression.

Our understanding of plant signal transduction, including hormonal signalling, has been informed by advances in animal systems and it is increasingly apparent that plants share much of the same cellular signalling machinery adapted to different ends. As in animal systems, plant signal perception involves ligand binding to protein receptors linked to ion-channels, kinases or G-proteins (Hooley, 1994; Zimmerman et al., 1997; Tori, 2000). The primed receptor-complex initiates protein phosphorylation or changes in the cytoplasmic concentration of a wide variety of second messengers such as Ca^{2+} or cGMP (McAnish and Hetherington, 1998; Newton et al., 1999). These changes in the cytoplasmic environment regulate the activity of transduction proteins such as kinases, phosphatases and 14:3:3 proteins (Luan, 1998; Hardie, 1999; Roberts, 2000). Downstream events comprise the activation of effectors including transcription factors, osmotic motors and ion channels (Ward et al., 1995; Reichmann and Ratcliffe, 2000). A survey of the *Arabidopsis* genome shows that signalling proteins account for as much as 10% of the genome and that, compared with animals, plants have greatly expanded families of some signalling proteins such as transcription factors and kinases (Chory and Wu, 2001). It is assumed that the explanation for this complexity lies in the additional developmental plasticity of plants necessitated by their sessile habit.

The physiology of the five 'classical' phytohormones, auxin, gibberellin (GA), ethylene, cytokinin and abscisic acid (ABA) as well as their newer counterparts, such as the brassinosteroids, has been studied intensively and it is clear that these compounds are key regulators of plant growth and development (Davies, 1995; Creelman and Mullet, 1997). By contrast, the signalling pathways which underpin the hormone responsiveness of plants are only more recently beginning to be understood, aided in large part by the advent of modern molecular techniques to study these events. Both the ethylene and cytokinin signalling pathways involve the operation of histidine-kinase receptors resembling the two-component histidine-kinase regulators from fungi and bacteria (Stepanova and Ecker, 2000; Mok and Mok, 2001). Relatively little is understood about brassinosteriod signalling but a major breakthrough came with the cloning of a brassinosteriod receptor gene from *Arabidopsis* encoding a leucine-rich repeat receptor kinase (He et al., 2000). Work on auxin signalling has centred on the role of the ubiquitin-proteosome pathway in the auxin-mediated degradation of repressors of the primary auxin response genes in *Arabidopsis* (del Pozo and Estelle, 1999). The delineation of GA- and ABA-signalling has progressed not only through molecular genetics in *Arabidopsis* but through biochemical studies in single cell types, including guard cells and the cereal aleurone layer. For example, the involvement of phosphoinositide signalling and phosphorylation in both ABA-regulated gene expression and stomatal closure has been demonstrated (Rock, 2000).

The work described in this dissertation is concerned with attaining a better understanding of plant hormone signal transduction through studies of the molecular basis of gibberellin signalling in the cereal aleurone layer. The gibberellins exert profound effects on plant growth and development including post-germination events in cereal grain and the aleurone layer, which is a secretory tissue surrounding the starchy endosperm, is a valuable system for investigating GA-signalling. The study of GA-response mutants has revealed that common elements mediate the GA-response in aleurone and other plant tissues and that there is conservation of these factors amongst diverse species. However, much more remains to be understood – a GA-receptor has not been identified and little is understood about how early signalling events are transduced to downstream targets such as transcription factors.

(1.2) Gibberellin signal transduction: current understanding

The gibberellins were first isolated in the 1930s from *Gibberella fujikuroi,* a fungus which causes abnormal elongation in infected rice seedlings, and were subsequently found to form a large class of endogenous steroid-like hormones and metabolites in plants (Takahashi, 1998). The phenotype of GA-deficient mutants and the observed response of plants to applied GAs has revealed that this class of hormone regulates diverse aspects of plant growth and development including stem elongation, floral induction, fruit development and post-germination events in cereal grains (Hooley, 1994). The signalling pathways underpinning the GA-control of these events are being unravelled in two systems. The cereal aleurone layer has proved an informative system for biochemical studies of GA-signalling and work to date in this field is reviewed in section 1.2.1. Genetic studies of GA-signalling, involving the analysis of dwarf or 'slender' (elongated) GA-response mutants have been conducted in both cereals and *Arabidopsis* and this work is reviewed in section 1.2.2. However, as will be emphasised in these sections, key advances in GA-signalling are increasingly products of a dynamic interplay between the two systems. A theme emerging jointly from this work is that GA-signalling appears to be constitutively repressed by negative regulators and the GA-signal operates at least in part through a mechanism of 'derepression' (Sun, 2000).

(1.2.1) GA-signalling in the cereal aleurone

The cereal aleurone layer is a secretory tissue some 1-4 cells thick which surrounds the starchy endosperm (Fincher, 1989). After the initiation of germination by water uptake, the embryo releases GA into the endosperm which stimulates the synthesis and secretion of hydrolases by the aleurone layer. It is these enzymes which mobilise starch and protein reserves to fuel growth of the developing seedling. The aleurone layer is a useful system for the study of GA-signalling because it is a uniform tissue with a defined and unified response to its two key regulators, GA and ABA. In addition, this tissue is highly responsive to exogenously applied hormone, can be cultured in isolation from endogenous sources of hormone and is amenable to manipulations such as microinjection and protoplast isolation (Bethke et al., 1997).

One of the characteristic responses of aleurone to GA stimulation, first noted in the 1960's, is an increase in synthesis of the starch degrading enzyme α-amylase (Paleg, 1960; Yomo, 1960). Consequently α-amylase synthesis is often used to monitor the GA-response in this tissue along with associated responses such as changes in the concentration of cytosolic Ca^{2+} and the degree of cellular vacuolation. In seeds, ABA is involved in embryo maturation, the onset and maintenance of dormancy and protection from pathogens (Fincher, 1989). However, mature aleurone layers retain ABA-sensitivity and ABA generally exerts counteractive effects on GA-regulated processes including hydrolase synthesis and secretion, presumably in order to slow down events after the initiation of germination if the environmental conditions are unfavourable (Lovegrove and Hooley, 2000).

Gibberellin Perception

The majority of evidence suggests that GA is perceived at the external face of the aleurone plasma membrane. Membrane impermeable forms of GA strongly elicit α–amylase expression while microinjection of GA fails to stimulate such characteristic GA-responses (Hooley et al., 1991; Gilroy and Jones, 1994). Also, anti-idiotypic antibodies which contain regions analogous to biologically active domains of GA, cause agglutination of aleurone protoplasts suggesting that GA ordinarily binds to the external surface of aleurone cells (Hooley et al., 1992). Likewise, ABA-inhibition of GA-induced α–amylase expression occurs only if ABA is available at the external surface of the cell (Gilroy and Jones, 1994), suggesting that this antagonist of GA is also externally perceived.

A variety of biochemical techniques have been used to search for GA-binding proteins with the aim of isolating a GA-receptor (for a review see Lovegrove and Hooley, 2000). To date a GA-binding protein with a proven receptor function has not been found however two unlinked polypeptides from aleurone plasma membrane which GA-photoaffinity label have been isolated, suggesting the receptor may be a multi-protein complex (Lovegrove et al., 1998). A receptor-like protein kinase which is induced by GA has been isolated from deepwater rice as has a GA-induced putative type 1a plasma

membrane receptor (Van der Knaap and Kende, 1998; Van der Knaap et al., 1999) but again direct functional evidence for a role in GA perception is lacking.

Evidence is accumulating which suggests that the GA-signal might be transduced from the activated receptor via a G-protein heterotrimeric complex, a highly conserved plasma membrane signalling pathway now also thought to be involved in plant hormone signal transduction (for a review see Hooley, 1998). In this pathway, a G-protein-coupled plasma membrane receptor undergoes a conformational change after ligand binding. This stimulates GTP-GDP exchange at the α-subunit of a plasma membrane heterotrimeric G-protein complex. The α-subunit then disassociates from the complex and activates downstream targets such as kinases or ion channels and is subsequently deactivated by GTP-hydrolysis. In wild oat aleurone the Mas7 molecule, which artificially stimulates GTP/GDP exchange by heterotrimeric G-proteins, elicits α-amylase expression (Jones et al., 1998). In addition, a hydrolysis resistant analogue of GDP which cannot be exchanged for GTP, inhibits GA-induction of α-amylase expression (Jones et al., 1998). Genetic evidence for this scenario has emerged with the discovery that the maize *Dwarf1* locus encodes the alpha subunit of a heterotrimeric G protein (Ashikari et al., 1999; Fujisawa et al., 1999). Mutations at this locus give rise to a partly GA-insensitive, dwarf phenotype.

Second messengers

Second messengers are small intracellular signalling molecules such as Ca^{2+} or cAMP which function as signalling intermediates according to their localised concentration within a cell. For example Ca^{2+}-binding can activate enzymes directly or through the activation of the calmodulin (CaM) protein (Snedden and Fromn, 1998). The concentration of a number of potential second messengers including Ca^{2+} and cGMP is rapidly altered by the GA-stimulation of aleurone and the role these molecules might play in GA-signalling has been investigated further (for a review see Bethke et al., 1997).

One of the earliest events observed in GA-stimulated aleurone is a rapid increase in the concentration of cytosolic Ca^{2}, $[Ca^{2+}]_i$. In barley aleurone protoplasts, $[Ca^{2+}]_i$ increases approximately 3-fold within 5 h of GA treatment (Gilroy and Jones, 1992).

In wheat aleurone protoplasts, $[Ca^{2+}]_i$ increases approximately 10–fold over 30-90 min and the response is initiated within 2-5 min of GA-stimulation (Bush, 1996). The calcium driving these changes in $[Ca^{2+}]_i$ is thought to derive from the apoplast because GA does not induce increases in $[Ca^{2+}]_i$ in isolated aleurone cells in the absence of exogenous Ca^{2+} (Bush, 1996). In addition, the increase in $[Ca^{2+}]_i$ in GA-treated aleurone cells is most concentrated in the peripheral cytoplasm (Gilroy and Jones, 1992; Bush, 1996). Accordingly, it is speculated that a GA-stimulated receptor might activate an aleurone plasma membrane Ca^{2+}-transporter, but a candidate transporter has not yet been identified (Lovegrove and Hooley, 2000).

The expression of CaM, a key Ca^{2+}-transduction protein, is also rapidly induced in aleurone in response to GA. In barley aleurone, CaM mRNA expression increases five-fold after 1 h and a sustained increase in CaM protein expression is observed for 8 h after GA-stimulation (Schuurink et al., 1996). These changes precede increases in α-amylase mRNA expression which shows a sustained induction from around 4 h, until 20 h, following GA-treatment (Schuurink et al., 1996). Treatment of barley aleurone protoplasts with ABA is known to prevent the GA-induced increases in $[Ca^{2+}]_i$ and the expression of CaM (Wang et al., 1991; Schuurink et al., 1996).

In isolated aleurone layers, the GA-induced synthesis and secretion of a number of α-amylase isoenzymes is dependent on the provision of Ca^{2+} but α-amylase mRNA expression is induced to the same extent by GA irrespective of whether Ca^{2+} is provided in the incubation medium (Deikman and Jones, 1985). In addition, the microinjection of caged Ca^{2+}-chelators into barley aleurone blocks the GA-stimulation of the α-amylase secretory pathway but not the GA-induction of α-amylase promoter activity (Gilroy, 1996). These results suggest that there are Ca^{2+}- independent and Ca^{2+}-dependent GA-signalling pathways in barley aleurone, controlling α-amylase expression, and synthesis and secretion respectively. This interpretation is consistent with the knowlege that α-amylase is a Ca^{2+}-requiring metalloprotein (Bush et al., 1989) and the observation that secretory vesicle fusion in aleurone is stimulated by Ca^{2+} (Zorec and Tester, 1992). Although elevated concentrations of Ca^{2+} and expression of CaM protein are not sufficient to induce α-amylase secretion, they can however block the ABA-inhibition of both α-amylase gene expression and α-amylase secretion

suggesting that ABA antagonism of GA-action might work largely through Ca^{2+} and/or CaM levels (Gilroy, 1996).

The cyclic nucleotide monophosphates are known to function as prokaryotic and eukaryotic second messengers, interacting with and activating downstream targets, such as kinases or ion channels and a role for these signalling factors is also emerging in plants (Newton et al., 1999). In GA-stimulated barley aleurone cells, the concentration of cGMP increases 3-fold within 3 h but when cGMP synthesis is prevented by the use of a guanyl cyclase inhibitor, GA stimulation fails to upregulate α-amylase synthesis (Penson et al., 1996). However cGMP analogues do not stimulate α-amylase expression suggesting that cGMP is necessary but not sufficient for the GA-induction of α-amylase expression.

In another early event in GA-stimulated aleurone, cytosolic pH (pH_i) is observed to drop by approximately 0.2 pH units after a 1 h GA treatment and, conversely, is observed to increase by a similar magnitude after a 1 h ABA treatment (Heimovaara Dijkstra et al., 1994). Artificially altering the pH_i does not affect the activity of an α-amylase promoter in transfected barley aleurone protoplasts (Heimovaara Dijkstra et al., 1995) indicating that pH_i changes alone are not sufficient to transmit the GA-signal to the α-amylase promoter. However both Ca^{2+} and pH_i are known to act as second messengers in the ABA-regulation of stomatal closure through the control of K^+ channels, an effect thought at least in part to occur through Ca^{2+} and H^+ regulation of protein phosphorylation (Blatt, 2000). It is as yet unclear what the role of pH_i might be in GA-signalling and whether there is cross talk with Ca^{2+} signalling. Certainly in some animal cell types, changes in pH_i are correlated with changes in Ca^{2+} levels, presumably due to an alteration in the activities of ion transporters, but this relationship is not yet well understood (Cabado et al., 2000).

Transduction proteins

The transient modification of proteins by phosphorylation has a well established role in the transmission of information in eukaryotic signal transduction pathways. Several lines of evidence implicate this type of modification in GA-signalling in aleurone. The treatment of wheat aleurone with the phosphatase inhibitor okadaic acid prevents a

number of characteristic GA-responses including the induction in α-amylase gene expression, suggesting that phosphatases are involved in GA-regulated gene expression (Kuo et al., 1996). The microinjection of Syntide-2, a specific inhibitor of Ca^{2+}- and CaM-dependent protein kinases, into barley aleurone protoplasts reduces the GA-induction of α-amylase mRNA expression, α-amylase secretion and cellular vacuolation but the GA-mediated increase in $[Ca^{2+}]_i$ is unaffected (Ritchie and Gilroy, 1998a).

A number of kinases with hormonally regulated expression have been identified in aleurone including a MAP-kinase like gene, Aspk9, from oat aleurone known which is downregulated by GA (Huttly and Phillips, 1995) and the abscisic-acid induced kinase, PKABA1, from wheat (Anderberg and Walker-Simmons, 1992). Subsequently it has been shown that transient expression of PKABA1 suppresses GA-regulated genes including α-amylase in aleurone (Gomez-Cadenas et al., 1999). A salt- and ABA-induced gene from wheat grass resembling a serine-threonine kinase, *Esi47,* has also been found to repress the activity of a low pI α-amylase promoter in barley when transiently expressed, but the barley homologue of this gene is not yet known (Shen et al., 2001). Despite these advances, the placement of these kinases in the GA-signalling pathway awaits identification of their immediate and downstream targets as well as information about their mode of regulation.

While it is clear that more remains to be understood about the precise role of kinases and phosphatases in GA-signalling, there exists relatively little information about the involvement of other transduction proteins in the transmission of the GA-signal. A role for CaM is indicated by the rapid induction of this protein in response to GA and a number of endomembrane targets for CaM action have been proposed (see below). Notwithstanding, little else is currently known about how the upstream events in GA-signalling translate into downstream responses. From our knowledge of other signalling pathways, factors such as the WD40-scaffold proteins (Smith et al., 1999) and 14:3:3 phosphopeptide-binding proteins (Roberts, 2000) might well be involved. Other types of transient, post-translational modifications such as acetylation (Bannister and Miska, 2000) and certain types of glycosylation (Reuter and Gabius, 1999) are

increasingly becoming understood as signalling mechanisms and might also apply within the GA-pathway.

Molecular targets of GA-signalling: endomembrane system

A number of endomembrane targets involved in the GA-control of hydrolase synthesis and secretion have been proposed, including the ER and tonoplast (for reviews see Bethke et al., 1997 and Ritchie and Gilroy, 1998b). The observed GA-induced acidification of the protein storage vacuoles (PSVs) in aleurone is thought to lead to protein mobilisation through the activation of pH-sensitive proteases (Bethke et al., 1996; Swanson and Jones, 1996). It is therefore highly likely that the activity of tonoplast H^+-pumps is targeted by the GA-signal (Ritchie and Gilroy, 1998b). In addition the activity of the slow vacuolar channel in the PSV, which is involved in the export of minerals mobilised by protease action, is positively regulated by Ca^{2+} and CaM (Bethke and Jones, 1994).

The maintenance of high levels of Ca^{2+} in the ER lumen is a requirement for the synthesis of α-amylase at the ER, since α-amylase is a Ca^{2+}-requiring metalloprotein (Bush et al., 1989), and Ca^{2+}-transport into the ER is known to be stimulated by GA in a CaM dependent fashion (Bush et al., 1993; Gilroy and Jones, 1993). A putative ER Ca^{2+}-ATPase has been cloned from rice which, when transiently expressed in rice, elicits α-amylase gene expression (Chen et al., 1997). This result suggests that there might be cross-talk in GA-signalling between the secretory pathways and pathways targeting gene expression but it has not been shown that the changes in α-amylase expression in these experiments were associated with corresponding changes in cellular concentrations of Ca^{2+}. One further membrane target for Ca^{2+}-CaM signalling in aleurone has been identified with the cloning of a barley CaM-binding transporter-like protein which localises to the plasma membrane (Schuurink et al., 1998). The possible role of this factor in hormonal signalling is as yet unclear.

Molecular targets of GA-signalling: transcriptional regulation

GA is known induce expression of numerous genes including calmodulin, nucleases and starch, cell wall and protein degrading enzymes (Bethke et al., 1997). To date the

search for GA-responsive *cis*-elements and their cognate transcription factors has centred on the α-amylase genes. The α-amylase genes form a multi-gene family in cereals and the wheat and barley genes fall into the *Amy1* and *Amy2* subclasses (Mitsui and Itoh, 1997). In barley and wheat the α-amylase genes are also classified on the basis of whether the encoded protein possesses a high or low isoelectric point.

Comparisons of the promoter regions from the family of cereal α-amylase genes reveal three highly conserved elements - a TAACA$^{A}/_{G}$A element, a TATCCA$^{C}/_{T}$ element and a pyrimidine box (Huang et al., 1990). Through mutagenesis and the analysis of truncated promoters it has been shown that these elements contribute to GA-response complexes (GARCs) and that the TACAA$^{A}/_{G}$A element is particularly important to the hormonal responsiveness of the α-amylase genes (Gubler and Jacobsen, 1992; Huttly et al., 1992; Lanahan et al., 1992; Rogers and Rogers, 1992; Tregear et al., 1995). Multimers of a 21 bp region containing the TAACAAA element from the barley *α-amy1/6-4* promoter confer GA-responsiveness to a minimal promoter, and consequently this element has been designated a GARE (gibberellin response element; Skriver et al., 1991). In a barley high pI α-amylase promoter, the GARE and the TATCAC together form a GARC (Gubler and Jacobsen, 1992). In the barley low pI α-amylase genes, an element corresponding to the Opaque-2 binding sequence (O2S) from maize (known to bind a leucine zipper transcription factor) has also been found to modulate GA-responsiveness and together with the GARE forms a GARC (Lanahan et al., 1992; Rogers and Rogers, 1992). The importance of the pyrimidine box in mediating the interaction between the O2S and GARE *cis*-elements has also been demonstrated (Lanahan et al., 1992). Work on a wheat low pI α-amylase promoter has also confirmed the importance of the O2S-like element to the structure of the GARC in the low pI α-amylase promoters (Tregear et al., 1995).

The conserved *cis*-elements which, according to functional assays, form the GARCs in the α-amylase promoters are highly likely to incorporate binding sites for *trans*-acting factors. Already a number of interactions between nuclear proteins and these elements have been defined. An aleurone nuclear factor has been isolated which binds to the GARE in the barley low pI, *Amy32b* promoter in a GA-dependent manner (Sutliff et al., 1993), but this factor has not yet been identified. The oat ABF1 and ABF2 proteins

bind to the O2S (box 2) coupling element from the wheat *α-Amy2/54* promoter but their role in the regulation of α-amylase gene expression remains to be determined (Rushton et al., 1995). DNase1 footprinting has identified nuclear factor binding at the GARE, O2S element and a CCATG/C element in the promoter of the wild oat *α-Amy2/A* gene, suggesting that at least three classes of transcription factor are involved in the regulation of this gene (Willmott et al., 1998). A barley zinc-finger protein, HRT, which binds to the GARE in the barley *Amy1/6-4* promoter, represses the activity of both *Amy1* and *Amy2* promoters in transient expression assays (Raventos et al., 1998). However the regulation of this factor by hormonal signals is not yet understood.

To date, the most intensively studied GARC binding factor is the barley GAMYB transcription factor, a GA-regulated activator of α-amylase expression known to bind to the GA-response element (GARE) in a barley high pI α-amylase promoter (Gubler et al., 1995). The MYB transcription factors were first identified in animal systems with the discovery of the v-Myb oncogene from avian myelobastosis virus and feature one to three repeats (R1/R2/R3) of a conserved 50 aa DNA binding domain (Lipsick, 1996; Martin and Paz-Ares, 1997). In *Arabidopsis*, the R2/R3 MYBs number over 90 and functional analysis suggests they are involved in diverse processes including the regulation of secondary metabolism, cellular differentiation and the cell cycle (Jin and Martin, 1999; Stracke et al., 2001).

Work leading to the discovery of GAMYB was prompted by the recognition of a consensus MYB binding site, TAACAAA, in the GARE of a barley high pI α-amylase promoter (Gubler et al., 1995). Screens of cDNA libraries for MYB candidates resulted in the isolation of a cDNA encoding a protein with conserved MYB motifs. The expression of GAMYB is hormonally regulated - in response to GA-treatment, expression of the GAMYB transcript in isolated barley aleurone increases approximately four-fold while GAMYB protein expression increases around 10–fold (Gubler et al., 1995; Gubler et al., 2002; F. Gubler, personal communication). GAMYB gene expression has also been detected in barley anthers and the expression of GAMYB-like genes has been detected in wheat internodes and *Lolium* floral meristem, suggesting that GAMYB may have a role in GA-regulated processes outside

the aleurone (F.Murray, personal communication; Gocal et al., 1999; Chen et al., 2001).

A number of results are suggestive of the functional importance of GAMYB to the transcriptional activation of the α-amylase promoter. In transient expression assays, GAMYB strongly transactivates a high pI α-amylase promoter fused to GUS in the absence of GA, but mutation of the GARE in this promoter abolishes this effect (Gubler et al., 1995). A tight correlation between GAMYB binding sequences contained in the this high pI GARE, defined *in vitro*, and sequences necessary for transcriptional activity of the entire α-amylase promoter in transient expression assays, has also been established (Gubler et al., 1995).

GAMYB is known to bind the sequence TAACAGAC *in vitro*, which is the GARE found in barley low pI α-amylase promoters (Gubler et al., 1995) and similar consensus MYB-binding sites exist in the promoters of other classes of GA-responsive grain hydrolases. Subsequently it has been shown in transient expression assays that GAMYB strongly transactivates the promoters from the barley high pI α-amylase, (1-3,1-4)- β-glucanase and cysteine protease *EPB-1* genes as well as the wheat β-capthesin-like protease promoter (Cercos et al., 1999; Gubler et al., 1999). These results suggest that GAMYB is a broad-acting transcriptional activator of GA-regulated genes in aleurone.

A functional map of the GAMYB protein has been generated through the analysis of the transactivation potential of a series of C-terminal GAMYB deletions co-expressed in barley aleurone both high pI- and low pI α-amylase promoter-GUS fusions (Gubler et al., 1999). Two transcriptional activation domains, spanning the regions in GAMYB from amino acid positions 150 to 230 and 356 to 490, have been defined along with two negative regulatory domains in the regions between amino acids 230-356 and 490-553. This domain organisation is supported by the results from an analysis of the transactivation potential of a series of COOH-terminal GAMYB deletions in a yeast one-hybrid system (Gubler et al., 1999).

Repressors of GA-signalling in aleurone

The cereal orthologues of the A*rabidopsis* GAI/RGA genes, including barley and rice SLENDER1 (SLN1/SLR1), are repressors of GA-signalling identified from work in GA-response mutants (see section 1.2.2) and encode putative plant transcription factors (Pysh et al., 1998; Ikeda et al., 2001; Chandler et al., 2002). Mutants at this locus exhibit both dwarf and elongated phenotypes representing dominant gain-of-function mutations or loss of function mutations respectively. In the aleurone layers of rice and barley *slender1* (elongated) mutants, expression of α-amylase is constitutive rather than GA-dependent (Chandler, 1988; Lanahan and Ho, 1988; Ikeda et al., 2001). Conversely, in a dwarf gain-of-function mutant at the barley slender locus (*Sln1d*), the GA-induction of α-amylase expression is substantially repressed (Gubler et al., 2002).

The molecular basis of the SLN1 repression of GA-signalling in aleurone is not yet fully understood. In aleurone, the barley SLN1 protein is constitutively expressed in the nucleus but rapidly degraded in the presence of GA (Gubler et al., 2002) suggesting that wildtype SLN1 acts as a GA-repressible, negative regulator of GA-signalling. GAMYB has been identified as a target for the repressive action of SLN1 because in the barley *Sln1d* dwarf mutant, the GA-induction of GAMYB expression in aleurone is significantly weaker than in wildtype (Gubler et al., 2002). This result suggests that GAMYB expression is controlled by events directly downstream of SLN1 action

The molecular basis of the ABA-mediated antagonism of GA-signalling in aleurone is also incompletely understood. Although exogenous ABA represses the induction of α-amylase gene expression by GA (Jacobsen and Beach, 1985), it does not repress GAMYB mediated transactivation of an *α-Amy1* promoter in transient expression assays in barley aleurone (Gomez-Candenas et al., 2001). Instead, like SLN1, ABA has been observed to inhibit GAMYB transcription rates and protein expression in aleurone (Gubler et al., 2002). However ABA appears to function downstream of SLN1 because the barley *sln1* (elongated) mutant retains ABA-sensitivity (Chandler, 1988; Lanahan and Ho, 1988), and ABA-treatment of wildtype barley aleurone layers has no effect on the GA-induced degredation of SLN1 (Gubler et al., 2002). The transcriptional activity of a 1031 bp GAMYB 5' flanking sequence fused to GUS is

induced two-fold by GA treatment in bombarded barley aleurone but is repressed by ABA (Gomez-Candenas et al., 2001). However, transient expression of the ABA-induced kinase, PKABA1, also suppresses this activity suggesting that ABA-repression of GAMYB expression is mediated by PKABA1 (Gomez-Candenas et al., 2001).

The relationship between SLN1 and the second messenger cGMP in GA-signalling in aleurone has also been investigated. Studies in the barley *slender1* background using a guanyl-cyclase inhibitor suggest that cGMP functions downstream of SLN1 action in the GA-signalling pathway. This is because α-amylase and GAMYB expression are repressed to similar degrees by this inhibitor treatment in both wild-type and mutant backgrounds (Gomez-Candenas et al., 2001).

A second molecular repressor of GA-signalling in aleurone was originally identified through studies of GA-response mutants at the *Arabidopsis SPINDLY* locus (*SPY*) which is thought to encode an *O*-GlcNAc transferase (see section 1.2.2 below). A SPY homologue was isolated from barley (HvSPY) and, in transient expression assays, has been shown to repress the GA-induction of α-amylase promoter activity in barley aleurone (Robertson et al., 1998). However it is as yet unclear whether this effect occurs through GAMYB or is mediated by an independent pathway.

(1.2.2) GA response mutants

Mutants altered in GA signal transduction are identified on the basis of reduced GA-responsiveness and altered GA-regulated growth characters such as internode elongation. These mutants are phenocopies of GA biosynthesis mutants but differ in that they exhibit reduced sensitivity to exogenously applied GA or GA-biosynthesis inhibitors. There are two main categories of GA response mutants - GA-insensitive dwarfs (decreased GA signal transduction) and constitutive GA response, "slender" mutants (increased GA signal transduction).

The analysis of GA-response mutants in *Arabidopsis* and cereals has traditionally provided a complementary approach to cereal aleurone studies of GA-signalling but a number of key advances have recently been generated through a dynamic interplay between the two systems. As described below, at least three factors, the GAI-

orthologues, SPY, and D1 are known to function as relatively broad acting GA-response regulators in a variety of species. The placement in GA-signalling pathways of factors identified in GA-response mutants is complicated by the fact that mutants exhibit pleiotropic phenotypes and the various GA-regulated processes can be differentially altered. Given that GA does influence diverse processes in diverse tissues, it is reasonable to expect that the upstream components in GA-signalling pathways, such as D1, SPY and the GAI-orthologues, are more likely to be conserved and that the pathways then diverge to activate separate molecular targets. A number of other interesting loci have also been identified in *Arabidopsis* GA-response mutants which could form part of a conserved upstream signalling system, and represent targets for further study in aleurone if cereal counterparts expressed in this tissue can be identified.

GA-insensitive dwarfs

The GA-insensitive dwarf mutants are phenotypically identical to GA deficient mutants – they are reduced in stature and have altered leaf colour and can have impaired seed germination, delayed flowering, abnormal flowers and reduced apical dominance in relation to wild-types. The dominant or semi-dominant members of this class, that is, heterozygotes which exhibit a mutant phenotype, are normally considered to represent gain-of-function mutations in a negative regulator of GA-signalling. Conversely recessive mutants in this class are generally assumed to contain lesions in potential positive regulators of GA-signalling.

The semi-dominant *Arabidopsis gai-1* (*gibberellin-insensitive 1*) allele, which gives rise to a severe GA-deficient-like phentoype, was the first allele to be cloned from the GA-insensitive dwarfs. The wildtype gene was found to encode a protein from a new class of putative plant GRAS transcription factors (Peng et al., 1997). GAI also contains features reminiscent of the STATS, a class of proteins with dual signalling functions which, upon plasma membrane receptor-mediated dimerisation in SH2-phosphotyrosine domains, translocate to the nucleus and initiate changes in gene expression (Hovarth, 2000). Subsequently, the dwarfing alleles *Rht-B1* and *Rht-D1* from high yielding wheat varieties developed during the so-called 'green-revolution', plus the maize *dwarf-8* locus were found to be altered in genes encode orthologues of

GAI (Peng et al., 1999). Likewise, the barley *Sln1d* dwarf mutant was found to be altered at a GAI-like locus (Chandler et al., 2002). The results of biochemical studies in aleurone (described in section 1.2.1 above) show that SLN1 is a repressor of GA-signalling in aleurone, as well as in vegetative tissues.

Recently a new GA-insensitive dwarf mutant which overexpresses a zinc finger protein known as SHI (for *Short Internodes*) has been isolated in *Arabidopsis* (Fridborg et al., 1999). Further molecular evidence for the involvement of this possible negative regulator in GA-signalling remains to be shown as the phenotype could be an artefact resulting from ectopic expression of SHI. The *Arabidopsis* mutant, *pickle* (*pkl*) is a recessive GA-insensitive dwarf thought to encode a potential positive regulator of GA-signalling. The PKL gene has been cloned and encodes a factor with sequence homology to CHD3, a chromatin-remodelling factor (Ogas et al., 1997; Ogas et al., 1999). However the *pkl* mutant phenotype is pleiotropic, complicating placement of this factor in GA-signalling. A role for PKL in GA-induced root differentiation has been proposed (Ogas et al., 1997). No cereal counterparts for SHI or PKL have yet been identified. An additional recessive GA-unresponsive semi-dwarf, *sleepy1* (*sly1*), has also been identified in *Arabidopsis* through suppressor screens in an ABA-insensitive mutant background, *abi1-1* (Steber et al., 1998). The *sly1* mutant displays the full spectrum of GA-related phenotypes and is unable to germinate even in an *ABI1* background, suggesting that this locus may encode a key upstream component of GA-signalling and consequently the cloning of SLY is much anticipated.

Like *sly1*, the barley *gse1 (GA sensitivity 1)* and rice *dwarf1* mutants are recessive semi-dwarfs with reduced sensitivity to GA which may contain lesions in loci which encode potential positive regulators of GA-signalling (Mitsunaga et al., 1994; Chandler and Robertson, 1999). The rice *Dwarf1* locus encodes the alpha subunit of a heterotrimeric G-protein supporting the biochemical evidence in aleurone that a G-protein signalling complex is a component of GA-signal transduction (Ashikari et al., 1999; Fujisawa et al., 1999). The *gse1* mutants, which are also genetically upstream of barley *sln1*, exhibit a 100-fold reduced sensitivity to applied GA as measured by α-amylase synthesis and rates of leaf elongation, and are thought to represent lesions in a positive regulator of GA-signalling in these tissues (Chandler and Robertson, 1999).

The rice *dwarf1* (*d1*) mutant also displays a dose-response relationship to applied GA with respect to α-amylase gene expression and is genetically upstream of rice *slr1*, hinting that the dwarf1 and gse1 loci might both encode the same Gα subunit (Mitsunaga et al., 1994; Ueguchi-Tanaka et al., 2000). The Gα subunit is encoded by a single gene in rice but in *d1*, the second leaf blade retains a sensitivity to GA which is comparable to the wildtype, indicating that the Gα subunit may not be involved in all GA-responses. The *Arabidopsis* genome contains only a single gene corresponding to a Gα–like protein however a loss-of-function mutation does not affect responses related to GA, suggesting that the Gα subunit is not uniformly important in GA-signalling in all tissues or in every species (Ullah et al., 2001; Wang et al., 2001).

Constitutive GA-response mutants

The second major class of potential GA signal transduction mutants is defined by a phenotype that resembles GA-treated plants. This "slender" phenotype involves elongated internodes, pale and narrow leaves and male/female sterility. Key examples of this class of mutant include barley *sln1* and rice *slr1* (Chandler, 1988; Lanahan and Ho, 1988), *Arabidopsis spy (*for *spindly)* and *rga (*for *repressor of ga1-3;* (Jacobsen and Olszewski, 1993; Silverstone et al., 1997). All members of this class contain recessive mutations in a putative negative regulator of GA-signalling.

The *SPINDLY* locus in *Arabidopsis* was originally isolated in supressor screens in the GA-deficient background, *ga1-2* (Jacobsen and Olszewski, 1993). The *spy-1* mutant could partially restore all of the vegetative and reproductive phenotypes associated with the *ga1-2* mutant. The *SPY* gene was cloned by T-DNA tagging and found to encode a protein with homology to a class of regulatory proteins, *O*-GlcNAc transferases, which add *O*-linked N-acetyl glucosamine residues to proteins (Jacobsen et al., 1996). This type of transient modification is thought to regulate protein function in a manner similar to phosphorylation (Comer and Hart, 1999), and as described above, the biochemical evidence from aleurone shows that the barley homologue of SPY is a repressor of GA-signalling in this tissue (Robertson et al., 1998). An additional *Arabidopsis* locus, *gar-2*, thought to encode a negative regulator of GA-signalling has also been isolated in supressor screens in the *ga1-1* background and this mutation

restores flowering time, height and germination frequencies to wildtype levels (Wilson and Somerville, 1995). The *gar-2* mutation is dominant and is therefore likely to encode a potential negative regulator of GA-signalling however the relevant gene has not yet been cloned.

The *Arabidopsis* locus, *RGA*, was isolated by screening for suppressor mutations which overcome the dwarf phenotype of the GA biosynthesis mutant, *ga1-3* (Silverstone et al., 1997). In this background the *RGA* mutation partially restores stem growth, flowering time, trichome initiation and apical dominance in the absence of exogenously applied GA but not the impaired germination and sterility phenotypes. The RGA gene has been cloned and is closely related to GAI (Silverstone et al., 1997). In stably transformed roots, RGA-GFP fusion proteins are localised to the nucleus and are rapidly degraded upon GA-treatment, suggesting that GA-regulates RGA function at the level of protein stability (Silverstone et al., 1998) as has been observed for the SLN1 protein in aleurone (Gubler et al., 2002).

It is apparent that mutations in the GAI-like genes give rise to two contrasting phenotypes. For example, the dominant gain-of-function barley mutant, *Sln1d* is a dwarf while its counterpart, the recessive loss-of-function mutant *sln1c*, is elongated. An analysis of the gain-of-function mutants, *gai-1* and *Sln1d* shows that they contain lesions in the highly conserved NH_3-terminus of these proteins (Peng and Harberd, 1993; Chandler et al., 2002) and it is known that the barley *Sln1d* mutant protein is resistant to GA-mediated degredation (Gubler et al., 2002), suggesting that this region ordinarily confers GA-sensitivity. The lesions in *RGA* and barley *SLN1* which give rise to loss-of function (elongated) phenotypes result in disruption to the ORFs meaning that functional, full-length transcripts are not produced in these backgrounds and that GA-signalling is derepressed (Silverstone et al., 1998; Chandler et al., 2002). Two further relatives of RGA have been identified in *Arabidopsis*, RGAL (RGA-like; Sanchez-Fernandez et al., 1998) and RGA1-like (GenBank accession number AC009895). It seems likely that the GAI-like genes in *Arabidopsis* have overlapping if not redundant functions. The phenotype of the *gai* and *rga* mutants suggests that GAI is the most broadly acting member of this family and that RGA may have a role in a subset of GA-regulated processes.

(1.3) Delineating the aleurone GA signal transduction pathway: scientific aims

The results of biochemical and genetic studies of GA-signalling are beginning to coalesce and are together providing a glimpse of the conserved nature of the early events in GA-perception and signal transduction. With the advent of increasingly sophisticated molecular technologies, the traditional advantages of cereal aleurone render it attractive for gene discovery projects. A biochemical model summarising current understanding of GA-signalling events in aleurone leading to the upregulation of α-amylase gene expression is presented in Figure 1.

In the proposed model, the GA signal stimulates an as yet unknown plasma membrane receptor and is transduced via a heterotrimeric G-protein complex. A central feature of this model is that the pathway is de-repressed through the rapid GA-mediated degradation of the upstream repressor SLN1. The genetic evidence from *Arabidopsis* suggests SPY is necessary for GAI/RGA function (Thornton et al., 1999) so HvSPY is placed at the level of SLN1 action. It is envisaged that the interaction between these factors could be mediated through an *O*-GlcNAc activity. The requirement for cGMP synthesis in positive transmission of the signal is indicated, the brackets merely noting that the cGMP signal is not sufficient at this point in the pathway. In more downstream events in this model, the de-repression of SLN1 by GA leads to an upregulation in GAMYB expression and GAMYB in turn transactivates the α-amylase promoter. Finally, ABA is shown to repress GA-signalling through PKABA1 which acts at the level of GAMYB expression. A number of potential routes through which GAMYB function might be regulated are also indicated.

Despite the recent advances in this field it is clear that much more remains to be understood about the molecular basis of GA-regulated gene expression in cereal aleurone. It is almost certain that large transcriptional complexes direct this process and little is known about how signalling intermediates target downstream transcription factors. For example, the possibility that GAMYB function is post-translationally regulated, or modulated by protein-protein interactions, is suggested by the fact that GAMYB protein is expressed at detectable levels in barley aleurone in the absence of GA without any corresponding α-amylase gene expression (Gubler et al., 2002). Also,

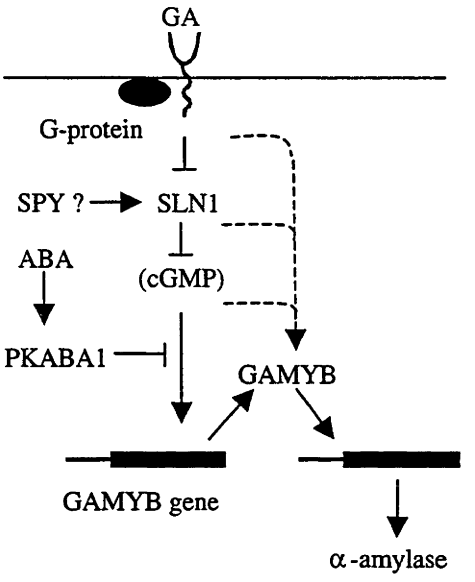


Figure 1. Current understanding of the GA-signalling pathway in cereal aleurone leading to α-amylase expression.

Routes through which GAMYB might be post-translationally regulated are indicted with dashed arrows.

these same protein gel blots show that multiple forms of GAMYB protein are expressed in aleurone, but the types of modifications giving rise to these isoforms are unknown. Certainly, reversible phosphorylation and acetylation are known to modulate the function of other MYB transcription factors and MYBs are increasingly understood to operate as part of multi-protein complexes along with other elements such as co-activators (Vorbrueggen et al., 1996; Colgin and Nyborg, 1998; Wong et al., 1998; Tomita et al., 2000). It can readily be inferred that there are multiple players in the aleurone GA-signalling pathway leading to α-amylase expression which remain to be identified.

The experimental work described in this doctoral thesis is designed to address some of the deficiencies in our understanding of GA-signalling in cereal aleurone through a new gene discovery thrust centred on the GAMYB transcription factor. Proteomics technologies such as the yeast-two hybrid system are proving useful tools for identifying new components in signal transduction pathways. Consequently, an experimental approach utilising a yeast two-hybrid system was devised with the goal of isolating aleurone GAMYB-binding proteins and determining their function in GA-signalling. The results and significance of this work are described in the following chapters.

Chapter 2

General Materials and Methods

(2.1) Plant materials

Barley vegetative and floral tissues were obtained from *Hordeum vulgare* L. cv Himalaya plants grown in natural light in the CSIRO Plant Industry Phytotron in Canberra, Australia. Daylight was extended to 16 h with incandescent light and a temperature control regime based on a sine function oscillating between 17°C and 9°C was used. Himalaya grain was obtained from 1985 and 1998 harvests, Department of Agronomy, Washington State University. Grain from the *Sln1d* Himalaya mutant (kindly provided by P. Chandler, CSIRO, Canberra) was obtained from plants grown in the Phytotron as described above. Isolated aleurone layers were obtained by surface sterilising embryo-less half seeds in 1 % w/v sodium hypochlorite pH 6.0, imbibing seeds on filter paper soaked in 5 mL of sterile water for three days and stripping away the starchy endosperm.

(2.2) Isolation of total RNA and mRNA from barley tissues

Aleurone layers were isolated as described above and incubated in groups of 10 for six hours at 25°C in 2 mL of incubation medium containing 10 mM $CaCl_2$, 50 units/mL nystatin and 150 µg/mL cefotaxime plus hormone as required. Unless otherwise specified GA_3 was used at a concentration of 10^{-6} M and ABA was used at a concentration of 5×10^{-5} M. Layers were then frozen in liquid nitrogen. Vegetative and floral tissues were harvested directly from the plant and frozen in liquid nitrogen. RNA was isolated from these tissues by grinding 20 aleurone layers or approximately 1 g of floral or vegetative tissues with acid-washed sand to a fine powder in liquid nitrogen in a mortar and pestle. Powder was vortexed vigorously in 1.3 mL NTES containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.1% SDS along with 2.5 mL phenol:chloroform:iso-amylalcohol (25:24:1). Samples were then centrifuged at 5000 x *g* at 4°C for 10 min. The supernatant was re-extracted with 2 mL phenol:chloroform:iso-amylalcohol (25:24:1) and nucleic acids were precipitated from the resulting supernatant by the addition of a 10% volume of 3 M sodium acetate and two equal volumes of absolute ethanol, followed by incubation for 16 h at –20°C. Nucleic acids were pelleted by centrifugation at 16 000 x *g* for 30 min at 4°C and washed with 70% ethanol. Pellets were resuspended in 700 µL of dH_2O and RNA was

precipitated by the addition of an equal volume of 4 M LiCl and 5 mM EDTA followed by incubation for 16 h at 4°C. RNA was pelleted by centrifugation at 21 000 x *g* at 4°C and samples were washed with 70% ethanol and solubilised in 30 µL dH_2O. Insoluble material was removed by centrifugation of the resuspension at 14 000 x *g* at 4°C for 10 min. For experiments requiring the isolation of mRNA, milligram quantities of total aleurone RNA were applied to Oligo(dT)-cellulose columns (Pharmacia Biotech, catalogue no. 27-9258-01) and bound mRNA was purified from columns according to the manufacturer's instructions.

(2.3) General molecular procedures

In general, standard molecular biology procedures as described in Sambrook and Russell (2001) were used for the manipulation of DNA and generation of constructs. The salient details of these methods are noted in the sections below. At the beginning of each results chapter further details are provided about the assembly of specific constructs.

(2.3.1) Generation and cloning of gene specific DNA fragments

Target DNA fragments were amplified with primers encoding unique restriction sites using the polymerase chain reaction (PCR). Typically, the high fidelity DNA polymerases *Pfu*Turbo (Stratagene) or Platinum *Pfx* (Gibco-BRL) were used. Ampli*Taq* gold (Perkin-Elmer) DNA polymerase was used when high fidelity amplification was not required. Thermocycling typically consisted of 35 cycles preceded by a 5 min pre-incubation at 95°C and finishing with a 2 min supplementary extension period. Each cycle incorporated a 30 s 95°C denaturing step, a 20 s annealing step and a 1min/kb extension time at 68°C or 72°C (for *Pfx* or *Pfx* and *Taq* respectively). The annealing temperature of reactions was optimised on a temperature gradient and 10% v/v DMSO was used to increase yields and specificity from GC-rich templates. In a typical reaction, the commercial polymerisation buffer was used and $MgCl_2$, primers and dNTPs were used at final concentrations of 1.5 mM, 200 µM and 2 pmol/µL respectively along with 10 ng of plasmid template. The resulting PCR products were cloned into pBluescript SK(+) (Stratagene) and subcloned into other

vectors using the methods recommended by Sambrook and Russell (2001). Restriction endonucleases and other DNA modifying enzymes were sourced from MBI (Fermentas). Plasmids were maintained in the XL1-Blue strain of *E. coli* (Stratagene) and were transformed into this strain via electroporation essentially as described in Sambrook and Russell (2001). Construct assembly was confirmed by sequencing and sequencing reactions were carried out by the dideoxynucleotide chain-termination method using the Big Dye terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Perkin Elmer) according to the manufacturer's instructions.

(2.3.2) Isolation of full length cDNAs

An aleurone tissue cDNA library in Lambda ZAP II from water-imbibed *Hordeum vulgare* L. cv Himalaya embryo-less half seeds (Stratagene catalogue no. 936003) and containing 2 x 10^6 independent clones was plated at a density of 50 000 pfu per 150 mM plate and lifted onto Hybond-N^+ filters (Amersham). DNA was UV cross-linked to filters and denatured in a solution containing 1.5 M NaOH and 1.5 M NaCl followed by a neutralisation step in 0.5 M Tris-HCl, pH 8.0 containing 1.5 M NaCl. Filters were prehybridised for 4 h at 65°C in modified Southern Buffer (MSB) containing 3 x SSC (0.045 M NaCl, 0.045 M sodium acetate), 0.2 % w/v Ficoll 400, 0.2 % w/v polyvinylpyrrolidone, 1% w/v BSA, 1 % w/v HEPES buffer and 20 µg/mL denatured salmon sperm DNA. Filters were hybridised with random primed radioactive probes in MSB at 65°C for 16 h. Probes were produced using the NEN extension labelling system (catalogue no. NEP103L) according to the manufacturer's instructions. Filters were washed twice for 30 min at 65°C in 2 x SSC and 0.1 % w/v SDS and once in 0.1 x SSC and 0.1% w/v SDS at 65°C for 30 min. Screens were repeated at a lower plaque density to isolate individual plaques hybridising to probes. The pBluescript SK(-) plasmids of interest were excised from Lambda ZAP II clones using the ExAssist/SOLR system (Stratagene catalogue no. 200253) according to the instructions of the manufacturer.

(2.3.3) RNA gel blot analysis

RNA samples were diluted in 5 x loading buffer containing 0.005% w/v bromophenol blue, 4 mM EDTA, 2.6 % formaldehyde, 20% v/v glycerol, 30% v/v formamide and 4 x MOPS buffer (80 mM MOPS, 20 mM sodium acetate, 4 mM EDTA, pH 7.0). Typically, 5 μg RNA samples were preincubated at 65°C for 5 min then separated by electrophoresis at 100 V for approximately 60 min on a 1% agarose gel containing 0.8% formaldehyde, 0.1 μg/mL ethidium bromide and 1 x MOPS buffer. Gel running buffer consisted of 1 x MOPS and 0.8% v/v formaldehyde. RNA was blotted to Hybond N filters (Amersham) according to the manufacturer's instructions. Radioactive complementary riboprobes were generated by linearising the gene of interest in pBluescript SK (+) at its 5' end and performing an *in vitro* transcription reaction using T7 or T3 DNA polymerase in the presence of α^{32}P- UTP. A total of 1 μg of template was used and reaction components were derived from the MBI (Fermentas) *in vitro* Transcription Kit (catalogue no. KO411). The manufacturer's protocol was used. Random primed radioactive DNA probes were produced using the NEN extension labelling system (catalogue no. NEP103L). For riboprobes, filters were pre-hybridised in 50% v/v formamide, 0.25 M phosphate buffer, 0.25 M NaCl, 1 mM EDTA and 7% SDS at 55°C for 4h and hybridised for 16 h at 55°C in freshly made probe diluted in pre-hybridisation solution. For DNA probes, filters were pre-hybridised for 7 h at 65°C in 5 x SSC, 0.1 % w/v Ficoll 400, 0.1 % w/v PVP (polyvinylpyrrolidone), 0.1% w/v BSA, 0.5% w/v and SDS 300 μg/mL denatured salmon sperm DNA then hybridised for 16 h at 65°C in freshly made probe diluted in pre-hybridisation solution. All filters were washed three times for 10 min in 2 x SSC and 0.1% SDS at 65°C and twice for 10 min in 0.1 x SSC and 0.1% SDS at 65°C. Blots were exposed to film at –80°C.

(2.4) Yeast strains and manipulations

(2.4.1) Composition of growth media

YPD media – 1% w/v yeast extract (DIFCO), 2% w/v bacto peptone (DIFCO), 2 % w/v D-glucose.

Ynb -glucose media: 2% (w/v) glucose, 0.5% (w/v) $(NH_4)_2SO_4$, 0.17% (w/v) yeast nitrogen without amino acids (DIFCO).

Ynb-galactose media: 3% (w/v) D-galactose, 2% (w/v) D-raffinose, 2% (w/v) glycerol and 0.17% (w/v) yeast nitrogen without amino acids (DIFCO).

Amino acid supplements – amino acid supplements giving rise to final concentrations of 50 μg/mL each of leucine, uracil, tryptophan, methionine, lysine, adenine and histidine were added to the Ynb- solutions to generate minimal media. For plasmid selection on the basis of nutritional dependency, the corresponding amino acid was omitted from the amino acid supplement.

Plates - bacto-agar (DIFCO) to 4% w/v was used to ensure good replica plating.

(2.4.2) Growth conditions

The *S. cerevisiae* temperature sensitive strain 352-15A2 (*MATα, ade5, cdc25-5, his7, lys2, met, trp1, ura3052*) referred to as *cdc25-2* was used in all experiments (a gift from A. Aronheim, Dept Genetics, Rappaport Faculty of Medicine, Technion, Israel). The *cdc25-2* line was cultured using YPD media at the permissive temperature of 25°C. Plasmid transfected lines were grown using either Ynb-glucose or Ynb–galactose medium supplemented with the appropriate amino acid drop-out mixture. These lines were grown at either the permissive temperature or restrictive temperature (36°C), depending on experimental requirements.

(2.4.3) Transfection of yeast with DNA

The transfection protocol used is a modified version of the lithium acetate TRAFO method (Gietz and Schiestl, 1995). For a typical plasmid transfection, cells were grown at 24°C in 200 mL of YPD to a density of 2-10 x 10^6 cells/mL and harvested by centrifugation. Cells were washed 3 times in LISORB – 10 mM lithium acetate, 1 M sorbitol, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Cells were resuspended in 3 mL of LISORB and rotated at room temperature for 30 min. A 200 μL aliquot of competent cells was added to a tube containing 2 μg of each plasmid plus 100 μg of

denatured salmon sperm DNA. A 1.2 mL aliquot of LIPEG containing 40% PEG3350, 100 mM lithium acetate, 10 mM Tris-HCl, pH 8.5, and 1 mM EDTA was added to the transfection mixture and samples were rotated at room temperature for 30 min then heat shocked at 42°C for 10 min. Following the heat shock, cells were harvested by centrifugation then plated in 150 µL of 1 M sorbitol on the appropriate Ynb- selective medium. For transfection of libraries, a *cdc25-2* line containing the pRAS-GAMYB expression plasmid (section 3.2) was cultured in 3 mL Ynb glucose minus leucine at 24°C for 24 h then subcultured to 200 mL and grown until the cell density reached approximately 3 x 10^6 cells/mL. Cells were harvested and transferred to 200 mL of YPD and grown for a further 3-5 hours to allow another two doublings. Transfections were then carried out as above using 1 µg of the plasmid library per transfection tube.

(2.4.4) Protein extraction from yeast lines

Total yeast protein for protein gel blotting was extracted from a 3 mL culture inoculated with a single colony and incubated for 40 h at 24°C. Proportions of each culture (around 1 mL) corresponding to approximately equal cell numbers were pelleted and cells were lysed by vigorous vortexing in 220 µL of 120 mM Tris-HCl pH 6.8, 2% w/v SDS, 20% v/v glycerol, 0.005% w/v/ bromphenol-blue, 5% β-mercaptoethanol, 1 mM EGTA, 10µg/mL leupeptin, 1µg/mL pepstatin A and 50% v/v 1 mm glass beads. For SDS-PAGE, 10 µL aliquots were boiled for 5 min then centrifuged at 21 000 x *g* for 10 min to remove insoluble materials prior to loading.

(2.4.5) Library screening using the RAS-recruitment system

A yeast *cdc25-2* line containing the pRAS-GAMYB expression construct was generated according to section 2.4.3. Expression of the fusion protein was confirmed by protein gel blotting according to the procedure described in section 2.5. The bait-expressing line was transfected with cDNA-expression libraries (section 3.2) according to section 2.4.3 until enough transformants were generated to encompass two to three times the size of the library. Primary transformants were grown on Ynb glucose minus leucine and uracil for 3-4 d at 25°C then replica plated to the equivalent galactose medium and grown for a further 7-10 days at the restrictive temperature of 36°C, until a

strong background of revertants emerged. Colonies were picked progressively and arrayed on Ynb glucose –leu, -ura plates, grown for a further 2 d at 25°C then replica plated to both Ynb-glucose and Ynb-galactose –leu, -ura to test for galactose dependent growth at 36°C. Clones were tested twice for galactose dependent growth.

(2.4.6) Recovery of plasmids from yeast lines

To extract plasmids from yeast lines transformed with library plasmids, 3 mL Ynb glucose -ura cultures were inoculated with a single colony and grown for 48 h at 24°C. Cells were pelleted and washed with 1 mL of dH_20 then vortexed vigorously for 5 min in 100 µL 8% sucrose, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% v/v Triton X-100 plus 0.2 g of 0.45 mm acid-washed glass beads (Sigma). A further 100 µl of buffer (without beads) was added and samples were mixed and boiled for 3 min. Insoluble material was removed by centrifugation at 21 000 x *g* for 10 min at 4°C. Ammonium acetate to a final concentration of 0.3 M was added and the sample was incubated at -20°C for 1 h before insoluble material was removed as above. Plasmid was precipitated from the supernatant by the addition of a 2x volume of 100% ethanol. The pellet was washed and resuspended in 20 µL of dH_2O. A 1 µL aliquot was used to transform XL1-Blue *E.coli* (Stratagene) by electroporation and plasmids in the resulting clones were sequenced.

(2.5) Protein gel blotting

Denaturing SDS-PAGE (SDS-polyacrylamide gel electrophoresis) was carried out according to Laemmli et al., (1970) and protein was blotted to a polyvinylidene fluoride membrane (PVDF) using a standard semi-dry procedure in a discontinuous buffer system. Membrane was blocked for 16 h in 1 x TBST containing 30 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20 plus 5% skim milk powder. It was then incubated for 1 h with an affinity purified polyclonal antibody raised in rabbits against amino acids 300-553 of barley GAMYB (kindly provided by F. Gubler, CSIRO, Canberra), diluted 1:1000 in 1 x TBST containing 1% w/v BSA. Membrane was washed 4 x 15 min in 1 x TBST. An anti-rabbit IgG secondary antibody conjugated to horse-radish peroxidase raised in donkey (Amersham) was used at a dilution of 1:10 000.

Incubation and washes were the same as those used for the primary antibody. Immunoreactive bands were resolved by chemiluminescence (NEN detection kit) according to the manufacturer's instructions.

(2.6) Transient expression assays

Preparation of barley half grains (*Hordeum vulgare* L. cv Himalaya) for particle bombardment and precipitation of plasmid DNA onto gold preparations was conducted essentially as described in Gubler *et al.* (1995 and 1999; for details of particular effector and reporter constructs, see the relevant sections in chapter 4 and chapter 5). Embryo-less half seeds were surface sterilised in 1 % w/v sodium hypochlorite and imbibed for two days on filter paper soaked in 7 mL of dH_2O after which the testa and pericarp were removed. A total of 2-2.5 μg of effector (balanced by Ubi1-cassette if necessary) and 1 μg of the reporter construct was precipitated onto 1.5 mg of gold beads suspended in 25 μL of 50% v/v glycerol by continuous vortexing of the DNA-gold mixture followed by addition of 25 μL of 2.5 M $CaCl_2$ and 20 s later by 10 μL of 1 M spermidine. In each preparation, 1 μg of a construct consisting of the luciferase reporter gene fused to the maize ubiquitin promoter was included as an internal standard (pACH18; Lanahan et al., 1992). Gold preparations were shaken vigorously for 20 min at room temperature, pelleted and washed with 70% ethanol. The final pellet was resuspended in 20 μL of 70% ethanol. A 6 μL aliquot of each gold preparation was used to shoot 4-6 prepared half seeds under a vacuum of 70 kPa using a helium gun at a pressure of 1750 kPa. In some experiments the bombarded half grains were cut into two equal quarter grains and incubated with or without 10^{-6} M GA_3 or 5 x 10^{-5} M ABA plus 10 mM $CaCl_2$, 50 units/mL nystatin and 150 μg/mL cefotaxime for 24 h at 25°C. In other experiments where hormonal treatments were not used, the half-grains were uncut and incubated in 10 mM $CaCl_2$, 50 units/mL nystatin and 130 μg/mL cefotaxime.

To assay luciferase and GUS activity, frozen seeds were ground to a paste in 0.5 mL of 0.2 M phosphate buffer, pH 7.8, 5 mM DTT and 1% Triton-X 100 with acid-washed sand. A further 1.5 mL of grinding buffer was added then samples were clarified by centrifugation at 20 000 x *g* at 4°C for 10 min. For GUS assays, a 25 μL aliquot of the

supernatant was added to 100 µL of GUS substrate - 1 mM MUG (4-methylumbelliferyl β-D-glucuronide), 10 mM EDTA, 50 mM sodium phosphate buffer, pH 7.0, 10 mM mercaptoethanol in 20% v/v methanol – and samples were incubated at 37°C for 3 h. Reactions were stopped by the addition of 125 µL 0.8 M sodium carbonate and the amount of fluorescent product was measured in a Labsystems Fluoroskan II (version 4) using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Luciferase activities were assayed by the addition of 100 µL of clarified extract to 100 µL of LAB/ATP buffer containing 30 mM Tris-SO_4, pH 7.7, 20 mM DTT, 20 mM $MgCl_2$, 20 mM EDTA and 2 mM ATP. Samples were assayed in a Lumat LB9501 (Berthold Instruments) according to the manufacturer's instructions using 0.28 mg/ml luciferin (Boehringer), 0.015 M Tris-SO_4, pH 7.7, 10 mM DTT, 10 mM $MgCl_2$ and 10 mM EDTA as the injectible substrate. Each experiment was highly replicated (n>12) and data was normalised on the basis of luciferase activities.

(2.7) Transfection of aleurone protoplasts with DNA

The method was adapted from that of Jacobsen (1985). Protoplasts were prepared by imbibing 120 barley *Hordeum vulgare* L. cv Himalaya quarter-grains in groups of 20 at 25°C for 16 h in 1.5 ml soaking buffer containing 10 mM arginine, 70 mM $CaCl_2$, 20 mM MES buffer (2-(N-morpholino)ethanesulfonic acid), pH 4.5, 50 units mL^{-1} nystatin and 150 µg/mL cefotaxime. Aleurone layers were stripped from the endosperm and incubated in groups of 20 in 1.5 mL PIM 1/1 solution containing 10mM arginine, 360 mM KCl, 90 mM $CaCl_2$, 20 mM MES, pH 4.5, and 1 % w/v PVP (polyvinylpyrrolidon) plus 4.5 % w/v Onazuka cellulase (Yakult Honsha Co.) for 24 h at 25°C after which cellulase medium was replaced with fresh solution and incubation continued for a further 18 h. Protoplasts were released by gentle swirling of flasks and manipulation of layers, which were then allowed to settle for 1 h at room temperature. The supernatant was removed and cells were washed in 20 mL of PIM 1/1 and resuspended in 5 ml PIM 1/1. Protoplasts were applied to a gradient consisting of 20 ml of 0. 4 x PIM1/1 plus 40% v/v percoll layered on 6 ml of 2.5 M sucrose. The gradient was centrifuged at 400 x g for 10 min and protoplasts were recovered from the top of the sucrose phase, diluted in 20 mL of PIM 1/1 and harvested by centrifugation at 50 x g for 5 min. Protoplasts were resuspended in 20 mL PIM 1/2 containing 10 mM arginine, 90 mM

Ca Cl_2, 180 mM KCl and 20 mM MES pH 4.5 and allowed to settle for 30 min. The supernatant was removed and protoplasts resuspended in 10 ml IM solution containing 55 mM sucrose, 14 mM arginine, 10 mM MES pH 4.5, 0.32% w/v Gamborg salts.

Protoplasts were harvested as above by centrifugation and resuspended in 3.3 mL IM solution. For each transfection, 1 mL of resuspended protoplasts was incubated for 20 min at room temperature with 25 μg of the target DNA construct plus 100 μg denatured salmon sperm DNA in 3.6 mL of PTM solution containing 670 mM mannitol, 130 mM $Ca(NO_3)_2$, 10 mM Tris-HCl pH 9.0 and 17% w/v PEG6000. A total of 40 mL of IM solution was added to the transfected protoplasts in 10 mL aliquots interspersed with two minute equilibration periods. Protoplasts were harvested by centrifugation and washed twice in 20 mL of IM solution. Finally transfected protoplasts were resuspended in 3.5 mL of IM solution plus 20 mM $CaCl_2$, 50 units/mL nystatin and 150 μg/mL cefotaxime and incubated for 16 h at 25°C.

(2.8) Sequence Analysis and Database Searching

The GenBank database was searched with protein and DNA sequences using the BLAST program (Altschul et al., 1990) on the National Center for Biotechnology on-line service. Protein sequences were analysed using the Simple Modular Architecture Research Tool (SMART) on the European Molecular Biology Laboratory on-line service (Schultz et al., 2000) or the Prosite tool in the Expert Protein Analysis System (ExPASy) on the Swiss Institute of Bioinformatics on-line service (Hoffman et al., 1999). Multiple protein sequence alignments were generated using a ClustalW1.8 program at the Baylor College of Medicine, Search Launcher on-line service (http://searchlauncher.bcm.tmc.edu).

Chapter 3 – Results 1

Use of a novel yeast two-hybrid system to explore gibberellin signalling in aleurone

(3.1) Introduction

The transmission of information through protein-protein interactions is central to the functioning of signalling pathways. Consequently the isolation of protein-binding partners for a known signal transduction component can reveal new and interesting components in a pathway. This approach to mapping signalling pathways has potential advantages over the more traditional approach of screening for signal transduction-deficient mutants, where lethal gene-disruption phenotypes and gene redundancy can render some signalling components invisible. As described in Chapter 1, the GAMYB transcription factor has a relatively well characterised function in GA-signalling in aleurone but much more remains to be understood about the pathway in which it operates. Accordingly, a strategy was devised for the isolation of GAMYB binding partners in order to generate candidate GA-signalling factors for further study.

A wide variety of techniques exist for the detection of protein-protein interactions (for a review see Phizicky and Fields, 1995). In general the biochemical methods involve the physical isolation of protein-complexes and the characterisation and identification of the separated components. However, the identification of peptide fragments from complexes does not immediately reveal the encoding genes in organisms for which no genome sequence is available. Also, many biochemical methods model protein-protein interactions outside intact cellular environments, limiting the possibility for detection of less stable complexes such as those consisting of transiently interacting factors which might be expected to constitute many of the protein-complexes in a signalling pathway. A number of alternative methods exist which do model protein-protein interactions inside cells and which also facilitate direct identification of genes encoding proteins of interest.

To date, the most developed system for high-throughput screening of protein-protein interactions inside cells is the yeast two-hybrid system (for a review see Brachmann and Boeke, 1997). The conventional yeast two-hybrid system (Fields and Song, 1989) relies on the modular structure of yeast transcription factors and proteins of interest are expressed as nuclear-directed fusions with the DNA-binding domain or transactivation domain of a yeast transcription factor. High throughput screening of cDNA libraries

for interacting proteins is possible and protein-protein interactions are monitored via the reconstitution of the activity of the yeast transcription factor, which transactivates the promoter of a reporter-gene construct.

Despite its many successful applications the conventional yeast two-hybrid system is unsuitable for studies of proteins such as GAMYB which have an inherent transactivating capability and for factors such as membrane proteins which are unable to fold correctly in the nucleus. Partly in response to this problem, a number of alternative two-hybrid systems have been developed (for a review see Brachmann and Boeke, 1997). The ubiquitin based split-protein sensor system (Johnsson and Varshavsky, 1994) tethers protein interactions in yeast to the reconstitution of a split ubiquitin moiety at a site in the cell where the proteins of interest would normally be expressed. The ubiquitin-linked interactions are monitored through the concomitant cleavage of a reporter protein which is detected by immunoassay, however this detection method is arguably unsuitable for routine use in high-throughput screens. A high-throughput bacterial two-hybrid system exists which tethers interacting proteins to the reconstitution of an adenylate cyclase activity linked to selective growth phenotypes through cAMP regulated gene expression (Karimova et al., 1998). A bacterial system would not be generally applicable to the modelling of eukaryotic protein interactions but the technology for a mammalian two-hybrid system does exist whereby fusions between proteins of interest and separable modules comprising a β-galactosidase activity are co-expressed in the native proteins' cellular compartments (Rossi et al., 1997).

In another yeast two-hybrid alternative, the RAS-recruitment system (RRS; Broder et al., 1998) and its progenitor, the SOS-recruitment system (SRS; Aronheim et al., 1997), protein interactions are monitored in a temperature sensitive yeast mutant which possesses a defective RAS-guanylnucleotide exchange factor (CDC25) ordinarily involved in plasma membrane signalling. In the RRS, potential protein-binding partners ('prey'), which can be encoded by cDNA libraries, are localised to the inner face of the plasma membrane. 'Bait proteins' are fused to the activated mammalian-RAS (mRAS) protein which can bypass the deficiency in RAS GTP-GDP exchange in the *cdc25* mutant provided it is membrane localised (Ballester et al., 1989).

Consequently, protein-protein interactions between bait and prey facilitate the recruitment of mRAS to the plasma membrane and permit growth of *cdc25-2* at the restrictive temperature.

For the study of transcription factors and lipophilic proteins, the RRS presents significant advantages over the conventional yeast two-hybrid system. This is because protein-protein interactions are not monitored on the basis of a transcriptional readout, which can be falsely activated by transcription factor baits. Also, interactions occur at the cytosolic face of the plasma membrane rather than in the nucleus, arguably a more favourable environment for the assembly of protein complexes containing lipophilic components. These advantages have been demonstrated with the detection of novel interactions between components of mammalian transcriptional complexes and plasma membrane-associated signalling complexes using the RRS (Aronheim et al., 1998; Takemaru and Moon, 2000). The RRS was therefore deemed the most useful system for high throughput screening of potential GAMYB binding proteins.

In this chapter, the results obtained from screens of barley aleurone cDNA expression libraries for GAMYB-binding partners using the RRS are described. It was found that with some adjustments, the system efficiently produced a sizeable number of interesting clones from quite modest libraries, including potential signalling factors and transcriptional regulators. Several factors were selected for further functional characterisation.

(3.2) Materials and methods: assembly of constructs used in the RAS-recruitment system

1. pRAS-GAMYB (bait construct)

The GAMYB ORF was amplified by PCR using the following primers – forward: 5' ATG AAT TCT GAT GTA CCG GGT GAA GAG CGA; reverse: 5' TAG AAT TCA TTT GAA CTC CTC CGA CAT TTG. The resulting PCR product was cloned into the pYESRAS(61)ΔF vector (described in Broder et al., 1998 and kindly provided by A. Aronheim) as an *Eco*RI fragment to generate a fusion with the carboxy- terminus of the

RAS protein. The resulting RAS-GAMYB cartridge was subcloned into the yeast expression vector pADNS (Colicelli et al., 1989) as a *Hind*III-*Not*I fragment.

2. pMyr-GBPs (prey constructs)

Two cDNA-expression libraries were constructed from 5 μg of poly(A^+) RNA derived from barley (*Hordeum vulgare* L. cv Himalaya) aleurone layers treated for 6 h at 25°C with 10^{-6} M GA or 5 x 10^{-5} M ABA. The +GA-library was generated from oligo-dT primed cDNA (Gibco-BRL cDNA synthesis kit) ligated to *Eco*RI containing adaptors and cloned directionally as 5' *Eco*RI-*Xho*I fragments into the pMyr vector (Stratagene) to produce fusions at the carboxy-terminus of the v-Src myristoylation sequence. The construct is driven by a galactose-inducible promoter. The +ABA-library was generated from random-primed, *Eco*RI-adapted cDNA (Stratagene cDNA synthesis kit) and cloned non-directionally into the pYESM vector (kindly provided by A. Aronheim and described by Aronheim et al., 1997) to generate a myristoylation-signal fusion, as above. Plasmid libraries were transformed by heat shock into the ultra-competent cell line XL10-gold (Stratagene) according to the manufacturer's instructions. The +GA-library contained 2 x 10^5 independent clones while the +ABA-library contained 1 x 10^5 independent clones. The clones contained in the libraries were collectively termed pMyr-GBPs (for myristoylated- potential GAMYB binding proteins).

(3.3) Results and Discussion

(3.3.1) Isolation of putative GAMYB-binding proteins from aleurone cDNA libraries

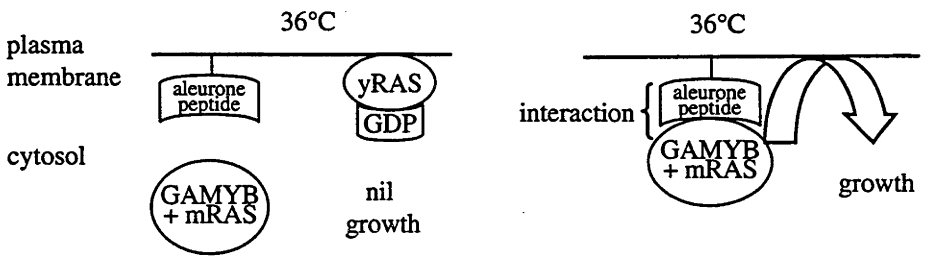
A schematic representation of how the RRS was used to screen aleurone cDNA-expression libraries for GAMYB-binding proteins is shown in Figure 3.1 (for details of procedures see section 2.4). To summarise, this approach utilises a membrane signalling mutant, *cdc25-2,* and interactions between galactose inducible, GAMYB-binding partners tethered to the plasma membrane and the GAMYB portion of a mRAS-GAMYB fusion results in recruitment of activated mammalian RAS (mRAS) to the plasma membrane. Plasma membrane localised mRAS is able to complement the *cdc25-2* mutation, giving rise in this system to a galactose-dependent growth phenotype

Figure 3.1. Use of the RAS-recruitment system (RRS) to screen aleurone cDNA libraries for GAMYB-binding proteins.

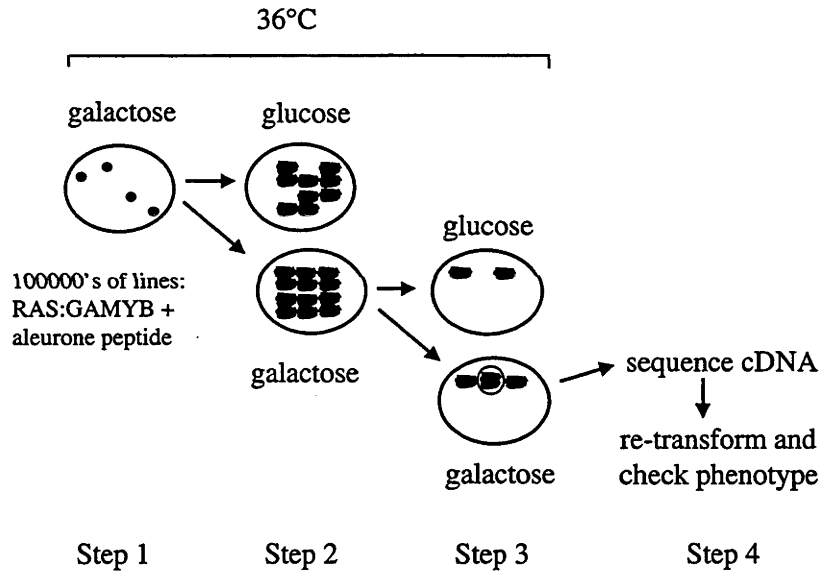
(A) In the absence of interactions between GAMYB and a membrane tethered, aleurone peptide (pMyr-GBP, encoded by the cDNA library), the *cdc25-2* mutant is unable to grow at the restrictive temperature (left panel). Interactions between the GAMYB portion of the GAMYB-RAS fusion and the aleurone peptide results in mRAS recruitment to the plasma membrane which promotes growth of the mutant at the restrictive temperature.

(B) Outline of the four-step process used to screen aleurone cDNA libraries for GAMYB-binding proteins. The *cdc25-2* lines constitutively expressing RAS-GAMYB and a galactose-inducible, myristoylated aleurone peptide were selected for growth on galactose at the restrictive temperature and then tested twice for galactose-dependency. The cDNA in each galactose-dependent line was isolated and sequenced and interesting clones were re-transformed with RAS-GAMYB into *cdc25-2* to confirm the phenotype.

A


plasma
membrane
cytosol
36°C
aleurone peptide
yRAS
GDP
GAMYB + mRAS
nil growth
36°C
interaction
aleurone peptide
GAMYB + mRAS
growth

B


36°C
galactose
glucose
100000's of lines: RAS:GAMYB + aleurone peptide
glucose
galactose
galactose
sequence cDNA
re-transform and check phenotype
Step 1
Step 2
Step 3
Step 4

at the restrictive temperature of 36°C. Screens of aleurone cDNA expression libraries for GAMYB binding proteins were conducted in four steps. Firstly, a *cdc25-2* line expressing RAS-GAMYB was transformed with aleurone cDNA-expression libraries encoding cDNA-myristoylation fusions. Enough transformants to encompass twice the size of each library were generated and growth of primary transformants at the restrictive temperature was selected for on galactose media. In the second and third phases the galactose-dependency of growth at the restrictive temperature for all promising lines was tested twice. In the fourth and final phase, plasmids encoding promising myristoylation-cDNA fusions were retransformed into a *cdc25-2* line expressing RAS-GAMYB to confirm the phenotype along with a variety of control plasmids to test the specificity of the interaction.

The expression of the RAS-GAMYB fusion construct was verified by probing total protein extracts from *cdc25-2* cells transformed with pRAS and pRAS-GAMYB with a GAMYB antibody (see sections 2.4.4 and 2.5). As shown in Figure 3.2A, an immunoreactive product of approximately 75 kDa, which was not expressed in the pRAS-transformed cells, was detected in the pRAS-GAMYB-transformed lines. This represents an increase in molecular mass of approximately 20 kDa compared to the endogenous GAMYB protein from GA-treated aleurone and equates to the approximate shift in size predicted for a protein fused to the 200 aa RAS protein. The quality of the aleurone cDNA libraries was assessed by profiling the size and numbers of inserts in 16-18 clones from each library (Figure 3.2B). The +GA-library, comprising 2×10^5 independent clones, possessed an average insert size of ~1 kb while the +ABA-library, comprising 1×10^5 independent clones, contained a smaller average insert size of ~ 0.7 kb due to the use of random primers. Over 90% of clones in each library contained inserts.

Initially, the screen of cDNA-expression libraries from hormonally treated aleurone for GAMYB-binding factors generated 32 promising clones. The 32 lines of interest exhibited a distinct galactose-dependent growth phenotype at 36°C after 5-7 days but were generally significantly slower growing than the positive control lines. The positive control lines were transfected with vectors encoding the known mammalian interactors c-Jun and JDP2, fused to the mRAS and myristoylation signals respectively

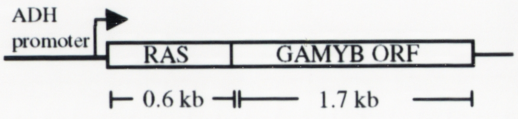
Figure 3.2. Analysis of 'bait' and 'prey' expression constructs used to screen for GAMYB-binding partners in the RAS-recruitment system.

(A) Constitutive expression of the RAS-GAMYB bait fusion is driven by the yeast alcohol-dehydrogenase (ADH) promoter in a pADNS-based vector *(i)* and was confirmed in yeast *(ii)* by probing a blot of total protein from GA-treated aleurone (lane 1) and from *cd25-2* transformed with pRAS (lane 2) and pRAS-GAMYB (lane 3) with GAMYB antibody.

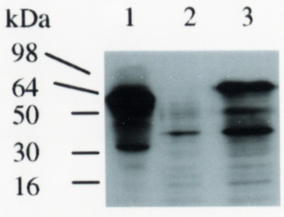
(B) The population of cDNAs from hormonally treated aleurone were cloned as fusions with the carboxy-terminus of the v-Src myristolyation sequence (*pMyr-GBP*, for potential GAMYB binding partner) under the control of a galactose inducible promoter in a pYES2-based vector *(i)*. The cDNAs from a subpopulation of each library were excised using the listed restriction enzymes and electrophoresed on a 1% agarose, 1 x TBE gel stained with ethidium bromode *(ii)*.

A

(i) *pRAS-GAMYB*

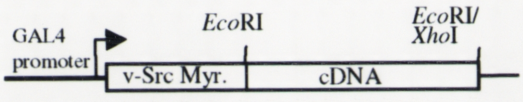


(ii)

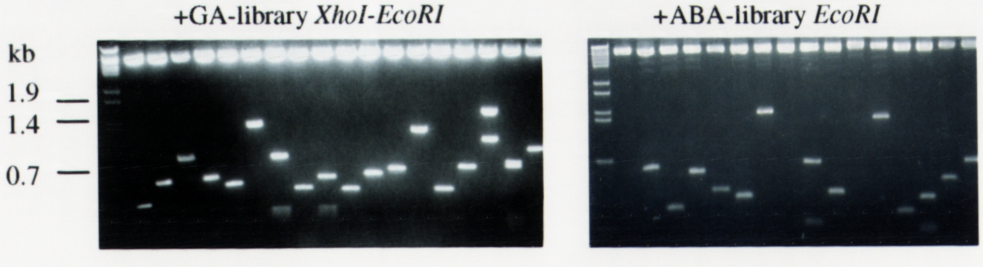


B

(i) *pMyr-GBP*



(ii)



(kindly provided by A. Aronheim and described in Aronheim et al., 1997) and these lines formed robust galactose-dependent colonies after 2-3 d at 36°C. One explanation for this phenomenon may be that the RAS-GAMYB fusion inhibits full mRAS function due to sub-optimal folding of the fusion protein, or that the bait and prey factors were expressed in yeast at relatively low levels. Another feature of the 32 clones of interest is that they were obtained exclusively from the +ABA-library. This may relate in part to the fact that this library was produced from random-primed rather than oligo-dT primed cDNA, and so may have presented a better range of GAMYB-binding epitopes.

The cDNA insert in the prey-expression vector in each of the 32 galactose-dependent lines was sequenced (see appendix) and the deduced in-frame protein sequence was compared with the contents of the gene databases. Features of each of the deduced protein sequences are summarised in Table 3.1. Of the 32 clones, 16 were found to be related to known stress or pathogen induced factors and a further 10 were related to metabolic enzymes. These two groups were considered unlikely to represent biologically relevant GAMYB-binding partners. The predominance of stress and pathogen induced factors probably reflects the abundance of these factors in ABA-stimulated tissue. However four of the remaining five types of factors, encoding variously a MAP-kinase like protein, two transcriptional regulator-like proteins and a novel WD40 protein were considered strong contenders for further study as GAMYB-binding partners functioning in GA-signalling. The histidine rich factor, clone 2, was eliminated from consideration as it aligned out of frame with a predicted protein in the *Arabidopsis* genome database. The expression plasmids encoding the four factors of interest, clones 1, 3, 4 and 5, were re-transformed into *cdc25-2* along with a variety of controls to test the specificity of the galactose-dependent complementation. As shown in Figure 3.3, only yeast lines transformed with plasmids encoding both the myristoylated-cDNA fusion and the RAS-GAMYB fusion showed galactose-dependent growth at the restrictive temperature.

(3.3.2) Isolation of full length cDNAs for clones of interest

In order to discover more about the four clones of interest, a larger lambda-based barley aleurone cDNA library (see section 2.3.2) containing approximately 2 x 10^6

Clone	Features of deduced protein*	Potential functional role in barley aleurone	No. of times clone was represented in screen**
1	66% (88%) similarity to Rat Mitogen Activated Kinase (MAK) – MAP kinase-like factor, GenBank acc. no. M35862	Signalling	2 (1)
2	Novel – deduced protein is histidine rich and hydrophilic	Unknown	1 (1)
3	Novel WD40 protein	Signalling?	1 (1)
4	Novel – full length protein contains a BTB/POZ domain and leucine acidic motifs common in types of transcriptional regulator	Transcriptional Regulation	1 (1)
5	38% (61%) similarity to human nuclear coactivator 'SKIP' GenBank acc. no. HSU51432	Transcriptional Regulation	1 (1)
6	Encodes barley chitinase – GenBank acc. no. HVU2287	Plant protection	7 (3)
7	73% similarity to rice thaumatin, pathogenesis/stress related protein, GenBank acc. no. OSU77656	Plant protection/ abiotic stress responses	3 (2)
8	Encodes barley glycine rich protein elicited by fungi/cold GenBank acc. no. Z48265	Plant protection/ cold stress response	4 (1)
9	95% similarity to wheat pathogen induced secretory protein, GenBank acc. no. AF079526	Plant protection	1 (1)

* amino acid identities are listed as percentages and amino acid similarities, when functional substitutions are considered, are listed afterwards in brackets

**figure in brackets represents number of independent cDNAs obtained for each type of clone

Table 3.1. Catalogue of putative GAMYB-binding proteins isolated from aleurone cDNA expression libraries using the RRS.

Clone	Features of deduced protein*	Potential functional role in barley aleurone	No. of times clone was represented in screen**
10	74% similarity to cotton callose synthase, GenBank acc. no. AF085717	Wounding/fungal response	1 (1)
11	89% similarity to sorghum PEP carboxylase	Metabolism	3 (1)
12	63% similar to rice metallothionein GenBank acc. no. OSU57638	Abiotic stress tolerance	2 (1)
13	94% similarity to maize cystathionine gamma synthase, GenBank acc. no. AF007785	Amino acid synthesis	1 (1)
14	75% similarity to sweet potato purple acid phosphatase, GenBank acc. no. AF200826	Metabolism	1 (1)
15	65% similarity to *Arabidopsis* flavoprotein monooxygenase, GenBank acc. no. ATH7588	Metabolism	1 (1)
16	Encodes barley α-amylase, GenBank acc. no. J01236	Starch degredation	1 (1)
17	31% similarity to soybean dihydroxyflavonal reductase, GenBank acc. no. AF202182	Metabolism?	1 (1)

* amino acid identities are listed as percentages and amino acid similarities, when functional substitutions are considered, are listed afterwards in brackets

**figure in brackets represents number of independent cDNAs obtained for each type of clone

Table 3.1. (continued) Catalogue of putative GAMYB-binding proteins isolated from aleurone cDNA expression libraries using the RRS.

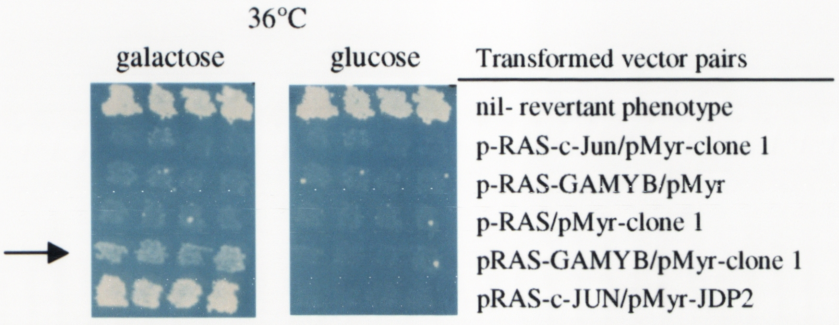


Figure 3.3. Galactose dependent complementation of *cdc25-2* lines transformed with vectors encoding RAS-GAMYB and putative GAMYB-binding proteins from barley aleurone.

Four independently transformed lines are shown. Identical phenotypes were observed for all cDNAs tested (clones 1,3, 4 and 5 from Table 3.1) after growth at 36°C for 7 d. Clone 1 is featured in this example. The *cdc25*-2 lines transformed with constructs expressing the RAS-GAMYB fusion and clone 1 fused to the myristoylation (myr) signal are noted with an arrow. The pRAS-c-Jun and pMyr-JDP2 vectors encode known interacting factors.

independent clones was screened for full-length cDNAs using the corresponding partial yeast-two-hybrid clone as a probe in each case. Although 5 x 10^5 clones were screened, homologues of clones 3 and 5 were rare and only partial cDNAs, extending beyond the 3' coding sequence but not upstream, were identified. A nested PCR-based approach utilising one gene specific and one vector anchored primer was also unsuccessful despite using a variety of barley cDNA-libraries as template (results not shown). Accordingly, further functional characterisation of clones 3 and 5 was not pursued and the features of these clones are instead noted in the following sections. However full length cDNAs were obtained for clones 1 and 4, encoding respectively a novel MAP-kinase like factor and a factor resembling a type of transcriptional regulator known in animals to be involved in chromatin remodelling. A detailed description of clones 1 and 4 is reserved for the beginning of chapters 3 and 4 respectively, where the results of the functional characterisation of these clones are described.

(3.3.3) Features of partial clones, encoding putative GAMYB-binding proteins, which were not pursued

Clone 3: a novel WD40 repeat protein

As shown in Figure 3.4, the putative GAMYB-binding protein encoded by the longest cDNA isolated for clone 3 (see appendix) contains two WD40 repeats in the C-terminal region. The domain formed by these repeats is a conserved protein-protein interaction motif found in a group of proteins with diverse functions, including signalling factors, such as the Gβ subunit of heterotrimeric G proteins and the TFIID subunit of the TATA-box binding complex (for a review see Smith et al., 1999). The WD40 repeat consists of a conserved 44-60 amino acid conserved sequence often flanked by an NH_3-terminal GH motif and a COOH-terminal WD motif. Crystal structures reveal that these repeats form a propeller-like structure which can act as a scaffold for anchoring reversible interactions between multiple proteins (Smith et al. 1999).

The only known full-length relative of the partial protein encoded by clone 3 is an uncharacterised mRNA from *Arabidopsis* (GenBank acc. no. AF360130), which is 64% identical to the barley clone at the amino acid level. A variety of uncharacterised monocot and dicot ESTs including wheat and rice homologues, correspond closely to

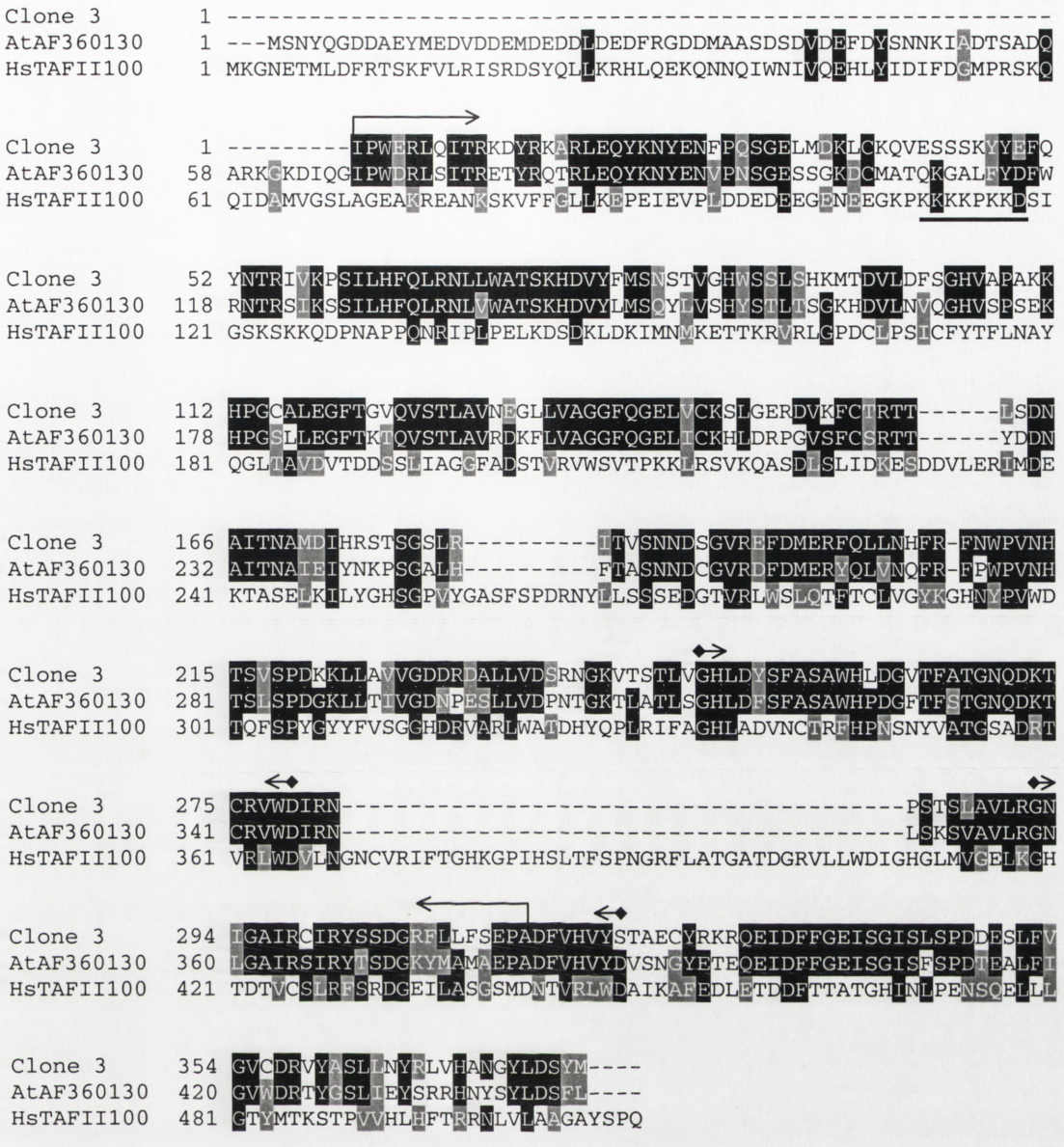


Figure 3.4. Features of the protein encoded by clone 3, a novel WD40 repeat protein, and alignment with a selection of related proteins.

The deduced protein sequence of the longest cDNA isolated for clone 3 is compared with the most closely related full-length plant sequence, an uncharacterised *Arabidopsis* protein (GenBank accession no. AF386950. The delimits of two WD40 repeats found in Clone 3 are marked with diamond-tailed arrows. The human TAFII100 protein (GenBank accession no. HSU80191), a more distantly related, classical WD40 protein, is also aligned for comparison and its nuclear localisation sequence is underlined. The portion of clone 3 corresponding to the original yeast two-hybrid clone is bracketed with arrows.

clone 3, while a number of more divergent barley ESTs have been cloned, suggesting that clone 3 might be part of a gene family in barley. The NH_3-terminus of the *Arabidopsis* homologue of clone 3 is strikingly enriched in charged, aspartic acid residues but the possible functional significance of this feature is unclear, theoretically it might facilitate interactions with other charged factors such as metal ions.

A comparison between clone 3, AF360130, and the human TAFII100 protein, a classical WD40 protein with some limited similarity to clone 3 (Figure 3.4), shows that the plant sequences are most similar to the human protein in their COOH-terminal half but are divergent in the NH_3-terminal region. The nuclear localisation sequence (NLS) typified by the SV40 large tumour antigen (Kalderon et al., 1984) which found is TAFII100, is absent in clone 3. The PSORT cellular localisation algorithm (Nakai, K., http://www.psort.nibb.ac.jp) predicts that the *Arabidopsis* homologue of clone 3 encodes a soluble, non-nuclear protein. With the knowledge that clone 3 (which encodes a near full-length protein) interacts reproducibly and specifically with GAMYB in yeast it is tempting to speculate that clone 3 might function to integrate some upstream event in the GA-signalling pathway involving the modification of GAMYB. In this scenario clone 3 might provide a platform for interactions of GAMYB with some sort of modifier. These ideas remain to be tested.

Clone 5: a homologue of the animal nuclear coactivator 'SKIP'

A search of the gene databases reveals that the longest cDNA obtained for clone 5 encodes a novel plant protein with 38% amino acid identity and 68% similarity (if functionally equivalent substitutions are considered) to the known human transcriptional co-activator, SKIP (for Ski-interacting protein). A small number of uncharacterised plant ESTs exist which correspond closely in sequence to clone 5 but only one full length relative has been identified to date, an *Arabidopsis* mRNA encoding an uncharacterised protein of 56% amino acid identity to clone 5 (GenBank acc. no. AF386950). The features of clone 5 and its relatives are noted in Figure 3.5.

Clone 5 contains a string of basic amino acids corresponding to the SV40-type NLS (Kalderon et al., 1984). The localisation of clone 5 in the nucleus would be consistent with a potential function as a nuclear coactivator. The nuclear coactivators are an

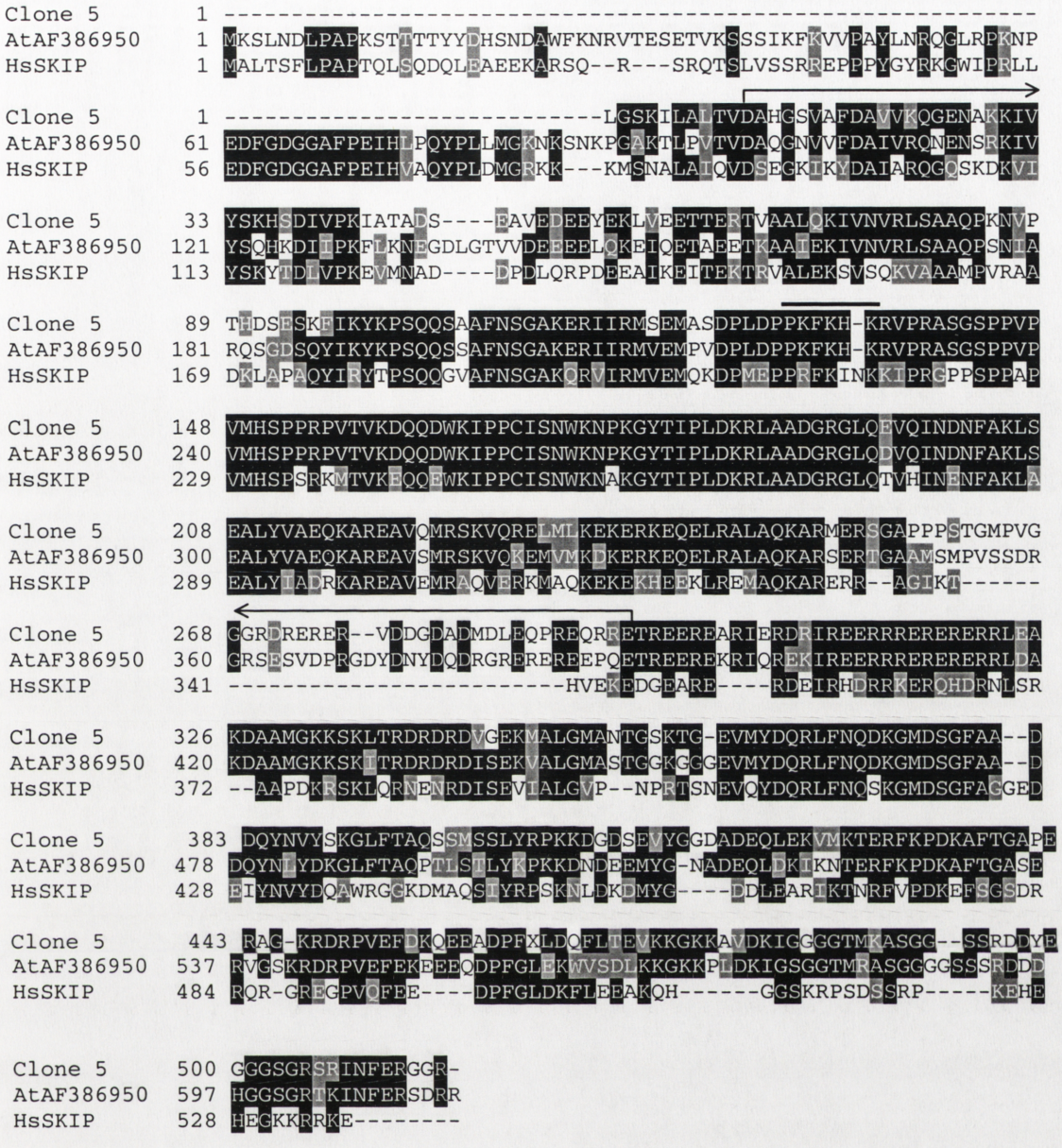


Figure 3.5. Features of the protein encoded by clone 5, a homologue of the SKIP-type coactivators, and alignment with a selection of related proteins.

The deduced protein sequence of the longest cDNA isolated for clone 5 is compared with the most closely related full-length plant sequence, an uncharacterised *Arabidopsis* protein (GenBank accession no. AF386950). The related human co-activator protein, HsSKIP, is also included in this comparison. The portion of clone 5 corresponding to the original yeast two-hybrid clone is bracketed and the position of a putative nuclear targeting sequence in clone 5 is indicated with a bolded line over the text.

emerging class of transcriptional regulators which mediate interactions between DNA-bound transcription factors and the general transcription machinery or direct chromatin remodelling activities which alter transcriptional activities (Xu et al., 1999). The SKIP protein was originally isolated as a binding partner of the Ski transcription factor in humans (Dahl et al., 1998). Subsequently, SKIP has been found to facilitate the recruitment to transcriptional complexes of a key regulator of *Drosophila* cell fate, the Notch transmembrane signalling factor which upon activation, translocates to the nucleus and initiates changes in gene expression (Zhou et al., 2000). SKIP was also independently cloned from humans as a coactivator (NCoA-62) which positively regulates vitamin D mediated gene expression through interactions with the nuclear localised vitamin D receptor (Baudino, 1998). Given that clone 5 is known to interact with the GAMYB transcription factor in yeast it seems highly likely that clone 5 is also a nuclear coactivator which forms part of a transcriptional complex involving GAMYB. Whether this interaction might have a stimulatory or repressive affect on transcriptional activity remains to be investigated.

(3.4) Concluding remarks

This work demonstrates the applicability of the RAS-recruitment system to the exploration of plant signalling pathways and transcriptional complexes using transcription factors as bait, and to the author's knowledge is the first reported use of this system to this end. An encouraging aspect of these results is that no false positives encoding endogenous guanyl-nucleotide exchange factors, which are potential false positives in this screen, were encountered. Instead, by relaxing the time-frame in which interactions were monitored a sizeable pool of primary GAMYB-binding candidates was obtained. Of these, four were obvious contenders for roles in GA-signalling and GA-regulated gene expression.

The two partial GAMYB binding proteins considered in detail in this chapter, clone 3 and clone 5, contain conserved sequence features which are highly suggestive of a role in signalling pathways leading to changes in gene expression. The WD40 domain in clone 3 suggests it could be a scaffold protein which mediates interactions between GAMYB and some sort of upstream modifier while the close sequence relatedness of clone 5 to known transcriptional co-activators implicates this factor in the modulation

of GAMYB function within a transcriptional complex. Unfortunately, it was beyond the scope of this study to test these ideas. Instead, the following chapters describe the results of investigations into the possible functional role in GA-signalling of two other GAMYB binding proteins isolated in the two-hybrid screen, the MAP-kinase like factor encoded by clone 1 and the transcriptional regulator-like protein encoded by clone 4.

Chapter 4 - Results 2

Characterisation of KGM *(clone1)*, a GAMYB-binding protein which negatively regulates GAMYB function

(4.1) Introduction

As described in the previous chapter, in order to uncover more about GA-signalling in aleurone, cDNA-expression libraries were screened for GAMYB-binding proteins. A sizeable pool of putative GAMYB binding partners was identified and several were selected for further characterisation. This chapter contains a description of the features of one of these promising clones, clone 1 - a MAP-kinase-like gene termed KGM (for *kinase associated with GAMYB*), along with the results of a series of experiments designed to assess the role of this factor in aleurone GA-signal transduction. It will be argued that these results show that KGM is a negative regulator of GA-signalling in aleurone and that KGM acts directly through the modulation of GAMYB function.

(4.2) Materials and methods

(4.2.1) Assembly of KGM constructs

1. pGEX.KGM

The KGM ORF was amplified by PCR from the full-length KGM cDNA using the following primers – forward: - 5' TAG GAT CCC CAT GGA GAG GTA TAA CAT TAT TAC 3'; reverse – 5' TAG AAT TCG CTG ACG AGC TTC CTT TGG 3'. The resulting PCR product was cloned as a *Bam*HI – *Eco*RI fragment into the pGEX-3X bacterial expression vector (Pharmacia) to produce an in frame fusion at the carboxy-terminus of GST (glutathione S-transferase).

2. Ubi1.KGM

The KGM ORF was amplifed by PCR from the full-length KGM cDNA using the following primers – forward: 5' TAG GAT CCA ATA AAA TGG AGA GGT ATA AC 3'; reverse – 5' TAG GTA CCT CAG CTG ACG AGC TTC 3'. The resulting PCR product was cloned as a *Bam*HI – *Kpn*I fragment downstream of the maize 1 ubiquitin promoter in pUbi1.cas (kindly provided by X. Li, CSIRO, Canberra) which is a monocot expression vector derived from pACH27 (Christensen et al., 1992).

3. Ubi1.mKGM

The $TEY_{(158)}$ motif in KGM was altered to $TEF_{(158)}$ using the Stratagene QuickChange Site-Directed Mutagenesis Kit (catalogue no. 200518). Procedures were as per the manufacturer's instructions. In brief, the 1.0 kb, 3' *Sac*I fragment of the full-length KGM cDNA was excised from Ubi1-KGM and cloned into the *Sac*I site of pBC SK(+) (Stratagene). The following complementary primers encoding the desired mutation relative to the wildtype KGM sequence were designed - forward: 5' CCA TAC ACA GAA TTT GTG TCA ACT CGC 3'; reverse: 5' GCG AGT TGA CAC AAA TTC TGT GTA TGG 3'. 16 cycles of PCR were performed using 50 ng of template, 250 ng of each primer, 200 μM dNTPs and 1 unit of native *Pfu* polymerase in the manufacturer's reaction buffer. Each cycle consisted of three steps - 95°C for 30 s, 55°C for 60 s and 68°C for 8 min 30 s. Parental DNA was then digested by incubation of the completed reaction with 20 units of *Dpn*I at 37°C for 1 h. A 1 μL aliquot of the resulting sample was used to transform XL1B by electroporation. Plasmids were extracted from the resulting clones and screened for the desired mutation by sequencing.

4. Ubi1.GFP:KGM

The KGM ORF from Ubi1.KGM was excised as a *Kpn*I –*Bam*HI fragment and cloned into the same sites in pUbi1.GFPT4 – a derivative of pUbi1.cas containing the coding sequence for GFP (kindly provided by F. Gubler, CSIRO, Canberra). The resulting construct produces an in-frame fusion of KGM with the carboxy-terminus of GFP.

(4.2.2) Production of recombinant protein

1. GST-KGM and GST

The pGEX.KGM and pGEX constructs were transformed into the *E. coli* strains BL21-CodonPlus-RP and BL21(Stratagene) respectively. A 10 mL LB starter culture containing 25 mM glucose plus appropriate antibiotic selection was inoculated with a single colony and incubated for 16 h at 37°C. A 2 mL aliquot was subcultured in 200 mL LB plus antibiotic at 37°C until the OD_{260} reached 0.5-0.6. Expression of the

recombinant protein was induced by the addition of IPTG (isopropyl-beta-D-thiogalactoside) to 0.1 mM and the culture was then incubated at 17°C for a further 13–16 h (for the GST-KGM-expressing line) or 3 h at 25°C (for the GST-expressing line). For SDS-PAGE analysis, total protein was extracted from frozen cell pellets equivalent to 150 μL of culture by vortexing the pellet in 100 μL of 2 x SDS-PAGE sample buffer containing 0.5 M Tris-HCl pH 6.8, 10 % w/v SDS, 0.1 % w/v bromophenol blue, 20 % v/v glycerol, 5% v/v β-mercaptoethanol. To prepare soluble protein extracts, a frozen cell pellet from 100 mL of culture was sonicated on ice for five 45 s bursts in 10 mL of 50 mM HEPES-KOH pH 7.8, 150 mM NaCl, 1 mM EDTA containing a dissolved Complete Mini protease inhibitor cocktail tablet (Roche, catalogue number 1 836 156). Following the addition of Triton X-100 to 1% v/v, sonicate was clarified by centrifuging the lysate at 21 000 x *g* for 10 min at 4°C. All subsequent procedures were carried out at room temperature.

The GST-KGM or GST protein was purified from soluble extract equivalent to 500 mL or 100 mL of culture respectively by incubating 1 mL of glutathione-agarose beads (Scientifix) sequentially with half aliquots of the cell lysate for 20 min. The beads were collected by centrifugation and packed into a column. Beads were washed three times in 5 mL of 50 mM HEPES-KOH pH 7.8 containing 1.5 M NaCl and once in wash buffer without salt. The bound protein was eluted by closing the column and incubating the beads three times in 1 mL of 50 mM HEPES-KOH pH 7.8 containing 10 mM reduced-glutathione for 10 min then collecting the fraction. Fractions were pooled and concentrated using an Ultrafree-CL Biomax-10 membrane filter (Millipore). Typically around 1 mg of purified KGM-GST and 15 mg of purified GST protein was obtained from this procedure. The variously purified fractions were analysed using SDS-PAGE.

2. GAMYB-HIS

A pET19b.GAMYB construct (kindly provided by F.Gubler, CSIRO, Canberra) consisting of the GAMYB ORF cloned into pET19b (Novagen) so as to produce an in-frame fusion at the COOH-terminus with a poly-histidine tag was transformed into the *E.coli* strain BL21 (Stratagene). Cultures were grown and induced with IPTG as

described for the KGM-GST fusion protein (see above). Soluble protein extracts were also prepared as described above and expression of the GAMYB-fusion protein in these fractions was confirmed by Western blotting using 1 μL of extract (as per section 2.5).

(4.2.3) *In vitro* binding assays

Aproximately 200 μL of glutathione-agarose beads were washed 3 x in 500 μL lysis buffer containing 50 mM HEPES-KOH pH 7.8, 150 mM NaCl and 1 mM EDTA. A 50 μL aliquot of beads was incubated with 50 μg of GST-KGM or GST for 30 min at room temperature. Beads were washed three times in lysis buffer for 5 min at room temperature (except the second wash also contained 1 % w/v BSA). A 20 μL aliquot of beads was then incubated with either 200 μL of soluble cell extract from an induced culture of *E.coli* transformed with pET19b.GAMYB (see above) or 200 μL of lysis buffer for 2 h at 4° C. Beads were washed 4 x in lysis buffer and bound proteins were eluted from beads in 25 μL of 50 mM HEPES-KOH pH 7.8 containing 10 mM reduced-glutathione. The elution procedure was repeated and the two eluted fractions for each sample were pooled. The protein contained in a 10 μL aliquot of the elution was separated using SDS-PAGE and probed with a GAMYB antibody on a Western blot as described in section 2.5.

(4.3) Results

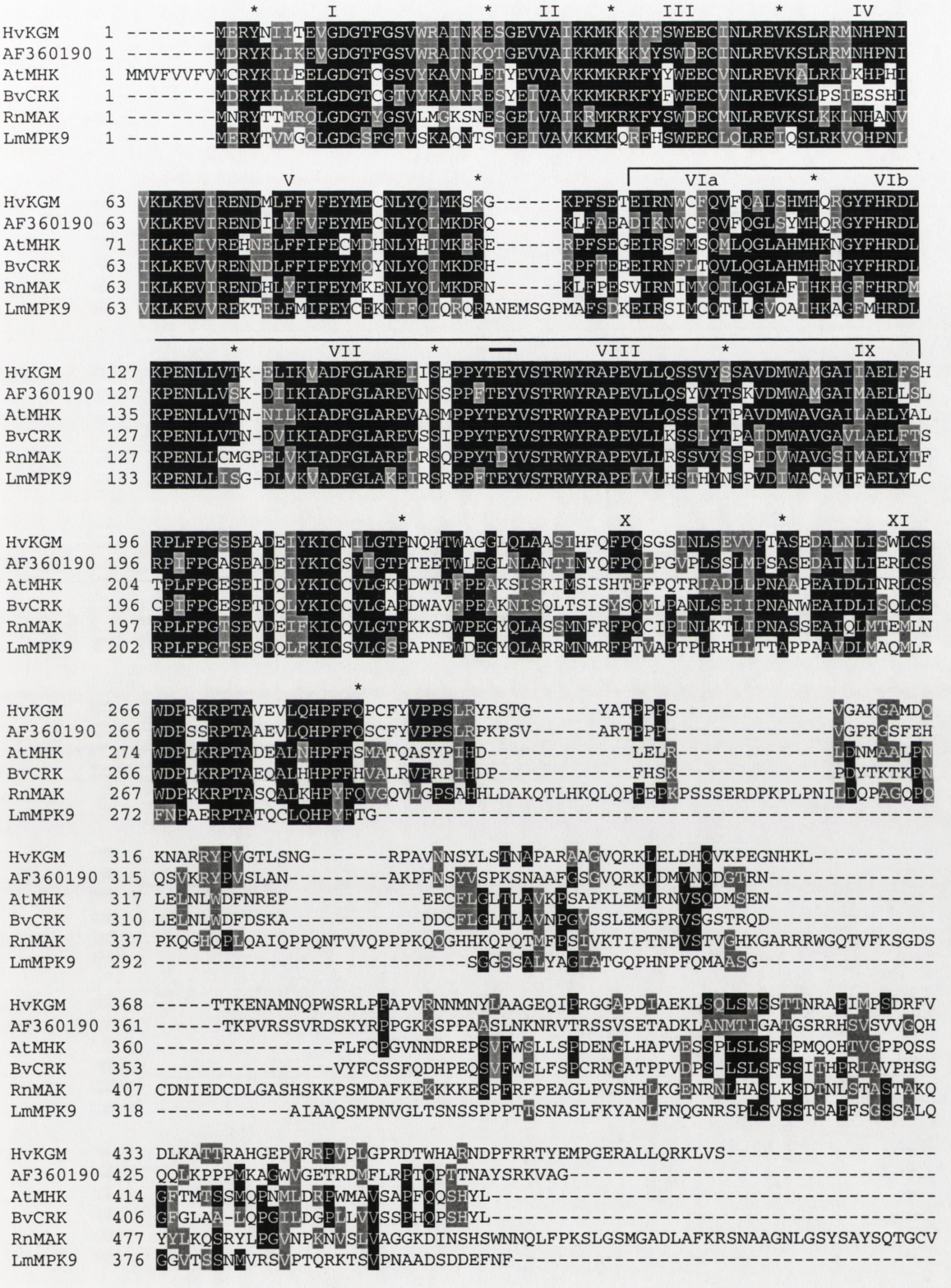
(4.3.1) Isolation of a full length cDNA for KGM

The partial cDNA for KGM derived from the two-hybrid screen (clone 1) was used as a probe to screen a Himalaya aleurone cDNA library for full-length clones (as per section 2.3.2). A 2.3 kb cDNA containing a 1.44 kb ORF and approximately 400 bp of both 5' and 3' untranslated sequence was obtained. On the basis of sequence homologies, searches of the gene databases reveal that KGM is a member of an emerging class of MAP-kinase related genes first identified in rats and termed MAKs (for *male germ cell associated kinase*; for a review see Miyata and Nishida, 1999).

The closest full-length relatives of KGM in a variety of taxa (on the basis of amino acid similarity) are aligned in Figure 4.1. In this grouping, KGM is most closely related to

Figure 4.1. Global alignment of the deduced KGM protein sequence with the MAK-homologues.

The eukaryotic kinase catalytic subdomains are indicated (I-XI) and their approximate limits are marked with an asterix. The conserved TXY motif in subdomain VIII, which is phosphorylated in active MAP-kinases is marked with a line above the text. The portion of KGM isolated in the original yeast two-hybrid screen is bracketed. HvKGM = barley KGM; AF360190 = GenBank accession number for an uncharacterised Arabidopsis cDNA; RnMAK = rat male germ cell-associated kinase MAK (COOH-terminal sequence extends beyond that shown); LmMPK9 = *Leishmania mexicana* gene for putative MAP-kinase 9; BvCRK = deduced protein from *Beta vulgaris* mRNA for Cdc2-related protein kinase CRK2; AtMHK = *Arabidopsis* MAK-homologous kinase, MHK.


HvKGM
AF360190
AtMHK
BvCRK
RnMAK
LmMPK9
I
II
III
IV
V
VIa
VIb
VII
VIII
IX
X
XI

an *Arabidopsis* cDNA clone, GenBank accession number AF36190 (amino acid identity of 60% and similarity of 69%), and approximately equally related to the other members, with amino acid identities and similarities ranging between 42-46% and 55-58% respectively. These more distant relatives include the progenitor of this class, MAK from rats (Matsushime et al., 1990), which was isolated in degenerate library screens for protein kinases and is known to be expressed in male germ cell tissue. (The human and mouse MAK-clones, MRK (Abe et al., 1995) and RCK (Bladt and Birchmeier, 1993) are highly homologous to rat MAK and are not included in this comparison). The *Arabidopsis* gene MHK (for *mak homologous kinase*) was isolated in low stringency library screens for kinases and is expressed in roots and rosettes (Moran and Walker, 1993) while other full-length MAK-like clones have been isolated from sugar beet (CRK2; Fowler et al., 2000), and *Leishmania mexicana* (MPK9; Genbank acc. no. AJ293287). The CRK2 transcript is highly expressed in the meristem of developing sugar beet storage organs (Fowler et al., 2000). A number of barley and other plant ESTs also correspond closely in sequence to KGM, however there is currently no data concerning the functional roles of any of the plant MAK-like genes. A third KGM-like ORF exists in the *Arabidopsis* genome, encoding a hypothetical protein (GenBank accession number BAB09171). As this is a very similar protein to that encoded by the *Arabidopsis* cDNA GenBank accession number AF360190, it was not included in this grouping.

The subdomain structure of eukaryotic protein kinases is defined on the basis of characteristic patterns of conserved amino acids. Structural studies have defined two functional lobes within the catalytic region, an NH_3-terminal lobe (subdomains I-IV), which anchors and orientates the nucleotide, and a COOH-terminal lobe (subdomains VIA – XI) which binds the peptide and initiates phospho-transfer (for a review see Hanks and Hunter, 1995). As shown in Figure 4.1, the portion of KGM isolated in yeast two hybrid screens corresponds to the region analogous to the COOH-terminal lobe in KGM. Although the conserved serine threonine catalytic domains in the MAK proteins are equally closely related to the MAP-kinases and cdc2-kinases (Miyata and Nishida, 1999) the MAKs, including KGM, contain a typical MAP-kinase regulatory motif, TXY, in subdomain VIII. In classical MAP-kinases, the phosphorylation of this motif by upstream kinases is a pre-requisite for full catalytic activity (Kultz, 1998).

(4.3.2) A full length GST-KGM fusion binds GAMYB *in vitro*

In order to independently verify the interaction observed in yeast between GAMYB and the partial KGM protein, an *in vitro* binding assay was performed using recombinant full-length GST-KGM fusion protein and a soluble protein extract from *E.coli* expressing a GAMYB-HIS fusion. The KGM protein is 483 aa in length (with a predicted molecular mass of approximately 50 kDa) and the GST tag would be expected to add approximately 26 kDa in molecular mass to a tagged protein. As shown in Figure 4.2A, the purified recombinant GST-KGM fraction used in the assay consists of two proteins, one of approximately 80 kDa and a second form of approximately 65 kDa (presumably a product of proteolytic activity).

As described in section 4.2.3, recombinant GST-KGM fusion protein was attached to glutathione-agarose beads and the beads were incubated with a soluble extract from *E.coli* cells expressing a GAMYB-HIS fusion protein. When bound proteins were separated by SDS-PAGE and probed on a protein gel blot with a GAMYB antibody, an immunoreactive band of approximately 65 kDa was detected (Figure 4.2B). This falls within the expected molecular mass range for full-length GAMYB protein, which is 553 aa in length, and corresponds exactly to the observed size of purified, full-length recombinant GAMYB-HIS protein after equivalent separation using SDS-PAGE (result not shown). The 65 kDa band was absent from the bound fraction eluted from GST-KGM linked beads which had been incubated with buffer alone. Likewise, when GST alone was complexed to beads and incubated with and without the GAMYB-HIS extract, no immunoreactive bands were detected. This result shows that the full-length KGM protein (as a GST-fusion) binds GAMYB from amongst a complex mixture of bacterial proteins.

(4.3.3) Transient expression of KGM in aleurone specifically represses α-amylase promoter activity

To assess the function of KGM in hormonal signalling in aleurone cells, the effect of transient expression of KGM on the activity of various promoters was tested in co-bombardment assays. In the first experiment (Figure 4.3), varying amounts of the Ubi1.KGM construct were co-bombarded into barley aleurone along with a reporter

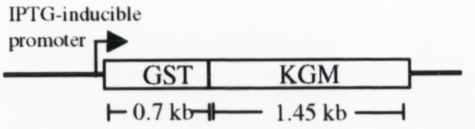
Figure 4.2. *In vitro* binding affinity of GST-KGM and GAMYB.

(A) Structure of the pGEX-KGM expression vector *(i)* and purification of recombinant GST-KGM *(ii)*. *E.coli* transformed with pGEX-KGM were grown to log phase at 37°C then induced with 0.1 mM IPTG for 13 h at 17°C. Total protein extracts after 0 h and 13 h induction, and a soluble protein extract after 13 h induction (equivalent to 100 μL of culture) were analysed using SDS-PAGE. The GST-KGM fusion was purified on glutathione-agarose beads and a 2 μg fraction of purified protein was also analysed by SDS-PAGE.

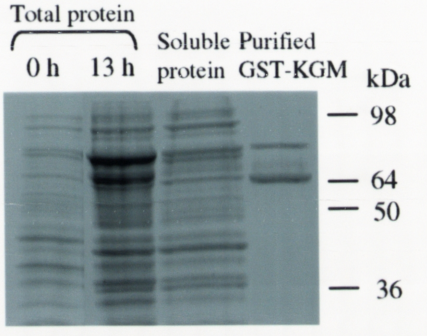
(B) The GST-KGM fusion protein or GST alone was attached to glutathione-agarose beads and incubated both with and without a soluble extract from *E. coli* cells expressing a GAMYB-HIS tagged protein. Bound proteins were eluted and a 10 μL aliquot of the elution was separated by SDS-PAGE and probed with a GAMYB antibody on a protein gel blot.

A

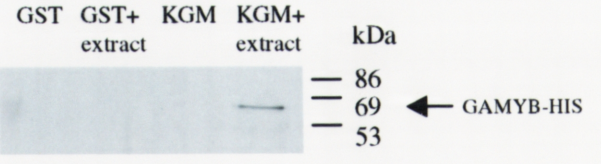
(i)


IPTG-inducible
promoter
GST
KGM
0.7 kb
1.45 kb

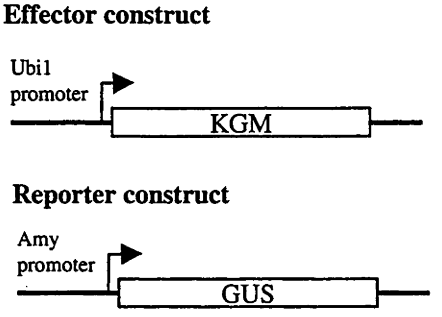
(ii)


Total protein
0 h
13 h
Soluble protein
Purified GST-KGM
kDa
98
64
50
36

B


GST
GST+ extract
KGM
KGM+ extract
kDa
86
69
53
GAMYB-HIS

A



B

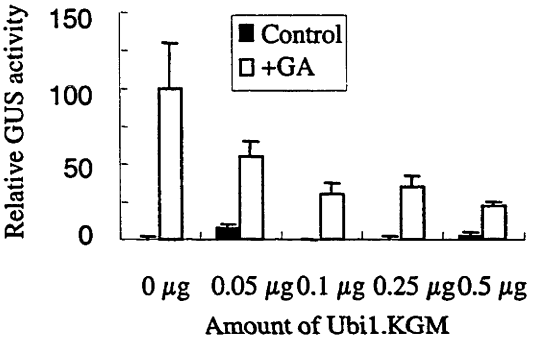


Figure 4.3. Effect of transient expression of KGM on α-amylase promoter activity in barley aleurone cells.

(A) Structure of the effector and reporter constructs used in the co-bombardment experiment.

(B) Embryo-less half seeds were co-bombarded with gold particles coated with 1 μg of the Amy.GUS reporter with or without varying amounts of Ubi1.KGM effector. Bars represent GUS activities ± standard error after a 24 h treatment with or without 10^{-6} M GA_3.

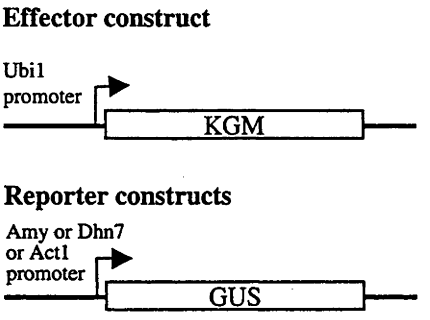
construct consisting of a barley low-pI α-amylase promoter fused to GUS (Amy.GUS; mlo22 in Lanahan et al., 1992). A reduction in GA-induced Amy.GUS activity of 40-75% was observed depending on the amount of Ubi1.KGM effector used. It was established that 0.1 μg of Ubi1.KGM effector precipitated onto gold particles (see section 2.6) was the lowest amount of effector required to produce a near maximal effect on Amy.GUS activity – this quantity was used in all subsequent experiments.

In a second co-bombardment experiment (Figure 4.4), the effect of transient expression of KGM on the activities of Amy.GUS, the ABA-inducible barley dehydrin7 promoter fused to GUS (Dhn7.GUS; Robertson et al., 1995) and the constitutive rice actin1 promoter fused to GUS (Act1.GUS; McElroy et al., 1990) was compared. While the expression of KGM reduced GA-inducible Amy.GUS activity by 60%, constitutive actin1 promoter activity and the ABA-inducible dehydrin7 promoter activity were unaffected. These results suggest that the effect of KGM on gene expression is specific to GA-signalling and not that of a 'global' repressor of transcription.

(4.3.4) Transient expression of KGM represses GAMYB-function in aleurone

The transient expression of GAMYB is sufficient to activate Amy.GUS activity in bombarded aleurone layers in the absence of GA (Gubler et al., 1995). Consequently, the possibility of a functional interaction between KGM and GAMYB was tested in this system. KGM and varying amounts of GAMYB were co-expressed in the presence of the Amy.GUS reporter in barley aleurone. When gold particles coated with 0.05 μg or 0.005 μg of an effector construct consisting of the GAMYB ORF under the control of the maize ubiquitin 1 promoter (Ubi1.GAMYB; Gubler et al., 1999) were co-bombarded into aleurone with constant amounts of Ubi1.KGM, there was a 55% and 85% reduction respectively in the GAMYB-mediated activation of Amy.GUS (Figure 4.5). At the highest level of GAMYB effector, 0.5 μg of Ubi1.GAMYB-coated gold particles, a statistically significant effect was not observed however this is probably because the ratio of Ubi1.GAMYB to Ubi.KGM effector is too high and there is insufficient KGM expressed to interact with the large pool of transiently expressed GAMYB.

A



B

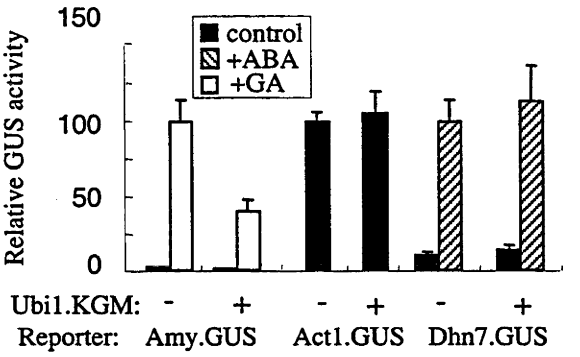
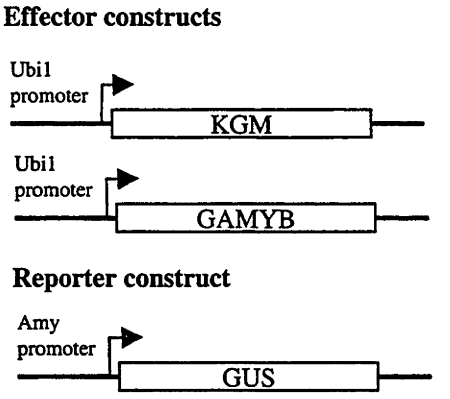


Figure 4.4. Effect of transient expression of KGM on various promoter activities in barley aleurone cells.

(A) Structure of the effector and reporter constructs used in the co-bombardment experiment. Amy = barley low pI α-amylase, Dhn7 = barley dehydrin 7, Act1 = rice actin1.

(B) Embyro-less half seeds were co-bombarded with gold particles coated with 1 μg of each reporter construct with or without 0.1 μg of the Ubi1.KGM effector. Bars represent GUS activities ± standard error after a 24 h treatment with or without 10^{-6} M GA_3 or 5 x 10^{-5} M ABA. Each promoter activity is normalised to the corresponding inducible or constitutive activity detected in the absence of the effector construct.

A



B

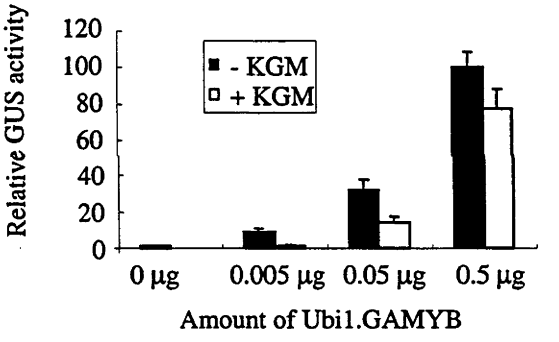


Figure 4.5. Functional interaction of transiently expressed KGM and GAMYB in barley aleurone cells.

(A) Structure of the effector and reporter constructs used in the co-bombardment experiment.

(B) Embryo-less half seeds were co-bombarded with gold particles coated with 1 μg of Amy.GUS reporter and varying amounts of Ubi1.GAMYB effector with or without 0.1 μg of the Ubi.KGM effector. Bars represent GUS activities ± standard error after a 24 h incubation without hormone.

The phosphorylation of serine/threonine residues by MAP-kinases is proline directed and the consensus sequence has been defined as (P)xxS/TP (Kultz, 1998). On the basis of their similarity to the MAP-kinase family of protein kinases, the MAKs might be expected to target similar motifs however nothing is yet known about the catalytic activity of the MAKs. The GAMYB protein was examined for the presence of the MAP-kinase phosphorylation consensus and five such motifs were found. Figure 4.6A shows their position on a functional map of the GAMYB protein (after Gubler et al., 1999). Three of these sites cluster in or near the conserved DNA-binding domain while the remainder are located in the COOH-terminal transactivation domain.

It has been demonstrated that certain COOH-terminal deletions of GAMYB can be made without abolishing its transactivating potential (Gubler et al., 1999). The functional relationship between KGM and GAMYB was therefore investigated further by testing in co-bombardment assays whether KGM represses the activity of a GAMYB protein with a 197 aa COOH-terminal deletion, a truncation which encompasses a number of the consensus phosphorylation motifs (construct kindly provided by F.Gubler, CSIRO, Canberra). As shown in Figure 4.6, the truncated from of GAMYB was repressed to the same degree as the full-length protein when co-expressed in barley aleurone with KGM. This suggests that the functional interaction between these two proteins occurs within the NH_3-terminal half of GAMYB.

(4.3.5) Mutation of a conserved regulatory motif in KGM alleviates the repression of GAMYB function in aleurone

By analogy to what is known about the regulation of MAP-kinase function, the importance of the conserved $TEY_{(158)}$ motif in catalytic subdomain VIII of KGM to KGM function was investigated. In classical MAP-kinases both the threonine residue and, most critically, the tyrosine residue in this motif must be phosphorylated for full catalytic activity of the enzyme. Structural studies suggest that phosphorylation of the tyrosine residue orientates a loop formed by sub-domain VIII to generate the mouth of the active site and threonine phosphorylation then facilitates the alignment of key catalytic residues (Hanks and Hunter, 1995).

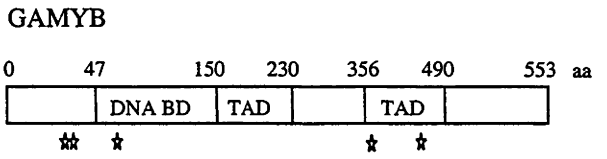
Figure 4.6. Functional interaction of transiently expressed KGM and truncated GAMYB in barley aleurone cells.

(A) Functional domains of GAMYB and position of consensus MAP-kinase phosphorylation sites[*]. DNA BD = DNA binding domain, TAD = transactivation domain.

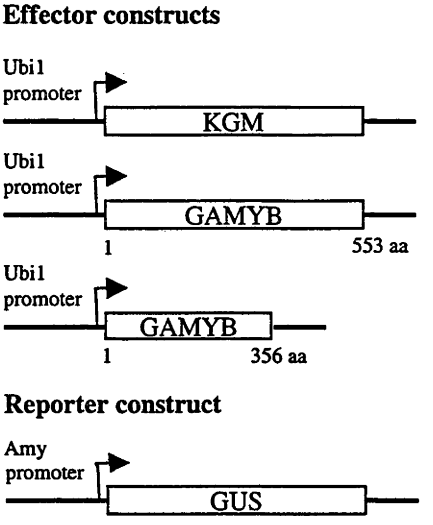
(B) Structure of the effector and reporter constructs used in the co-bombardment experiment.

(C) Embryo-less half seeds were co-bombarded with gold particles coated with 1 μg of Amy.GUS and 0.05 μg of effectors encoding full-length or truncated GAMYB, with or without 0.1 μg of Ubi1.KGM. Bars represent GUS activities ± standard error after a 24 h incubation without hormone.

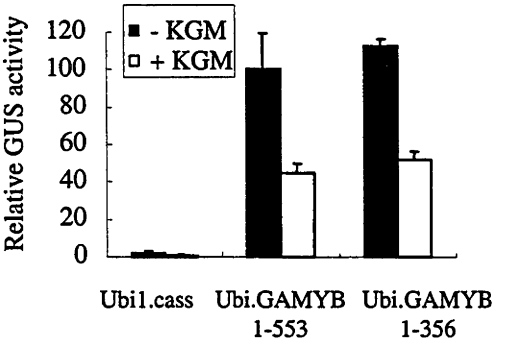
A


GAMYB
0
47
150
230
356
490
553 aa
DNA BD
TAD
TAD
=(P)xxS/TP

B


Effector constructs
Ubi1 promoter
KGM
Ubi1 promoter
GAMYB
1
553 aa
Ubi1 promoter
GAMYB
1
356 aa
Reporter construct
Amy promoter
GUS

C


Relative GUS activity
120
100
80
60
40
20
0
- KGM
+ KGM
Ubi1.cass
Ubi.GAMYB 1-553
Ubi.GAMYB 1-356

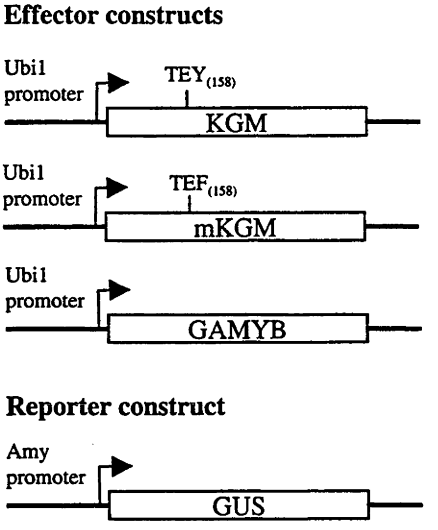
The $TEY_{(158)}$ motif in KGM was altered to $TEF_{(158)}$ by site-directed mutagenesis, a mutation in other plant MAP-kinases which has been shown to substantially reduce the *in vitro* catalytic activity of the enzyme (Takezawa, 1999). The effect of this mutation was assessed by co-bombarding barley aleurone with constructs expressing wildtype or mutant forms of KGM along with GAMYB in the presence of the Amy.GUS reporter. As previously observed, expression of wildtype KGM repressed the GAMYB transactivation of Amy.GUS activity by over 60% (Figure 4.7). However the mutant form of KGM was a substantially weaker repressor, able to repress GAMYB transactivation of Amy.GUS by only 24%.

Despite the functional interaction observed between GAMYB and KGM in transient expression assays, in this study recombinant GST-KGM was not observed to phosphorylate GAMYB in *in vitro* kinase assays using a method adapted from that of Takezawa (1999; results not shown). However it is possible that these test assay conditions were unrepresentative of the *in vivo* situation and that a better *in vitro* assay could be devised. Certainly there are currently no reports concerning the *in vitro* kinase activity of the MAK subgroup of kinases, even against site-rich substrates such as MBP (myelin basic protein) and conventional assay procedures may requires optimisation.

(4.3.6) A GFP:KGM fusion localises to the cytosol and nucleus in barley aleurone protoplasts

Although the mechanisms are not yet fully understood, activated MAP-kinases which target transcription factors are known to translocate from the cytosol to the nucleus in a phosphorylation dependent manner (Chen et al., 1992; Hulleman et al., 1999) however nothing is yet known about the cellular distribution of the MAKs. To investigate the cellular distribution of KGM, a construct containing the KGM ORF fused to the carboxy-terminus of GFP under the control of the maize ubiquitin promoter was transfected into barley aleurone protoplasts. Protoplasts were incubated for 20 h then examined using fluorescence microscopy (Figure 4.8). A cytosolic and nuclear distribution of fluorescence was observed in transfected aleurone protoplasts which was largely absent in the numerous large protein storage vacoules which are characteristic of this cell type. This distribution was unchanged by the inclusion of a 6 h treatment of

A



B

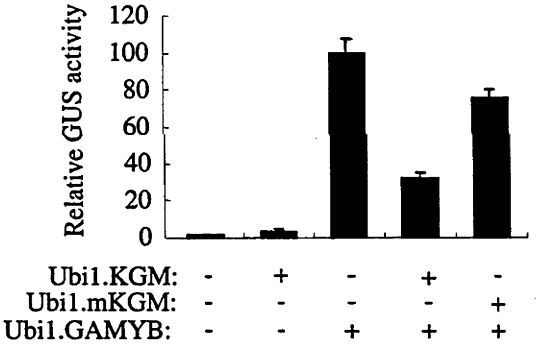


Figure 4.7. Effect of $TEY_{(158)}$ → $TEF_{(158)}$ mutation on KGM repression of GAMYB activity in barley aleurone.

(A) Structure of the effector and reporter constructs used in the co-bombardment experiment.

(B) Embryoless half seeds were co-bombarded with a 1 μg equivalent of Amy.GUS with or without various combinations of the three effectors - 0.01 μg of Ubi1.KGM or Ubi1.mKGM or 0.05 μg of Ubi1.GAMYB effector. Bars represent GUS activities ± standard error after a 24 h incubation without hormone.

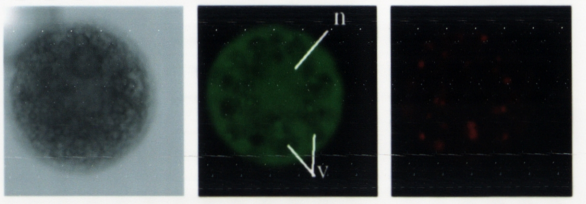


Figure 4.8. Cellular localisation of KGM in barley aleurone protoplasts. Distribution of fluorescence in a single protoplast transfected with a construct expressing a GFP:KGM fusion protein (centre panel). The left panel contains a bright field image of the same protoplast while autofluorescence is shown in the right panel. n = nucleus, v = protein storage vacuole.

protoplasts with 10^{-6} M GA or 5 x 10^{-5} M ABA at the end of the 20 h incubation period (results not shown).

(4.3.7) Expression of the KGM transcript in barley

The expression of the KGM transcript was investigated in hormone-treated aleurone tissues from Himalaya as well as the *Sln1d* mutant, to assess whether there is any interaction between this mutation and control of KGM mRNA expression. A variety of other Himalaya tissues were also tested. RNA gel blots of total RNA from these tissues were hybridised with a DNA probe corresponding to the 3' half of the full-length KGM cDNA (nucleotide positions 1141 to 2120 - see appendix). This probe incorporates around 400 bp of untranslated sequence and spans a less conserved region of the cDNA, downstream of the region encoding the conserved catalytic domain.

As shown in Figure 4.9A, similar amounts of a single transcript of around 2.2 kb (comparable in size to the KGM full-length cDNA) were detected in Himalaya aleurone tissues treated for 6 h with or without 10^{-6} M GA_3, or 5 x 10^{-5} M ABA or with both hormones. However, a two-fold induction in the amount of KGM transcript relative to the other treatments was observed in aleurone tissue treated with 10^{-9} M GA_3. In the *Sln1d* background the transcript was barely detectable in aleurone tissue treated without GA_3 but was present in the same amount in tissues treated with 10^{-9} M and 10^{-6} M GA_3 as was found in wildtype aleurone. As a positive control for hormone treatments, the same RNA samples were hybridised with a DNA probe corresponding to the high pI barley α-amylase clone, HV19 (Chandler et al., 1984). As previously observed, the α-amylase transcript was strongly induced in wildtype aleurone by GA and repressed by applied ABA. The *Sln1d* mutant possesses a reduced sensitivity to GA, and correspondingly the α-amylase transcript was induced less strongly by equivalent GA treatments in this tissue.

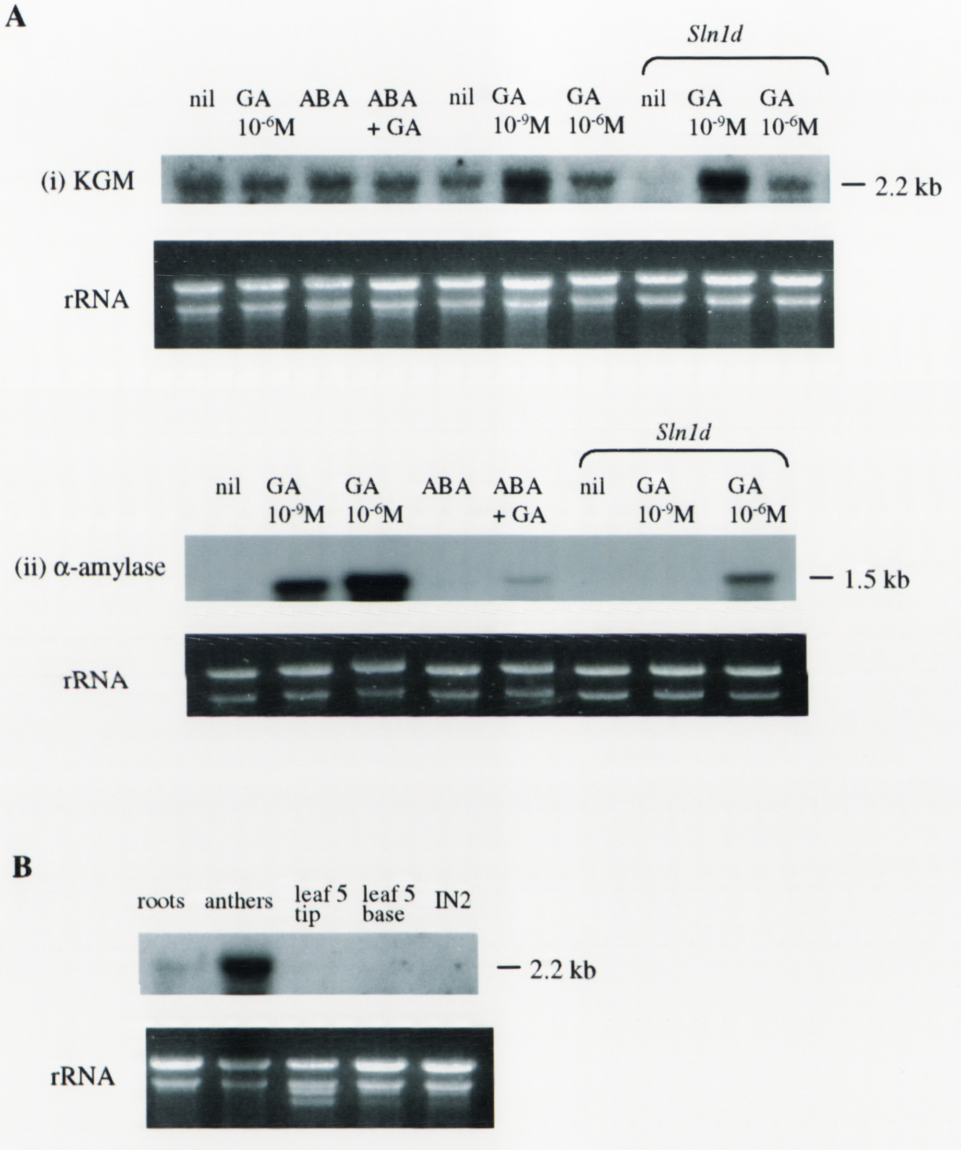
An RNA gel blot of total RNA from Himalaya roots, developing anthers (approximately 5-7 mm long), elongated internode (second from base of stem) and the tip and base of leaf 5 was also hybridised with the 3' KGM probe described above. (The barley plant from which non-root tissues were derived was 7 weeks old, while root material was derived from seeds germinated on water for 5 d in the dark). As

Figure 4.9. Expression of the KGM transcript in barley tissues.

(A) RNA gel blot of RNA from hormonally treated wildtype barley and *Sln1d* aleurone layers probed with a 1 kb fragment from the 3' end of the the KGM full-length cDNA *(i)* or a barley α-amylase partial cDNA (HV19) *(ii)*. Hormonal treatments represent 6 h incubations and GA_3 and ABA were used at concentrations of 1 x 10^{-6}M or 5 x 10^{-5}M respectively unless otherwise specified.

(B) RNA gel blot of RNA from a variety of barley tissues probed with a 1kb fragment from the 3' end of the the KGM full-length cDNA.

IN2 = elongated internode (second from base of stem).


A
Sln1d
nil
GA 10-6M
ABA
ABA + GA
nil
GA 10-9M
GA 10-6M
nil
GA 10-9M
GA 10-6M
(i) KGM
2.2 kb
rRNA
Sln1d
nil
GA 10-9M
GA 10-6M
ABA
ABA + GA
nil
GA 10-9M
GA 10-6M
(ii) α-amylase
1.5 kb
rRNA
B
roots
anthers
leaf 5 tip
leaf 5 base
IN2
2.2 kb
rRNA

shown in Figure 4.9B, the KGM transcript was detected in very high amounts in developing anthers and was faintly detectable in roots. (For purposes of comparison, the RNA gel blot containing the aleurone RNA samples probed with KGM was exposed to film for twice as long as the RNA gel blot of RNA from non-aleurone tissues.)

(4.4) Discussion

This chapter describes the cloning and characterisation of a new MAP-kinase like factor from barley aleurone which interacts with GAMYB and, when transiently expressed in aleurone, specifically represses the GA-induction of α-amylase gene expression at the level of GAMYB action. It is proposed that GAMYB is phosphorylated or otherwise modified by KGM in a manner which negatively regulates the activity of the transcription factor. This would provide an important mechanism for control of GAMYB function, transcending the controls on GAMYB expression which are not tightly linked to GA (Gubler et al., 2002). It is envisaged that KGM might function to prevent the basal amount of GAMYB protein in non-GA stimulated aleurone tissue from initiating a concomitant amount of precocious α-amylase synthesis.

The isolation of KGM from barley aleurone as a GAMYB-binding protein led to a series of experiments designed to test the possible involvement of KGM in GA-signalling. The first line of evidence for a functional role of KGM in this pathway came with the observation that when KGM is transiently expressed in aleurone, GA-induced α-amylase promoter activity is suppressed by 60% while other hormonally regulated and constitutive promoters are unaffected. Consistent with the finding that KGM interacts with GAMYB in yeast and *in vitro*, a functional interaction between these two proteins was demonstrated by the observation that the transactivation of the α-amylase promoter by constitutively over-expressed GAMYB in aleurone is strongly repressed by co-expression with KGM.

The KGM protein possesses the conserved sub-domain structure of the eukaryotic protein kinases and sequence comparisons place this protein squarely within an emerging group of MAP-kinase like genes, first identified with the cloning of the rat

MAK gene (Matsushime et al., 1990). By contrast with what is understood about the functioning of MAP-kinases, very little information exists about the functional roles of the MAK-homologues. With the knowledge that classical MAP-kinases depend on dual phosphorylation of the TXY motif in subdomain VIII for full catalytic activity (Hanks and Hunter, 1995) the critical tyrosine residue in this motif in KGM was mutated from TEY_{158} to TEF_{158}. In transient expression assays it was found that this single amino acid change significantly alleviated KGM repression of transactivation of the α-amylase promoter by GAMYB. If previous data about the importance of this tyrosine-residue to MAP-kinase activity is considered with this finding, namely that Y_{158} is central to the ability of KGM to repress GAMYB-action, it seems likely that an intact KGM kinase activity is fundamental to the interaction between KGM and GAMYB.

Although *in vitro* phosphorylation of GAMYB by KGM was not demonstrated in this study, the possibility that GAMYB is a kinase-substrate for KGM is given indirect support by a number of further findings. Firstly, the observed cytosolic and nuclear distribution of the GFP:KGM fusion in aleurone protoplasts is consistent with that of a MAP-kinase like factor which targets transcription factors. Studies of animal MAP-kinases, such as $p42^{MAPK}$, and ERK (extracellular signal related kinase 2) show that that these signalling factors are activated in the cytoplasm and subseqently translocate to the nucleus (Chen et al., 1992; Hulleman et al., 1999). Secondly, the fragment of KGM which was isolated as a GAMYB-binding protein in the yeast two-hybrid screen spans many of the conserved subdomains which in structural studies of other protein-kinases, comprise the COOH-terminal lobe. It is the COOH-terminal lobe which is known to participate in substrate binding (Hanks and Hunter, 1995). Finally, the GAMYB protein contains five consensus MAP-kinase phosphorylation motifs which could represent targets for a MAK-type kinase activity.

RNA gel blot analysis of expression of the KGM transcript in aleurone revealed that comparable amounts of transcript were expressed in tissue treated for 6 h with and without 10^{-6} M GA_3, 5 x 10^{-5} M ABA or a combination of the two hormones. A 10^{-9} M concentration of GA_3 resulted in a two-fold increase in expression of the amount of KGM mRNA compared to the other treatments. In the *Sln1d* background, GA-treated

aleurone tissue contained similar amounts of transcript as the wildtype although in non-hormonally treated tissue, the transcript was barely detectable, suggesting that there is some interaction between controls on KGM expression and SLN1 function in the minus-GA situation. Notwithstanding, this concentration-dependent expression pattern for the KGM transcript in response to GA, in both wildtype and *Sln1d* backgrounds, is complex and difficult to interpret without examining a broader range of concentrations and timepoints. Given that the expression of the KGM transcript is not downregulated by GA in aleurone tissue at 6 h after GA stimulation and that α-amylase gene expression has ordinarily already commenced at this point (Schuurink et al., 1996), it seems most likely that KGM, as a repressor of this process, is controlled at the post-transcriptional level.

Concluding remarks

The isolation of a kinase which binds to GAMYB and represses GAMYB function provides the first direct evidence for post-translational regulation of this transcription factor. This study also presents the first data concerning the functional role of a MAK-type kinase in plant or animal systems. However, a number of questions remain about the timing and co-ordination of KGM function in aleurone cells. The dual cytosolic and nuclear localisation of KGM suggests that this factor might integrate signalling events between these two compartments. Given the importance of the conserved TEY regulatory motif in subdomain VIII to KGM function, an upstream modifier in the cytosol directed toward this site might be a part of this process. These issues are canvassed in more detail in Chapter 6 where a broader view of these results and how they inform our current understanding of GA-signal transduction is presented.

Chapter 5 – Results 3

Characterisation of GMPOZ *(clone 4)*, a BTB/POZ domain protein which interacts with GAMYB and is required for α-amylase expression

(5.1) Introduction

This chapter describes the results of investigations into the possible functional role in GA-signalling of a second GAMYB-binding protein, clone 4, obtained in the yeast two-hybrid screen (see Chapter 3). As detailed below, isolation of a full-length cDNA for clone 4, termed GMPOZ, revealed a number of interesting features characteristic of certain types of transcriptional regulators and experiments were designed to test whether GMPOZ might be involved in hormonally regulated gene expression in barley aleurone. It will be argued that the results of this work suggest that GMPOZ is a nuclear-located activator of GA-regulated gene expression but that its role with respect to GAMYB function remains unclear.

A key strategy employed in these experiments was the use of transient expression of dsRNA (double stranded RNA) from the GMPOZ gene to activate post-transcriptional silencing of this gene in aleurone co-bombardment assays. The role of RNA as a target for gene silencing in plants (post-transcriptional gene silencing, or PTGS) is well established and is believed to represent part of an endogenous mechanism for protection against invading RNA, such as occurs during viral infection (Kooter et al., 1999; Finnegan et al., 2001). The importance of dsRNA in PTGS has been demonstrated through the specific silencing of genes by the expression of inverted-repeat sequences predicted to form dsRNA in transgenic plants (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000).

Recently, this gene silencing technology has been successfully adapted for use in transient expression systems in cereals, paving the way for the characterisation of gene function in these systems without the need to produce stable transformants. It has been shown that microinjection of dsRNA or expression of inverted-repeat RNA in bombarded maize and barley interferes with the function of the cognate, endogenous gene but does not silence transiently expressed reporter genes (Schweizer et al., 2000). This result held true for all genes tested including included two members of the anthocyanin biosynthetic pathway and a powdery mildew resistance gene. This technology has also been tested in barley aleurone (Zentello et al., 2001). A construct driven by a ubiquitin promoter consisting of approximately a 500 bp fragment of the

GUS gene present as an inverted-repeat separated by a spacer of several hundred base pairs was bombarded into aleurone. Rapid silencing of a transiently expressed GUS-reporter construct but not other, unrelated reporter constructs was observed. These same workers showed that transient expression of inverted-repeat RNA from the GAMYB gene resulted in a dramatic reduction in the GA-inducible activity of the α-amylase promoter, demonstrating that GAMYB is necessary for the GA-regulated expression of this gene.

(5.2) Materials and methods: assembly of GMPOZ constructs

1. Ubi1.GMPOZ

The GMPOZ ORF was amplified by PCR from the GMPOZ full-length cDNA using the following primers – forward: 5' TAG GAT CCA ATT TTA TGT TCT GTG CTT G 3'; reverse – 5' TAG GTA CCC TAT GAT TGC TTT ATG GTC 3'. The resulting PCR product was cloned as a *Bam*HI – *Kpn*I fragment downstream of the maize ubiquitin 1 promoter in pUbi1.cas (kindly provided by X. Li, CSIRO, Canberra) which is a monocot expression vector derived from pACH27 (Christensen et al., 1992).

2. S4.GMPOZir

Identical portions of the GMPOZ gene corresponding to nucleotides 514-882 of the full-length cDNA (see appendix) or amino acids 55-180 within the full-length protein were amplified by PCR using AmpliTaq DNA polymerase (Perkin-Elmer). The GMPOZ cDNA was used as template along with two sets of primers designed to incorporate unique restriction sites to facilitate cloning of the fragments as an inverted repeat. For the sense orientation - forward: 5' TAC TCG AGT ACG AGT TTG CCT TCA ACT CG 3'; reverse: 5' TAG GTA CCT CTC AAA ACC TGA TTA CAC TC 3'. For the antisense orientation - forward: 5' TAC CTA GGT ACG AGT TTG CCT TCA ACT CG 3'; reverse: 5' TAC CCG GGT CTC AAA ACC TGA TTA CAC TC 3'. The resulting PCR products were cloned into pGEMT (Promega) and subcloned as a *Xho*I – *Kpn*I fragment (for the sense orientation) and an *Xma*I – *Avr*II fragment (for the anti-sense orientation) into the monocot expression vector CLARICE I (kindly provided by C. Horser, CSIRO, Canberra). As shown in Figure 5.3, the resulting

cassette is driven by a constitutive promoter derived from the potato stunt 4 virus and contains the two PCR products as an inverted-repeat separated by a 710 bp intron from a cereal cyst nematode resistance gene (*cre*).

3. Ubi1.GMPOZ:GFP

The GMPOZ ORF from Ubi1.GMPOZ was excised as a *Kpn*I –*Bam*HI fragment and cloned into the same sites in pUbi1.GFPT4 – a derivative of pUbi1.cas containing the coding sequence for GFP (kindly provided by F. Gubler, CSIRO, Canberra). The resulting construct produces an in-frame fusion of GMPOZ with the COOH-terminus of GFP.

(5.3) Results

(5.3.1) Isolation of a full length cDNA for GMPOZ

The partial cDNA for GMPOZ (clone 4), derived from the two-hybrid screen, was used as a probe to screen a barley aleurone cDNA library for full-length clones (as per section 2.3.2). A 2.28 kb cDNA (see appendix) containing a 1.74 kb ORF and approximately 350 bp and 180 bp of 5' and 3' untranslated sequence respectively, was obtained. As shown in Figure 5.1A, the deduced full-length protein was found to feature both a BTB/POZ domain and the leucine-rich, acidic domains (LADs) evident in the truncated yeast-two hybrid clone. Consequently this factor was termed GMPOZ (for *GAMYB associated POZ protein*). The GMPOZ protein also features a short sequence of basic amino acid residues resembling a common nuclear localisation sequence typified by that found in the SV40 large tumour antigen (Kalderon et al., 1984).

The BTB domain (named for Broad-Complex, Trimtrack and Bric-a-brac) or, as it is also known, the POZ domain (for poxvirus zinc finger) is an NH_3-terminal motif of about 120 aa which has been found in a variety of transcriptional regulators and also in proteins involved in the modification of cytoskeletal architecture such as the Drosophila kelch actin-binding protein (for a review see Collins et al., 2001). The BTB/POZ domain is traditionally thought to function as a protein interaction interface, providing a scaffold for the organisation of higher-order structures such as the

Figure 5.1. Features of the GAMYB-binding protein, GMPOZ.

(A) *Full-length GMPOZ protein sequence.* The portion of protein comprising the original GAMYB-binding yeast two hybrid clone (clone 4) is indicated with arrows. The BTB/POZ domain is boxed, the leucine-acidic domains are bolded and the position of a consensus nuclear localisation sequence is underlined.

(B) *Comparison of BTB/PZ domains from GMPOZ and a selection of related proteins.* According to crystal structure determinations of the BTB/POZ domain protein PLZF from humans (Ahmad et al., 1998), the asterixed glutamate/aspartate and phenylalanine residues are conserved components of the ligand binding groove and the asterixed tyrosine is a conserved component of the monomer core of BTB/POZ protein homodimers. The boxed amino acid cluster forms an alpha-helical domain in PLZF, contributing to the monomer core and surface and the dimer interface. This sequence is the most widely conserved region in this class of protein. AtPOB1 – Arabidopsis thaliana POZ/BTB containing protein GenBank acc. no. AF292397; HsSPOP – human speckle-type POZ nuclear protein (Nagai et al., 1997); DmKELCH – Drosphila melanogaster KELCH actin-binding protein (Xue and Cooley, 1993); HsTIP – human Transcription Terminator Factor I interacting peptide GenBank acc. no. AF000561.

A

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1   MFCACQSRCLGCKFGVWEVAARSRILGLDWIGGKRARWWVGMEPDFSRAS
51  GGPSYEFAFNSVNFSDRVLRIEIVAGDDAPGAKGAAGEGCSSIADWARHR
101 KRRREDLRREKGGEEYGKYMFEPSNVKIEAEEHDTYEETGEEPVAMIEES
151 PPDIGQDGEDGENSDSSWNMECNQVLRVKSIYISSAILAAKSPFFYKLFS
201 NGMKESDQRHATLRITTSEESALMELLSFIYSGKLTTNEPTLLLDILMIS
251 DKFEVVSCMRHCSQLLRSLPMTTESALLYLDLPSSISMAAAVQPLTDAAK
301 EFLANKYKDLTKFQDEVMNIPLAGIEAILCSNDLQVASEDAVYDFVIKWA
351 RAQCPRTEERREILGTRLLPLVRFSHMTCRKLRKVLACSDLDHEQASKSV
401 TDALLYKADAPHRQRALAADVLTCRKYTERAYKYRPLKVVEFDQPYPQCI
451 AYLDLKREECSRLFPSGRIYSQAFHLAGQGFFLSAHCNMDQQSAFHCFGL
501 FLGMQEKGSTSVTVDYEFAARTRPSGDFVSKYKGYYTFTGGKAVGYRNLF
551 AIPWPSFMADDSLFFIDGVLHLRAELTIKQS
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B

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GMPOZ     86  AGEGCSSIADWARHRKRRREDLRREKGGEEYGKYMFEPSNVKIEAEEHDTYEETGEEPVA
AtPOB1    69  EVEGCTSIADWARHRKRRREDIKKESGVTISDIVACPEEQILTDEQPDMDGCPGGENPDD
HsSPOP    108 NAKGEETKAMESQRAYRFVQGKDWGFKKFIRRDFLLDEANGLLPDDKLTLFCEVSVVQDS
DmKELCH   91  AAEGSGLERGSCLLRYASQNSLDESSQKHVQRPNGKERGTVGQYSNE-------------
HsTIP     2   RAAAARGSDQGSATRAERAPRTPEASGRGSPVPTSGPGPARPGPAPRGLGAEDGRRRGRPI

                                                  *
GMPOZ     146 M--------IEESPPDIGQDGEDGENS-DSSWNMECNQVLRVKSIYISSAILAAKSPFFYK
AtPOB1    129 E--------GGEAMVEEALSGDEEETSSEPNWGMDCSTVVRVKELHISSPILAAKSPFFYK
HsSPOP    168 VNISGQNTMNMVKVPECRLADELGGLWENSRFTDCCLCVAGQEFQAHKAILAARSPVFSA
DmKELCH   138 --------QHTARSFDAMNEMRKQKQ-----LCDVILVADDVEIHAHRMVLASCSPYFYA
HsTIP     62  G------IPFPDHSSDILSGLNEQRIQG--LLCDVVILVEGREFPTHRSVLAACSQYFKK

          *                                       *
GMPOZ     198 LFSNGMKESDQRHATLRITTSEESALMELLSFIYSGKLTTNEPTLLLDILMISDKFEVVS
AtPOB1    182 LFSNGMRESEQRHVTLRINASEEAALMELLNFMYSNAVSVTTAPALLDVLMAADKFEVAS
HsSPOP    228 MFEHEMEESKK--NRVEINDVEPEVFKEMMCFIYTGKAPN-LDKMADDLLAAADKYALER
DmKELCH   185 MFT-SFEESRQ--ARITLQSVDARALELLIDYVYTATVEV-NEDNVQVLLTAANLLQLTD
HsTIP     114 LFTSGAVVDQQ--NVYEIDFVSAEALTALMDFAYTATLTV-STANVGDILSAARLLEIPA

GMPOZ     258 CMRHCSQLLRSLP
AtPOB1    242 CMRYCSRLLRNMP
HsSPOP    285 LKVMCEDALCSNL
DmKELCH   241 VRDACCDFLQIQL
HsTIP     171 VSHVCADLLDRQI
```

cytoskeleton and chromatin. However the BTB/POZ domain in the Drosphilia Bric-a-brac proteins has recently been reported to interact with elements of the basal transcriptional machinery suggesting that this domain can perform varied functions in transcriptional complexes (Pointud et al., 2001).

Although the protein databases list nearly 80 plant BTB/POZ domain-containing proteins there exists functional information for just two of these factors, both *Arabidopsis* proteins. The NPR-1 protein interacts with a b-ZIP transcription factor and is thought to function as a transcriptional regulator in the induction of systemic acquired resistance (Zhang et al., 1999) while the RPT2/NPH3 factors are related signalling factors recently found to be involved in the control of phototropism (Sakai et al., 2000). In animal proteins, the BTB/POZ domain forms homo- and hetero-dimers resulting in a peptide binding groove known to mediate interactions with other proteins. For example the BTB/POZ domain-dimer formed by the human transcriptional repressor PLTZ is known to recruit histone deacetylase 1 (Lin et al., 1998). A comparison of the BTB/POZ domain of GMPOZ with a selection of related full-length plant and animal relatives is shown in Figure 5.1B. Multiple, partial cDNAs related to GMPOZ have been identified in cereals, tomato, soy and cotton however no experimental work has been conducted on these genes. A number of conserved residues in the BTB/POZ domain of GMPOZ have been mapped as key components of the crystal structure of the PLTZ protein dimer (Ahmad et al., 1998) and the structural significance of these residues is also indicated in Figure 5.1.

The leucine-acidic domains of GMPOZ are similar to the LXXLL motifs which feature in animal steroid receptor coactivators (SRCs) and the general nuclear co-activators such as CREB-binding protein (CBP). These motifs have been characterised as the invariant residues in protein docking domains which mediate interactions of the co-activator with its cognate nuclear receptor, in a manner which promotes the transcriptional activity of the nuclear receptor (for a review see Leo and Chen, 2000). The presence of these LXXLL motifs and the BTB/POZ domain in GMPOZ, coupled with the knowledge that the COOH-terminal half of GMPOZ binds to a transcription factor, GAMYB, in yeast-two-hybrid assays suggest that GMPOZ may be a

transcriptional regulator of some kind involved in GA-signalling. The results of a series of experiments designed to test this idea are described below.

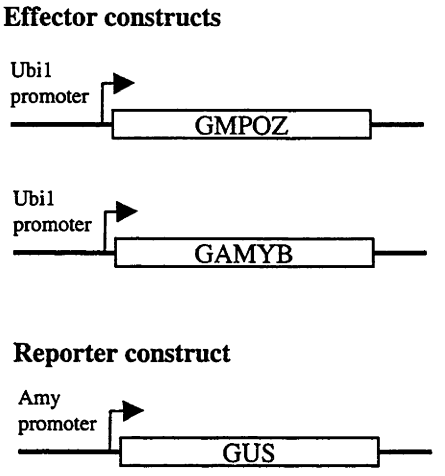
(5.3.2) Transient expression of GMPOZ does not influence α-amylase promoter activity or GAMYB function in barley aleurone

To assess whether GMPOZ influences GA-signalling leading to α-amylase gene expression, the effect of transient expression of GMPOZ on α-amylase promoter activity and GAMYB function was tested in co-bombardment assays. The Ubi1.GMPOZ construct was co-bombarded into barley aleurone with either the Amy.GUS reporter construct (described in section 4.3.2) or with the Amy.GUS construct plus the Ubi1.GAMYB effector construct (also described in section 4.3.2.). As shown in Figure 5.2, the GA-induced activity of the α-amylase promoter was unaffected by transient overexpression of GMPOZ and the GAMYB-mediated transactivation of the α-amylase promoter was likewise unaffected in both GA- and non-GA treated aleurone tissue. These results do not necessarily preclude GMPOZ from a role in GA-regulated gene expression because GMPOZ levels may not be limiting under these experimental conditions. In this case, overexpression of GMPOZ would not be expected to alter the processes under consideration.

(5.3.3) Transient expression of inverted-repeat RNA from the GMPOZ gene alters hormonally-regulated promoter activity in barley aleurone.

As described above (section 5.1) it has recently been shown in cereals that it is possible to selectively silence gene expression by transient expression of inverted-repeat RNA structures, predicted to form dsRNA, against the gene of interest (Schweizer et al., 2000; Zentello et al., 2001). Given that the overexpression of GMPOZ does not influence the processes of interest a gene-silencing approach was adopted. The effect on various reporter genes of the expression of inverted-repeat RNA from the GMPOZ gene in bombarded barley aleurone was tested. A construct was assembled (S4.GMPOZir; section 5.2) which produces inverted-repeat RNA from a 368 bp fragment of the GMPOZ gene from the 5' end of the ORF, upstream of the conserved regions in the BTB/POZ domain (Figure 5.3A). The effect of transient expression of inverted-repeat GMPOZ RNA on the activities of Amy.GUS, the ABA-inducible

A



B

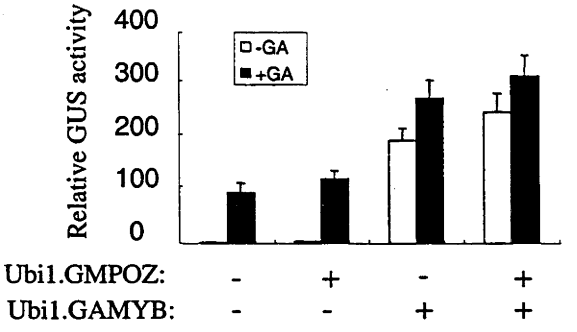
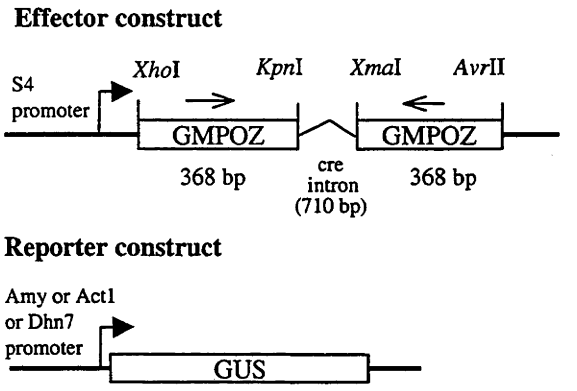


Figure 5.2. Effect of transient expression of GMPOZ on the activity of the α-amylase promoter and GAMYB function.

(A) Structure of the effector and reporter constructs used in the co-bombardment experiment.

(B) Embryo-less half seeds were co-bombarded with gold particles coated with 1 μg of the Amy.GUS reporter with or without 0.5 μg of the Ubi1.GMPOZ effector and/or 1 μg of the Ubi1.GAMYB effector constructs. Bars represent GUS activities ± standard error after a 24 h treatment with or without 10^{-6} M GA_3.

A



B

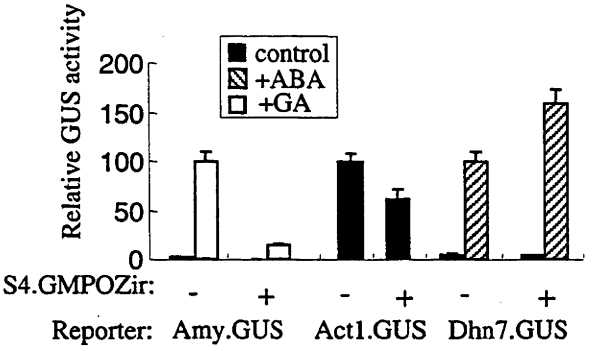


Figure 5.3. Effect of transient expression of inverted-repeat RNA from the GMPOZ gene on promoter activities in barley aleurone cells.

(A) Structure of the effector and reporter constructs used in the co-bombardment experiment. The effector s4.GMPOZir comprises a 368 bp cDNA fragment corresponding to amino acids 55-180 of GMPOZ cloned in opposing orientations on either side of a 710 bp cre intron. Transcription of the resulting inverted repeat RNA (ir) is driven by a constitutive promoter from the potato stunt 4 virus.

(B) Embryo-less half seeds were co-bombarded with gold particles coated with 1 μg of the reporter construct with or without 2.0 μg of the S4.GMPOZir effector. Bars represent GUS activities ± standard error after a 24 h treatment with or without 10^{-6} M GA_3 or 5 x 10^{-5} M ABA. Each promoter activity is normalised to the corresponding inducible or constitutive activity detected in the absence of the effector construct.

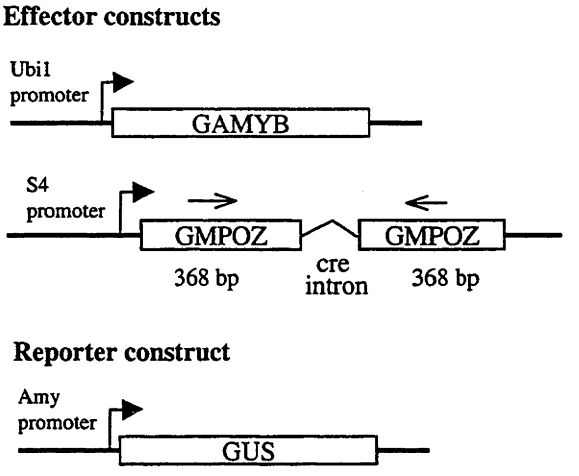
barley dehydrin7 promoter (Dhn7.GUS) and the constitutive barley actin1 promoter (Act1.GUS) was tested by co-bombardment assays in barley aleurone (reporter constructs are described in section 4.3.2).

As shown in Figure 5.3B, transient expression of GMPOZ inverted-repeat RNA reduced GA-induced Amy.GUS activity by 85% and Act1.GUS activity by 38%. By contrast the ABA-induced activity of the Dhn7.GUS reporter construct increased by 59%. These results indicate that GMPOZ is involved in hormonally-regulated gene expression in aleurone and has opposing roles, as an activator and repressor respectively, in the expression of GA- and ABA-regulated genes. The effect of GMPOZ inverted-repeat RNA on the constitutive activity of the actin1 promoter is relatively small compared to the effect on the α-amylase promoter but is significant. However this effect is unlikely to be a result of non-specific repression of transcription in this system because Dhn7 promoter activity was enhanced in the presence of inverted-repeat RNA from the GMPOZ gene.

(5.3.4) Transient expression of inverted-repeat RNA from the GMPOZ gene does not alter GAMYB function

In order to try and more precisely define at what level the GMPOZ factor operates in the GA-signalling pathway leading to α-amylase expression, and with the knowledge that GMPOZ binds to GAMYB in yeast, the effect of GMPOZ inverted-repeat RNA on GAMYB function was tested. The S4.GMPOZir effector construct and the Ubi1.GAMYB effector construct were co-bombarded into barley aleurone along with the Amy.GUS reporter construct. As shown in Figure 5.4B, in the presence of GA, the expression of inverted-repeat GMPOZ RNA did not alter the degree to which the α-amylase promoter was transactivated by overexpressed GAMYB, irrespective of the amount of Ubi1.GAMYB effector used. In the absence of GA, expression of GMPOZ inverted-repeat RNA resulted in an increase of 47% in GAMYB transactivation of the α-amylase promoter. Although the two results differ by virtue of an unknown effect of GA on the experimental system, they do both show unambiguously that the repression of α-amylase promoter activity by GMPOZ inverted-repeat RNA does not occur at the level of GAMYB function

A



B

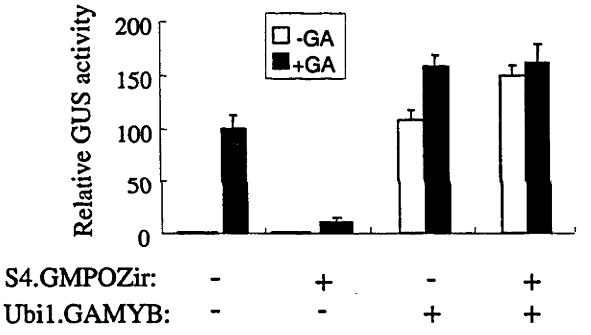


Figure 5.4. Effect of transient expression of inverted-repeat RNA from the GMPOZ gene on GAMYB function in barley aleurone cells.

(A) Structure of the effector and reporter constructs used in the co-bombardment experiment.

(B) Embryo-less half seeds were co-bombarded with gold particles coated with 1 μg of the Amy.GUS reporter construct with or without 2.0 μg of the Ubi1.GMPOZir effector and/or 0.5 μg of Ubi1.GAMYB effector. Bars represent GUS activities ± standard error after a 24 h treatment with or without 10^{-6} M GA_3.

(5.3.5) A GFP:GMPOZ fusion localises to the nucleus in barley aleurone protoplasts

The cellular distribution of GMPOZ was investigated by transfecting barley aleurone protoplasts with a construct expressing a GFP:GMPOZ fusion under the control of the maize ubiquitin 1 promoter (Ubi1.GFP:GMPOZ; section 5.2). Protoplasts were incubated for 20 h then examined using fluorescence microscopy. As shown in Figure 5.5, in protoplasts transfected with the GFP:GMPOZ expression construct, the GFP signal was predominantly localised in the nucleus. By contrast, protoplasts transfected with a construct producing GFP alone showed a characteristic distribution for this protein (Haseloff and Amos, 1995), with signal detected in cytosolic and nuclear regions, but not within the prominent protein storage vacuoles. The distribution of the GFP:GMPOZ fusion protein was unchanged by treatment of the transfected protoplasts with 10^{-6} M GA_3 or 5 x 10^{-5} M ABA during the final 6 h of the 20 h incubation period (results not shown).

(5.3.6) Expression of the GMPOZ transcript in barley

The expression of the GMPOZ transcript was investigated in hormonally treated aleurone tissues from Himalaya as well as the *Sln1d* mutant, given that both α-amylase expression and GAMYB expression are known to be altered in this background (Gubler et al., 2002). A variety of other Himalaya tissues were also investigated. RNA gel blots of total RNA from these tissues were hybridised with an RNA probe corresponding to the full-length GMPOZ cDNA (see appendix).

As shown in Figure 5.6A, a single transcript of around 2.2 kb (comparable in size to the full-length GMPOZ cDNA), which was induced strongly by ABA or GA treatment, was detected in Himalaya aleurone. In the *Sln1d* background the transcript was induced to a similar degree in aleurone treated with GA at a concentration of 10^{-9} M. However, at a GA concentration of 10^{-6} M induction was around 75 % weaker than that observed in wildtype tissue. As a positive control for hormone treatments, the same RNA samples were hybridised with a DNA probe corresponding to the high pI barley α-amylase clone, HV19 (Chandler et al., 1984). As previously observed, the α-

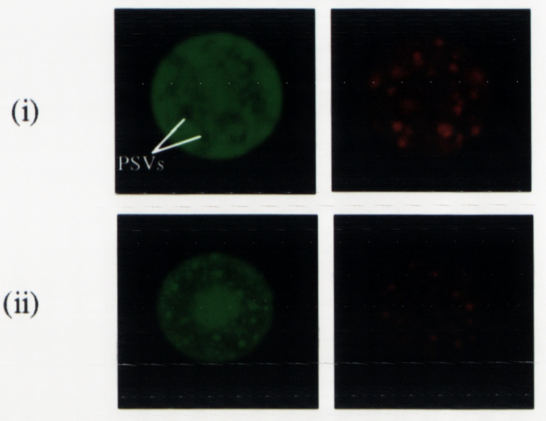


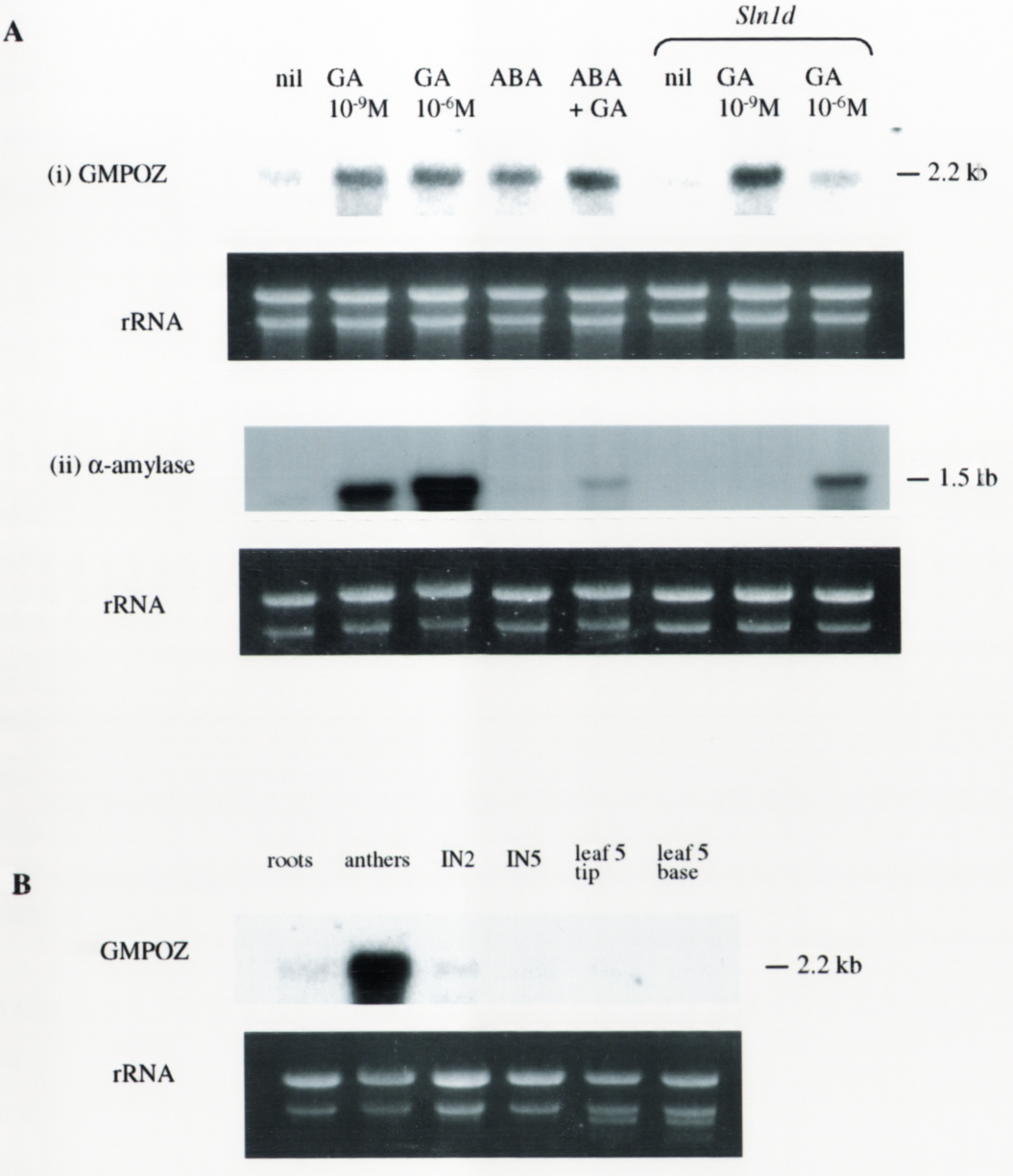
Figure 5.5. Cellular localisation of GMPOZ in barley aleurone protoplasts.

Distribution of fluorescence in a single barley aleurone protoplast transfected with a construct encoding GFP alone (i) or a GFP:GMPOZ fusion protein (ii). Right panels show distribution of auto-fluorescence. PSVs = protein storage vacuoles

Figure 5.6. Expression of the GMPOZ transcript in barley tissues

(A) RNA gel blot of RNA from hormonally treated wildtype barley and *Sln1d* aleurone layers probed with the full-length GMPOZ cDNA *(i)* or a barley α-amylase partial cDNA (HV19) *(ii)*. Hormone treatments represent 6 h incubations and GA_3 and ABA were used at concentrations of 1×10^{-6} M or 5×10^{-5} M respectively unless otherwise specified.

(B) RNA gel blot of RNA from a variety of barley tissues probed with the full length GMPOZ cDNA. IN2 = elongated internode (second from base of stem), IN5 = elongating internode 5 (harvested from top of stem).


A
Sln1d
nil
GA 10-9M
GA 10-6M
ABA
ABA + GA
nil
GA 10-9M
GA 10-6M
(i) GMPOZ
— 2.2 kb
rRNA
(ii) α-amylase
— 1.5 kb
rRNA
B
roots
anthers
IN2
IN5
leaf 5 tip
leaf 5 base
GMPOZ
— 2.2 kb
rRNA

amylase transcript was strongly induced in wildtype aleurone by GA treatment but repressed by applied ABA. In the *Sln1d* mutant, the aleurone possesses reduced sensitivity to GA, and correspondingly the α-amylase transcript was induced only when treated with higher concentrations of GA. To ensure the pattern of GMPOZ expression in aleurone was reproducible, the RNA gel blot which had been hybridised with the α-amylase probe was stripped and rehybridised with the GMPOZ probe. Identical results, as per Figure 5.6.A, were obtained (not shown).

A Northern blot of total RNA from Himalaya roots, developing anthers (approximately 5-7 mm long), an elongated internode (second from base of stem), an elongating internode (top-most internode) and the tip and base of leaf 5 was also hybridised with a full-length GMPOZ DNA probe. The barley plant from which non-root tissues were derived was 7 weeks old, while root material was derived from seeds germinated on water for 5 d in the dark. As shown in Figure 5.6B, the GMPOZ transcript was detected at very high levels in developing anthers and, on the original blot, was faintly detectable in roots and the elongated internode samples (not visible in reproduction). The non-aleurone and aleurone RNA samples in Figure 5.5A and Figure 5.5B were hybridised on the same blot so the relative expression levels between these tissues are directly comparable.

(5.4) Discussion

This chapter describes the cloning and characterisation of a new BTB/POZ nuclear factor from aleurone, GMPOZ, which appears to be involved in hormonally regulated gene expression. This work was prompted by the initial finding that GMPOZ, as a partial clone, interacts with GAMYB in yeast and the observation that the GMPOZ protein contains a number of features characteristic of a transcriptional regulator, including a BTB/POZ domain and leucine-acidic domains.

Two key lines of experimental evidence were obtained which support the idea that GMPOZ functions as a transcriptional regulator in hormonal signalling in aleurone. Firstly, the transient expression of inverted-repeat RNA from the GMPOZ gene, which would be expected to silence the expression of this gene, substantially repressed the

activity of the α-amylase promoter while it enhanced the ABA-induction of the dehydrin7 promoter. This result strongly implicates GMPOZ in the transmission of hormonal signals leading to gene expression in this tissue and suggests that GMPOZ ordinarily functions as an activator of GA-regulated gene expression and a suppressor of ABA-regulated gene expression. The mechanisms which might underpin this dual activity are discussed in the following chapter. The second line of evidence that is consistent with the idea that GMPOZ operates as part of transcriptional complexes lies in the cellular localisation observed for a GFP:GMPOZ fusion in barley aleurone protoplasts. The strong nuclear compartmentalisation of this fusion protein places GMPOZ directly in the vicinity of transcriptional events.

With the knowledge that the COOH-terminal half of GMPOZ interacts with GAMYB in yeast it was predicted that GMPOZ might be involved in regulating α-amylase promoter activity at the level of GAMYB function. However, the transient expression of inverted-repeat RNA from the GMPOZ gene did not repress the transactivation of the α-amylase promoter by GAMYB, suggesting that GMPOZ functions upstream of these events. To reconcile this result with the yeast two-hybrid data would require that GAMYB itself also operates upstream of α-amylase transactivation – an intriguing possibility that remains untested. One possible scenario is that GAMYB activates its own expression in conjunction with GMPOZ, which would be consistent with the observed requirement that GMPOZ must be expressed in order for the α-amylase promoter to be responsive to GA. Certainly there are known instances of MYBs autoactivating their own promoter or participating in feedback loops that enhance their own gene expression (Schaffer et al., 1998; Sala et al., 1999).

On RNA gel blots, the GMPOZ transcript in Himalaya aleurone is expressed at barely detectable levels but is strongly induced in tissue which had been treated for 6 h with either ABA or GA. This is a somewhat unexpected expression pattern for a factor that exerts counteractive effects on ABA- and GA-regulated gene expression but equally it is possible that a wider sampling of hormonal concentrations, and time points, may reveal more differentiated expression patterns for this gene. It could be that the repressive function of GMPOZ on certain ABA-regulated genes might be alleviated after this 6 h time point, or alternatively, that post-transcriptional controls on GMPOZ

gene expression or GMPOZ function are involved. If the observed pattern of hormonal regulation of GMPOZ RNA is preserved in aleurone tissue under various conditions, such as variable durations of hormone treatment, a novel pattern of gene expression would have been identified, that is, two antagonistic hormones upregulating expression of a common factor. Discussion of this possibility is reserved for the following chapter.

The expression of the GMPOZ transcript in aleurone tissue derived from *Sln1d* grain reveals a potentially interesting interaction between the GA-control of GMPOZ expression and SLN function. Like the wildtype, in *Sln1d* the GMPOZ transcript was strongly induced in aleurone tissue treated for 6 h with 10^{-9} M GA. However unlike the wildtype, an inhibition in expression of the GMPOZ transcript was observed in tissues treated with GA at the highest concentration used, 10^{-6} M. By contrast the effect of *Sln1d* on α-amylase gene expression is relatively straightforward, that is the reduced GA-sensitivity of this mutant means that the induction in α-amylase mRNA is correspondingly less GA-sensitive. The complexity of the interaction between *Sln1d* and control of GMPOZ gene expression may in part relate to the fact that both ABA and GA appear to influence GMPOZ gene expression but the mechanisms possibly underlying these events remain for the moment opaque. Alternatively, the complexity of this gene expression profile may be the result of differential expression of closely related members of a gene family, but this has yet to be established.

Concluding remarks

While these results clearly implicate GMPOZ in hormone-signalling leading to gene expression in barley aleurone they also raise a number of questions as to the exact mechanisms through which this occurs, particularly with respect to possible functional interactions between GMPOZ and GAMYB. Likewise, as described in the previous chapter, the precise involvement of another GAMYB-binding factor, KGM, in the regulation of GAMYB function also awaits a fuller characterisation.

It is apparent that more remains to be unravelled in aleurone about GA-signalling and the transcriptional complexes targeted by this pathway. The identification of a new set of potential signalling factors and transcriptional regulators, including KGM and

GMPOZ, provides a framework for developing a more sophisticated understanding of these processes. The following chapter contains a general discussion of the broader significance of the results described in this dissertation to our understanding of GA-signalling. Some avenues for further work in this field, which are suggested by this study, will also be considered.

Chapter 6

General Discussion

(6.1) Isolation of novel GA-signalling factors from barley aleurone

The delineation of plant hormone signalling pathways provides a mechanistic insight into the powerful effects hormones exert on plant growth and development and suggests ways in which plants might be manipulated for agronomic advantage. The investigation of the hormonal control of cereal aleurone layer function is a dynamic area of research within this field. The experimental work described in this dissertation was directed toward the identification of novel components of the signalling pathway in aleurone leading to GA-regulated expression of the gene encoding the key hydrolytic enzyme, α-amylase. A common strategy to identify novel factors in a signalling pathway involves searching for proteins which interact with known components from that pathway. Accordingly, a well characterised transcriptional activator from this pathway, the barley GAMYB transcription factor, was selected for this purpose. A yeast two-hybrid screen of barley aleurone cDNA expression libraries for GAMYB-binding proteins was conducted.

The yeast two-hybrid screen generated a sizeable pool of GAMYB-binding partners and the sequence of four of these factors, all of them newly described genes, revealed features consistent with a potential function in signalling pathways which target transcriptional complexes. The four clones of interest included a MAP-kinase like factor, a protein resembling the SKIP animal nuclear co-activator and proteins containing a WD40 domain or a BTB/POZ domain, domains both known to provide interfaces for protein-protein interactions. Full-length cDNA clones encoding the MAP-kinase like factor, termed KGM (*kinase associated with GAMYB*) and the BTB/POZ domain protein, termed GMPOZ (*GAMYB associated POZ-domain factor*) were isolated and accordingly the potential functional role of these factors in GA-signalling was investigated further (see Chapter 3 and Chapter 4). The following sections contain a general discussion of these results and their potential significance to our understanding of GA-signalling.

(6.2) Role of KGM in GA-signalling in barley aleurone

As described in Chapter 3 the GAMYB-binding protein, KGM is located both in the nucleus and cytosol of barley aleurone cells and appears to function in this system as a repressor of the GA-signalling pathway leading to α-amylase expression. The observed repression occurs directly through an inhibition of GAMYB function. As yet, the phosphorylation of GAMYB by KGM *in vitro* has not been demonstrated. The presence of a conserved TXY regulatory motif in subdomain VIII of KGM, which must be phosphorylated in catalytically active MAP-kinases, is necessary to the repressive effect of KGM on GAMYB function. However, it is unknown to what extent MAKs parallel MAP-kinases in their mode of activity and regulation. The transcription of the KGM gene is not strongly regulated in aleurone tissue treated with GA or ABA for 6 h and it is therefore inferred that KGM is likely to be controlled through post-transcriptional mechanisms.

There now exist multiple precedents for the regulation of MYBs by kinases. In animals, serine/threonine phosphorylation of B-Myb affects its transactivation potential and DNA binding activity (Johnson et al., 1999) while protein stability of c-Myb has been shown to be modulated by phosphorylation (Bies et al., 2001). The phosphorylation of c-Myb by a MAP-kinase has also been demonstrated *in vitro*, and *in vivo* evidence suggests that this modification is functionally important in mediating interactions with a negative regulator (Vorbrueggen et al., 1996). There is also evidence for the phosphorylation of plant MYBs. Interactions between subunits of the *Arabidopsis* protein kinase CK2 and the MYB, CCA1 (circadian clock- associate 1) have been demonstrated in yeast (Sugano et al., 1998). The CCA1-CK2 interactions were found to stimulate DNA binding of CCA1 *in vitro* while inhibition of CK2 kinase activity was found to inhibit CCA1-DNA binding in plant cell extracts, suggesting that both protein interactions and phosphorylation of the MYB are integral to this particular MYB-kinase interaction. In *Antirrhinum*, two MYBs with different transactivation potentials target the same promoter sites in genes involved in phenylpropanoid metabolism but differential phosphorylation is thought to alter the competitive DNA-binding of these factors resulting in subtle modulations of gene expression (Moyano et al., 1996).

These observations about the regulation of MYBs by kinases suggest a number of mechanisms through which KGM might negatively regulate GAMYB function. These include phosphorylation-mediated changes in protein stability, DNA-binding or transactivation potential, and the promotion of interactions with other negative regulators. Phosphorylation is also a key mechanism in the control of nuclear import of proteins by altering the affinity of 'cargo' proteins for their cognate receptor within the nuclear pore complex (Kaffman and O'Shea, 1999). It is possible that import of GAMYB into the nucleus might be inhibited by phosphorylation. A number of consensus MAP-kinase phosphorylation sites, which by analogy might be targets for a MAK-like activity, are distributed throughout the GAMYB protein. Transiently expressed KGM can still repress the transactivation potential of a COOH-terminal truncated form of GAMYB suggesting that the DNA-binding activity of GAMYB might be the target of KGM action because the consensus phosphorylation sites in the truncated GAMYB protein cluster in or near the NH_3-terminal DNA-binding domain. Alternatively, protein-protein interactions between KGM and GAMYB might be sufficient to modulate GAMYB function, such as seems to be the case with the CCA1-CK2 interaction (see above).

A key finding of this work regarding the operation of KGM in GA-signalling is that there appear to be two pathways through which GAMYB is negatively regulated. It was previously known that GAMYB expression is repressed at the transcriptional level both by SLN1 and through the ABA regulated kinase, PKABA1 (Gomez-Candenas et al., 2001; Gubler et al., 2002). A second, post-translational pathway involving direct repression of GAMYB function by KGM has now been identified. The existence of multiple negative controls on GAMYB, both at the level of expression and function, probably highlights the importance in aleurone of the tightly co-ordinated expression of hydrolytic enzymes after the onset of germination.

While the 'derepression' model of GA-signalling in aleurone centres on the GA-mediated degredation of the SLN1 protein, a parallel understanding of how KGM is regulated is lacking. Indeed, the exact window of time in aleurone in which KGM is ordinarily active is not yet known although it would be predicted that KGM is likely to function early in post-germination events before being deactivated by a GA-related

signal. The substantially unregulated pattern of KGM mRNA expression in hormonally treated aleurone suggests that this control is post-transcriptional. A GFP:KGM fusion in transfected aleurone protoplasts was unaffected by GA-treatment indicating that KGM is not regulated at the level of protein either, although this result needs to be confirmed where expression of the fusion protein is directed by the KGM promoter rather than a ubiquitin promoter.

It is possible that a phosphorylation cascade might instead regulate KGM function. Relatively little is understood of kinase cascades in plants although the components of a candidate MAP-kinase signalling module have been identified in *Arabidopsis* and many individual plant MAP-kinases have been identified (Meskiene and Hirt, 2000). By analogy, known mechanisms of MAP-kinase inactivation may inform predictions about what the mechanism of KGM inactivation might be. Work in animal and yeast systems suggests that dephosphorylation of the TXY motif in subdomain VIII by dual-specificity phosphatases (DsPTPases), tyrosine phosphatases or serine/threonine phosphatases (of the PP2A and PP2C types) is a central mechanism for MAP-kinase inactivation (Keyse, 2000). Plant phosphatases of this kind are now being described which can dephosphorylate and inactivate plant MAP-kinases *in vitro* (Meskiene and Hirt, 2000).

Although little is yet known about how the MAKs are regulated, given the importance of the $TEY_{(158)}$ motif in transiently expressed KGM to the repression of GAMYB function, it does seem likely that kinase and phosphatatase activities directed towards this motif in KGM might regulate the activity of this protein. The fact that the PP1, PP2A and PP2B inhibitor, okadaic acid inhibits many of the characteristic GA-responses in wheat aleurone, including α-amylase gene expression (Kuo et al., 1996) provides biochemical evidence consistent with the idea that a PP2A-type MAP-kinase inactivator directed toward KGM could be involved in derepressing GA-signalling. A protein phosphatase expressed in cereal aleurone has not yet been conclusively identified. A rice gene encoding a PP2A and which is expressed in embryoless half grains has been cloned, however the expression of this gene is not GA-regulated (Chang et al., 1999).

The regulated localisation of a kinase within a cell necessarily modulates its function yet nothing is currently known about the subcellular localisation of MAKs. The observed nuclear and cytoplasmic localisation of a GFP:KGM fusion protein in barley aleurone protoplasts is identical to that of a classical MAP-kinase which targets transcription factors. One of the most widely studied animal MAP-kinases, ERK2 (extracellular signal related kinase 2) undergoes signal-dependent translocation from the cytoplasm to the nucleus (Chen et al., 1992). Structural studies suggest that active MAP-kinases, including ERK2, dimerize upon dual phosphorylation (Canagarajah et al., 1997). Microinjection studies of mutant forms of ERK2 showed that phosphoryation at the conserved TXY motif in subdomain VIII is a requirement for translocation and that unphosphorylated forms of ERK2 are only transiently located in the nucleus (Khohklatchev et al., 1998). These authors suggest that unphosphorylated, inactive MAP-kinases might generally be small enough to diffuse freely into the nucleus but are then rapidly exported. The larger, active MAP-kinase dimers might instead to be translocated through a nuclear import mechanism and retained in the nucleus, possibly by virtue of concealment of a putative nuclear export signal within the dimer structure. Like KGM, the MAP-kinases do not possess a recognisable nuclear localisation signal (NLS) but it is possible that kinase dimers bind directly to the nuclear import machinery or associate with other proteins that possess a NLS.

Relatively little is known about the cellular localisation of MAP-kinases in plant cells. One study has reported nuclear and cytoplasmic immunofluoresence in onion root meristem cells and pepper vacuolate pollen microspores incubated with a polyclonal antibody raised against a tobacco MAP-kinase (Prestamo et al., 1999). It would be of interest to monitor the distribution of the GFP:KGM fusion protein, expressed under the control of the KGM promoter, in hormone stimulated aleurone protoplasts. Also, it is possible that mutations in the $TEY_{(158)}$ regulatory motif in subdomain VIII of KGM, which would be predicted to prevent phosphorylation at these sites, might prevent stable nuclear localisation of this protein, such as is the case for ERK2. While more remains to be understood, the observed cytoplasmic and nuclear distribution of KGM does clearly suggest that this factor could transmit signals between these two cellular compartments, and provides some insight into GA-signalling in aleurone at a level in the pathway that has previously been poorly understood.

A revised model of aleurone GA-signalling, which incorporates the findings presented in this dissertation about the regulation of GAMYB function by KGM is presented in Figure 6.1. KGM is shown as operating within an independent pathway, possibly a phosphorylation cascade, which directly represses the GAMYB transactivation of the α-amylase promoter. The possibility that a GA-stimulated phosphatase inactivates KGM is indicated. In section 6.4 some further avenues for the investigation of KGM function, which are suggested by these results, are considered.

(6.3) Role of GMPOZ in GA-signalling

As described in Chapter 4, a second GAMYB-binding protein from the yeast two-hybrid screen, the BTB/POZ domain protein GMPOZ, was found in aleurone to function both as a nuclear localised activator of GA-inducible α-amylase promoter activity, and a repressor of ABA-inducible dehydrin7 promoter activity. However, within the GA-signalling pathway, the effect of GMPOZ on α-amylase gene expression appears to occur upstream of GAMYB transactivation of the α-amylase promoter. The expression of GMPOZ mRNA was found to be induced in aleurone tissues treated for 6 h with both ABA and GA_3.

This work has identified a new factor which is likely to function as a co-activator within a transcriptional complex that includes GAMYB, and which is required for GA-inducible α-amylase gene expression. Since GMPOZ does not itself possess a recognisable DNA-binding domain it is envisaged that GMPOZ operates within such a transcriptional complex at a higher order level of the structure. A number of possible mechanisms through which GMPOZ might mediate GAMYB function are suggested by observations about BTB/POZ domain transcriptional regulators in other systems. The BTB/POZ domain in the Drosphila Bric-a-brac proteins is known to interact with elements of the basal transcriptional machinery while the human BTB/POZ domain, PLZF, is a transcriptional regulator known to recruit histone deacetylase 1 (Lin et al., 1998; Pointud et al., 2001). By implication, it is possible that GMPOZ might operate by mediating interactions between GAMYB and the basal transcriptional machinery or by perhaps recruiting a chromatin remodelling activity.

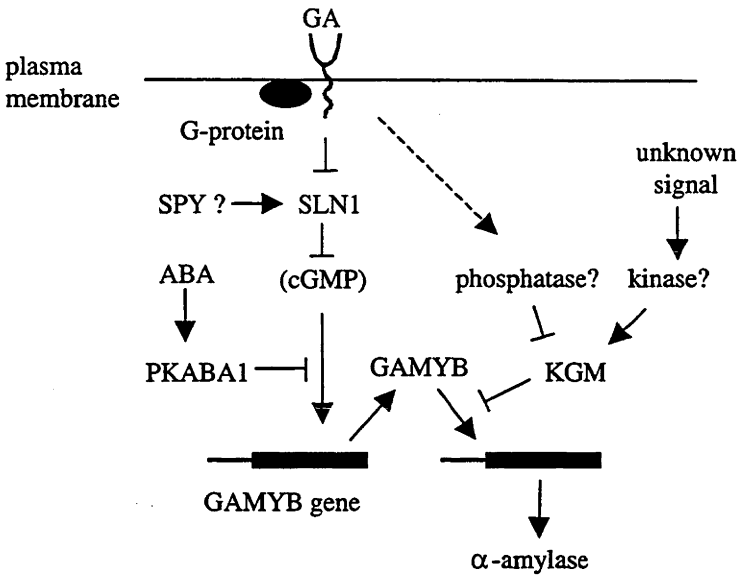


Figure 6.1. Proposed role of KGM in GA-signalling in aleurone.

The presence of a second pathway which negatively regulates GAMYB, through KGM-mediated repression of GAMYB function is shown. The possible involvement of a GA-stimulated phosphatase in the inactivation of KGM is indicated.

The immediate target of the proposed GMPOZ-GAMYB transcriptional complex remains uncertain because GMPOZ is not itself required for GAMYB transactivation of the α-amylase promoter, suggesting that another upstream promoter may be the site of action of this complex. As previously noted, this raises the possibility that GAMYB is involved in a positive feedback loop which targets the GAMYB promoter. The 5' flanking sequence 1.5 kb upstream of the GAMYB transcription start site has been isolated from barley (F. Murray, CSIRO Canberra, personal communication). An eight nucleotide GAMYB consensus binding site has been defined on the basis of *in vitro* interactions between GAMYB and oligonucleotides (Gubler et al., 1999) and a number of sites corresponding closely to the consensus (C/T A A C C/G G/A A/C C/A) occur within the GAMYB promoter (Figure 6.2A). One of these GAMYB-like binding sites, TAACAAAT, is situated within a region of the GAMYB promoter which strongly resembles the structure of the GARC from the promoter of the barley high pI α-amylase gene, HV19 (Gubler and Jacobsen, 1992). Like the HV19 promoter, this region in the GAMYB promoter contains a TATCCAC-like box 11-bp downstream of the MYB binding site (the GARE in HV19) and a pyrimidine box around 20 bp upstream. Also consistent with the idea that GAMYB might transactivate its own promoter is the observation that low amounts of GAMYB protein are expressed in non GA-treated barley aleurone tissues (Gubler et al., 2002). It is possible these basal levels of GAMYB could initiate autoactivation of GAMYB provided GMPOZ is present.

The mode of regulation of GMPOZ is for the moment somewhat uncertain although there may be important levels of control operating at the level of transcription because GMPOZ mRNA expression was strongly induced by 6 h in GA- and ABA-treated aleurone. The relative timing of expression of GMPOZ and GAMYB in aleurone is not yet known. It may be important to determine whether GMPOZ expression precedes or coincides with GAMYB expression, which, at the mRNA level, reaches 75% of its maximum expression in GA-treated barley aleurone after 3 h (Gubler et al., 1995). Certainly, the expression of a number of animal BTB/POZ domain factors is known to be regulated at the transcriptional level. For example, expression of the mammalian MAZR and Bach 2 transcripts is differentially regulated according to the stage of B-cell development in mice cells (Kobayashi et al., 2000).

Figure 6.2. Proposed role of GAMYB-binding proteins in GA-signalling in aleurone.

(A) Position of three GAMYB consensus binding sites and GARC-like elements in the 5' flanking sequence of the GAMYB gene. Numbering refers to distance in bp from the transcription start site. Score relative to consensus: 1 - 7/8 matches; 2 - 8/8 matches; 3 - 6/8 matches. Consensus: C/T A A C C/G G/A A/C C/A. The pyrimidine box and TATCCAC-like element are bolded.

(B) Simplified model of GA-regulation of α-amylase gene expression highlighting the proposed role of GMPOZ and KGM. A positive feedback loop for GAMYB expression involving autoactivation by GAMYB in concert with GMPOZ is shown. This loop could be stimulated by GA-induction of GMPOZ expression. KGM represses GAMYB transactivation of the α-amylase promoter. It is not yet understood how known repressors of GAMYB expression such as SLN1 or ABA might interact with this pathway.

A

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-1018  GATGCACGGA GATACCCAAG ATGGCAATGC ATCTCTAGCT TTTACTAAGT
-968   TTTAACCCAA[1] GTTGGGCCTA GTTTGTGTGT CTAGTTACAT CCATGTACTA
-918   CCTTGTCTCT ACCACCGGCG CGCGTCTCTT TCTTGCAAAC [2]CAACCAACAC
-868   CCGGTAACAA[3] ATGGCCTTCT TCTCATCCAC TGTTCTCTCG ACGTATACTT
-818   ACTTACTATA CAAAACAAAG ACTGGCGTAG GTAGGCGCTA TTGACGTCGC
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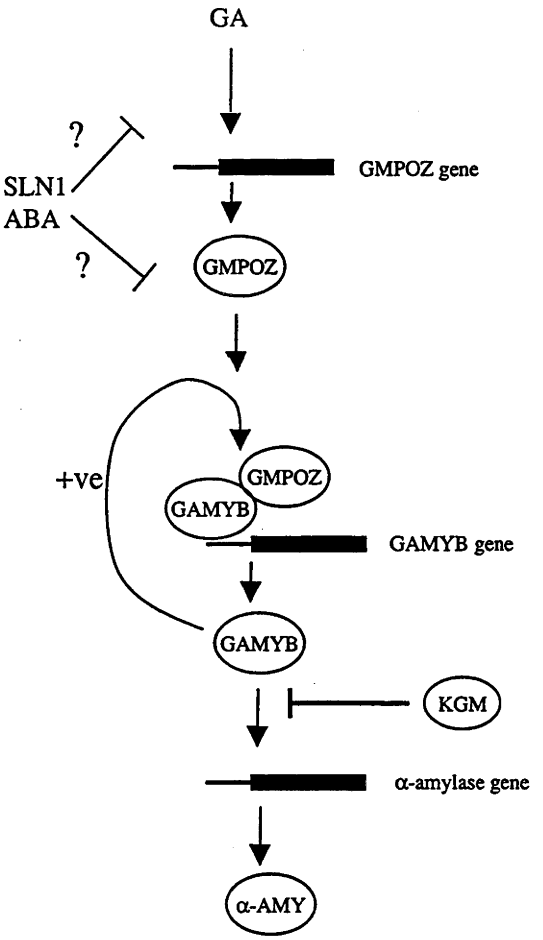
B



That ABA and GA, two antagonistic hormones, induce transcription of a common gene in aleurone is a novel expression pattern although the possibility that differential control might be exerted at other timepoints cannot be excluded. In this study GMPOZ was observed to exert counteractive effects on hormonally regulated gene expression in barley aleurone – it is required for the GA-induction of α-amylase promoter activity but conversely, appears to repress the ABA-induction of the dehydrin7 promoter. This parallels a recent finding in animal systems. The mammalian Bach2 BTB/POZ protein functions on its own as a transcriptional repressor of the IgH (immunoglobulin heavy chain) gene but, as a heterodimer with the MAZR BTB/POZ protein, functions as a transcriptional activator of a gene involved in limb bud morphogenesis (Muto et al., 1998; Kobayashi et al., 2000). This indicates that the transcriptional activity of a particular BTB/POZ domain protein can be mediated by the formation of heterodimers with other BTB/POZ domains, in effect a mechanism for a type of combinatorial control on gene expression. It is possible that GMPOZ along with other BTB/POZ domain proteins in aleurone could function as so-called architectural transcription factors, that is transcription factors which instead of possessing an intrinsic repressive or promotive activity, instead function as a product of the architecture of a particular transcriptional complex. By implication, BTB/POZ proteins may target diverse arrays of genes and consequently might be expected to exhibit complex patterns of expression.

In Figure 6.2B a simplified model of GA-signalling in aleurone is presented which combines these ideas about the potential role of GMPOZ with the model of KGM function. In the proposed pathway, expression of the GMPOZ factor is induced by GA and GMPOZ then participates in a positive feedback loop with GAMYB which autoactivates its own promoter. GAMYB then transactivates the α-amylase promoter, a process which is negatively regulated by KGM. It cannot yet be predicted how the known negative regulators of GAMYB expression including SLN1 and PKABA1 might interact with this process.

(6.4) Further avenues for work in GA-signalling

The augmented model of GA-signalling in aleurone presented in this chapter, which incorporates the proposed function of the newly isolated GAMYB binding proteins,

generates a number of testable ideas and predictions. In addition, there exist obvious opportunities to extend the scope of this work beyond the aleurone, into other tissues and other plant species. Some suggestions for future work, which would represent a logical extension of the results described in this dissertation, are presented in the following sections.

Defining the role of GAMYB binding proteins within the aleurone

To better understand the role of the GAMYB binding proteins KGM and GMPOZ in GA-signalling in aleurone requires knowledge both of the precise nature of their functional interaction with GAMYB and of the upstream mechanisms which regulate these factors. Two key proposals about KGM function in aleurone have been generated. Firstly, that KGM represses GAMYB function probably by phosphorylating the transcription factor in a manner which inhibits its ability to transactivate GA-regulated promoters. Secondly, that KGM is regulated by opposing phosphatase and kinase activities that operate within a novel pathway, possibly a phosphorylation cascade, which represses GA-signalling. Two central ideas about the operation of GMPOZ have also arisen. Firstly that GMPOZ is required for GAMYB expression and participates with GAMYB in a positive feedback loop at the GAMYB promoter and secondly, that this process might be initiated by expression of GMPOZ very early in GA-stimulated aleurone tissue.

A number of experiments could be conducted to develop the idea that GAMYB is negatively regulated by phosphorylation by KGM. Firstly, the demonstration of phosphorylation of GAMYB by KGM *in vitro* would garner support for this idea but the conditions necessary to assay a MAK-type activity would need to be defined. If this could be achieved, an obvious experiment would be to determine whether phosphorylated GAMYB has an altered DNA-binding activity *in vitro*. Secondly, it is of some interest to determine in aleurone whether GAMYB is phosphorylated *in vivo* and if so, under what conditions. In this study, a preliminary experiment was conducted involving immunoprecipitation of GAMYB from non-GA treated, ^{32}P-labelled aleurone tissue but results were not presented because sensitivity was marginal, possibly because of the large endogenous pools of phosphate in this tissue. More work needs to be done to refine this experiment. Alternatively, further

correlative evidence for this idea might also be obtained by testing whether mutagenesis of the consensus MAP-kinase phosphorylation sites in GAMYB render it immune to repression by KGM in transient expression assays in aleurone.

Knowledge of the regulation of KGM should indicate under what conditions KGM ordinarily represses GAMYB function. A quest to identify the phosphatase and kinase which are proposed to regulate KGM activity is an obvious starting point. Given that the biochemical evidence suggests that a PP2A activity is necessary for positive transmission of the GA-signal in aleurone, this class of factor would be a first target. Candidate phosphatases could be identified on the basis of database searches or through aleurone cDNA library screens using degenerate probes or primers against conserved motifs. Alternatively, a second yeast two-hybrid screen using KGM as bait could reveal, among other potential regulators, a likely candidate. These candidates could be readily characterised using transient expression assays in aleurone to assess whether KGM repression of GAMYB function is alleviated by co-expression with the phosphatase of interest.

Although somewhat speculative, the hypothesis that GMPOZ participates in GAMYB autoactivation can readily be tested in co-bombardment assays in aleurone. The ability of transiently expressed GAMYB to activate the GAMYB promoter fused to a reporter gene could be assessed as could the effect of transient expression of GMPOZ inverted-repeat RNA on activity of the GAMYB promoter. The operation of the proposed GMPOZ-GAMYB transcriptional complex could also be investigated further by trying to identify the factors with which GMPOZ interacts. The results of a yeast two-hybrid screen could also provide clues about the proposed combinatorial control on gene expression that BTB/POZ domain proteins might exert in aleurone, especially since GMPOZ is also implicated in ABA-regulated gene expression. It is difficult to predict how GMPOZ might be controlled owing to the scarcity of data about the regulation of BTB/POZ domain proteins in other systems. The observed hormonal controls on GMPOZ transcription in aleurone are certainly avenues for further investigation, especially since transcriptional controls on the genes encoding BTB/POZ domain proteins have been identified in animal systems.

A further two GAMYB-binding proteins with a potential function in signal-dependent gene expression were also identified in the yeast-two hybrid screen (clones 3 and 5, Chapter 3), but a characterisation of these factors was beyond the scope of this study. The WD40 domain in clone 3 suggests this protein could function as a scaffold, perhaps mediating interactions between GAMYB and an upstream modifier. The close sequence relatedness of clone 5 to a known animal transcriptional co-activator indicates this factor could modulate GAMYB function within a transcriptional complex. It is envisaged that a similar experimental strategy as that used to characterise KGM and GMPOZ in this study could be adopted to assess the significance of these two factors in GA-signal transduction in aleurone.

Defining the role of GAMYB binding proteins beyond the aleurone

Given that both GMPOZ and KGM are expressed in barley tissues other than the aleurone it would be of interest to determine what roles, if any, these genes play in other plant processes, especially those known to be regulated by GA. GAMYB gene expression has previously been detected in barley aleurone (Gubler et al., 1995), and at high levels in the tapetum of developing anthers (F. Murray, personal communication). It has also been detected in wheat internodes and *Lolium* floral meristem (Gocal et al., 1999; Chen et al., 2001). On RNA gel blots, the KGM gene in barley is expressed in intermediate concentrations in aleurone, is very highly expressed in anthers and relatively lowly expressed in roots. The GMPOZ gene is also expressed in aleurone, is very highly expressed in anthers and is expressed in low but detectable amounts in roots and internodes. Floral meristems were not examined. It is apparent that the expression pattern of the GAMYB binding proteins overlaps with the pattern determined for GAMYB, with the most striking similarity residing in the strong anther expression of these three genes.

In an intriguing development, stably transformed barley lines overexpressing a GAMYB genomic clone have been found to be male sterile due to abnormal anther development (F. Murray, personal communication). In addition, an *Arabidopsis* double mutant, with lesions in two GAMYB-like genes is also male sterile, and exhibits abnormal anther development (A. Millar, personal communication). Anthers are a GA-rich tissue with a high carbon demand because pollen formation requires a steady

supply of various proteins, lipids and carbohydrates (Takahashi et al., 1991; Goldberg et al., 1993). The tapetum is critical in controlling the secretion of these substances into the locule. A GA-deficient mutant, *male-sterile stamenless-2* (*sl2/sl2*), from tomato has arrested anther development and abnormal amylolytic activity and carbohydrate levels in stamens – the levels of soluble sugars remained low and starch contents did not decrease during stamen development as was found to be the case in the wildtype stamens (Jacobsen and Olszewski, 1991). These observations provide a link between GA and sugar hydrolase activity during development in anthers. An obvious prediction from this data is that GAMYB and GAMYB-binding proteins such as KGM and GMPOZ might be involved in the regulation of hydrolase gene expression in anthers. Like the aleurone, the tapetum also possesses a hydrolase secretory function, (Goldberg et al., 1993) releasing callase, which breaks down callose in the thick walls of the tetrad and facilitates release of the microspores. Although GA has not been implicated in this process, the potential parallels between these systems, namely GAMYB-mediated activation of the expression of genes encoding secreted hydrolases, are obvious.

The existence of KGM- and GMPOZ-like genes in *Arabidopsis* raises the possibility that this model genetic system could be used for studying the function of the GAMYB-binding proteins in tissues outside the aleurone. There are three KGM-related genes in *Arabidopsis*, including two genes which map to chromosome 4, AtMHK (Moran and Walker, 1993) and an uncharacterised cDNA (GenBank accession number AF360190) as well as a gene encoding a hypothetical protein from chromosome 5 (GenBank accession number BAB09171). These genes are 46%, 60% and 56% identical respectively to KGM at the amino acid level and while AtMHK is quite distinct within this grouping, the other two *Arabidopsis* KGM-like genes share 75% identity at the amino acid level. There also exist two closely related GMPOZ-like genes in *Arabidopsis*, an uncharacterised cDNA termed AtPOB1 which maps to chromosome 3 (GenBank accession number AF292397) and a hypothetical protein encoded by a gene on chromosome 2 (GenBank accession number At2g46260). These genes encode proteins with 65% and 64% amino acid identity to GMPOZ respectively. It would be of interest to screen for tagged mutants corresponding to all of these loci in *Arabidopsis*. The existence of possible multigene KGM- and GMPOZ-like families in

Arabidopsis could generate functional redundancy and might explain why mutants at these loci have not yet been described. The phenotype of dual or triple knockouts at the KGM- and GMPOZ-like loci in *Arabidopsis* may provide considerable insight into the likely function of these genes in a variety of tissues, including anthers.

Concluding remarks

The work described in this dissertation was designed to advance our understanding of GA-signalling in cereal aleurone, particularly with regard to how more upstream signalling events result in changes in gene expression. A new and powerful proteomics technology was utilised to facilitate gene discovery within a system that is also highly amenable to the study of hormonally regulated events. A number of novel candidate signalling factors and transcriptional regulators were generated for further study, and the involvement of two of these factors in GA-signalling events in barley aleurone was established. A concerted effort is currently being made within this field to understand how our biochemical observations about hormonal signalling in aleurone translate to the molecular level and the identification of potential new players in these pathways expands the framework within which this scientific enterprise can be conducted.

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Appendix - cDNA sequences

Putative GAMYB-binding proteins obtained in the yeast two-hybrid screen

Note – sequences read 5′ to 3′ and have been trimmed of vector sequence. For the longer cDNA inserts, only the first 600 nt were determined if clones were not pursued after the primary screen.

Clone 1 – similar to rat MAK (mitogen activated kinase)

```
1                    CCCGCA GCTTTCGCGC TCTCCCTCGC CAACATTTTC
37   GTAACTTTTG CCGTGGCACT GGTCAGCCAG CAAAAGTTAC CATCAGAACT
87   TCTTTGGACA CTTGGGATCT AACCCGTAGA TCCTCCCTGT CGGTTACAAG
137  CTCTAGGTTC TGGTTCTCTT TGCTTCTGTC AGCTCTCTTC TCCCCTATCA
187  CTGCGAGTTT TCAGTTTGTC CGGCTTGATC TTTGGTGTGT ATAGTGTGCA
237  CTGTTCTTCA TCAGGCTTCA CTTCTGATGG CGTGACGCGA GGGCAAAGGT
287  GTTCAAGTTT ATTTGTCACA AAGTATGGGT ATTGCCTACA GACGTTTGAA
337  AAAAGCCTGC AGTGACTTTG GTGACCTACT CTCCAAAGAC ATTCCGGCAA
387  TACGCGTATG AGTCTTGTTT GTGTTTATAG GACTGTTCAA AAATAAGGAC
437  AGCGTTGCAT TCTAGCTAAT AAAATGGAGA GGTATAACAT TATTACGGAA
487  GTGGGTGACG GTACGTTTGG TAGTGTTTGG CGTGCAATCA ATAAAGAAAG
537  TGGCGAAGTG GTTGCAATCA AGAAAATGAA GAAAAAATAT TTTTCTTGGG
587  AGGAGTGCAT CAATCTCCGT GAAGTGAAGT CCCTCCGAAG GATGAACCAT
637  CCAAACATTG TGAAGCTCAA GGAGGTCATA AGAGAGAATG ACATGCTATT
687  CTTTGTTTTT GAATACATGG AATGCAATCT CTATCAGCTG ATGAAGAGCA
737  AGGGCAAGCC CTTTTCGGAG ACTGAAATCC GGAACTGGTG CTTTCAAGTA
787  TTTCAAGCTC TTAGTCACAT GCATCAACGT GGATACTTTC ACCGTGACCT
837  TAAGCCTGAA AATTTGCTGG TTACAAAGGA GCTCATCAAG GTAGCTGATT
887  TTGGGCTTGC TAGGGAGATT ATTTCTGAAC CACCATACAC AGAATATGTG
937  TCAACTCGCT GGTATCGTGC CCCAGAGGTT CTGCTTCAAT CTTCTGTTTA
987  CAGCTCAGCA GTTGACATGT GGGCTATGGG CGCCATTATT GCAGAGCTTT
1037 TTTCACACCG ACCTCTTTTC CCTGGCTCAA GTGAAGCGGA TGAGATTTAC
1087 AAAATCTGTA ACATTCTTGG CACTCCAAAT CAGCATACTT GGGCTGGAGG
1137 GCTGCAGCTA GCAGCATCTA TCCATTTTCA GTTTCCTCAG TCTGGAAGCA
1187 TAAATCTTTC AGAAGTGGTT CCCACAGCAA GTGAAGATGC GCTGAACCTT
1237 ATTTCGTGGC TTTGCTCATG GGACCCACGT AAAAGGCCAA CCGCTGTGGA
1287 GGTTTTGCAG CATCCCTTCT TTCAGCCATG CTTCTATGTC CCCCCTTCAC
1337 TTCGTTACAG ATCGACAGGA TATGCAACAC CACCGCCATC AGTCGGGGCT
1387 AAAGGAGCTA TGGACCAGAA GAATGCTAGG AGATACCCTG TGGGGACTTT
1437 ATCCAATGGG AGGCCGGCAG TTAATAACTC CTACCTTAGC ACCAACGCCC
1487 CAGCAAGAGC AGCTGGCGTG CAAAGGAAGC TAGAGTTGGA TCATCAGGTG
1537 AAACCAGAGG GCAACCATAA GCTGACGACG AAGGAGAACG CAATGAACCA
1587 GCCTTGGTCC AGACTACCTC CAGCACCAGT GAGGAACAAC ATGAACTACC
1637 TTGCCGCCAA NGAGCAGATC CCTCGTGGTG GCGCGCCAGA CATAGCCGAG
1687 AAGCTGTCCC AGCTGTCGAT GTCCTCCACC ACAAACCGGG CGCCGATCAT
1737 GCCTTCCGAC AGGTTTGTGG ACCTGAAGGC CACTACCAGA GCCCACGGGG
1787 AGCCCGTGAG GCGGCCCGTG CCTCTGGGAC CCAGGGACAC GTGGCATGCC
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1837 CGAAACGACC CCTTCCGGCG CACCTATGAG ATGCCGGGCG AGAGGGCTCT
1887 CCTCCAAAGG AAGCTCGTCA GCTGAGGACC ATCCCACCGA CGCCCGCCCT
1937 TCTGCCATCC ATCTGCCTCT TCGCATCATC GACACCACCA CCACCACCCC
1987 GACCACAATA ATCCGGCGAT CTCCTTCCTG GCGGTCGCTG CCAAGTTTGT
2037 GTCTCTCTTT CATCGCTTGT GTTGTGATTG AAAATAAAAT CCAGTCTCCC
2087 CCGGTCCTCG CTTGGCATTG CAGCGGGGAG AGTGAAGAGC ACTGCTGTGT
2137 TTCCTTTGGG CTTTCGAGTG TGAGAAACTT GGACGATGAT ATATATGCTT
2187 TGTGTTATTT GTGTGCGAGC CCAAGCGGCC ATGTTACTTC CCAGTGCTGT
2237 CTCATGGTTA AACCTCTTGA TCGCTCTACC CGTTGATCAT CGAAACTGAA
2287 ATGAAATGTT GCCGTGAGAT CTTTGGAGCA AGGA
```

Clone 2 – likely out of frame peptide

```
1                                       G AACAAATGGT CCCAATGGAC
22   CGGCACCGGG AACAGGCATC AATATCGGTG GAGCAGGGAA TTCAGCCGAA
77   AAAGGAGCAT TGGAGCCTCT TCCACGGAAG GAATCAAACA TCTGATCGTC
127  AGGTCCATCA CGGCTTGGAG AGCCATCACC TCTACCATGT CTGCCTCCAT
177  CTTTATCTGC CCTATCATAT TCTCTGCGAT TGCCACGGTC ATATCTCAGA
227  CGACTATCCA TACCATTGTC AGATCTCCGC
```

Clone 3 – novel WD40 protein

```
1    ATACCATGGG AGAGGTTGCA AATTACCAGG AAGGATTACA GAAAAGCTAG
51   GTTG7GAACAG TACAAGAACT ATGAAAATTT CCCTCAATCC GGAGAGCTCA
101  TGGATAAGTT GTGCAAGCAA GTGGAGAGCA GCAGCAAGTA CTATGAATTT
151  CAGTACAACA CTCGGATAGT GAAACCATCA ATTCTCCATT TTCAGCTTCG
201  GAATTTGTTA TGGGCAACTT CAAAGCATGA TGTCTATTTC ATGTCGAATT
251  CCACAGTAGG CCACTGGTCG TCATTGTCTC ACAAAATGAC AGATGTTCTT
301  GATTTCTCAG GGCATGTTGC TCCAGCAAAG AAACACCCTG GCTGTGCACT
351  AGAAGGGTTT ACTGGCGTTC AAGTTAGCAC ACTTGCAGTA AATGAGGGTT
401  TATTGGTCGC TGGTGGTTTT CAAGGAGAAC TAGTTTGCAA GAGTCTAGGA
451  GAACGTGATG TTAAGTTCTG CACAAGGACT ACTTTGAGCG ACAATGCTAT
501  CACAAATGCT ATGGATATTC ACAGATCTAC AAGTGGAAGC TTGCGCATTA
551  CGGTATCAAA TAATGATTCT GGTGTTCGTG AATTCGACAT GGAAAGATTC
601  CAGCTCTTGA ATCACTTCCG TTTTAACTGG CCAGTGAATC ACACATCCGT
651  GAGCCCGGAC AAGAAACTGT TGGCAGTAGT TGGAGATGAC CGGGACGCTC
701  TTCTTGTTGA TTCACGAAAT GGCAAGGTAA CTTCCACCCT AGTTGGCCAT
751  CTGGACTACT CATTTGCCTC GGCGTGGCAC CTGGACGGTG TCACCTTTGC
801  AACCGGGAAC CAGGACAAGA CCTGCCGGGT GTGGGACATC CGGAACCCGT
851  CGACCTCCCT TGCGGTCCTG AGGGGCAACA TCGGCGCGAT CCGGTGCATC
901  CGCTACTCCT CGGACGGGCG GTTCCTGCTA TTCTCGGAGC CCGCCGACTT
```

Clone 3 (continued)

```
951  TGTGCACGTC TACAGCACCG CCGAGTGCTA CCGGAAGCGG CAGGAGATCG
1001 ACTTCTTCGG CGAGATCTCG GGGATCTCGC TCAGCCCAGA CGACGAGTCC
1051 CTGTTTGTGG GGGTGTGCGA CCGCGTCTAC GCCAGCCTGC TGAATTACAG
1101 GTTGGTGCAC GCCAACGGGT ACCTGGACTC ATACATGTAG AGGAATAAAA
1151 CCCTTTGTGT TTGATGTGAA AGAAAGAGCT CCGGAACTGC CGAAAATCTG
1201 AAGATCTCGC TCGGCCCGGG CGACGAGCCC CTGTTGTTCG AGGGGCGAGA
1251 TATGCCAGCG GGCTCCATTA CAGGATAGTC CAGGTGTGCC TTCGGGCGCC
1301 TGGACTCTGT ACATGTAGAG AGAGAGAAAG AAAGCTTGGT GCTAAGTTTA
1351 GCGTGTTCGT GTAAAGGAAG AGCGGAATTG CTAAAGATCT GCCGAACTGT
1401 AACTTTAATG AGACTCAGTA TATGGAATTT ATACCATCTT TCA
```

Clone 4 – novel BTB/POZ domain protein

```
1                          CCGCCAAGCC CAAGGCCAAG GTCGCCAAGA
31   AGACCGCTGC CAAGTCCCCG GCCAAGAAGG CCGCCGCAAA GCCCAAGGCC
81   AAGACCCCTG CCAAGGTCAA GGCCGTCGCA AAGCCCAAGG CGGCAGCGAA
131  GCCCAAGGCA GCCGCCAAGC CCAAGGCCAA GGCAGCCGCC AAGAAGGCGC
181  CGGCCGCCGC AACCCCCAAG AAGCCTCGCC AGGAAGGCGC CCACCAAGCG
231  GGCGACCCCG GTGAAGAAGG CTGCGCCGGC CAAGAAGCCC GCGGCGAAGA
281  AGGCCAAGAA GTAGAGGCCT GATGGATGGA TCTGCCTAGC GGTTGGCGTT
331  AGTTTCCACT AGTGGAATTT TATGTTCTGT GCTTGTCAGT CAAGATGCCT
381  TGGCTGTAAA TTTGGTGTTT GGGAGGTGGC TGCGCGTTCG CGGATTTTGG
431  GATTGGATTG GATTGGGGGG AAGAGGGCGC GGTGGTGGGT CGGGATGGAG
481  CCGGACTTCT CGCGGGCGAG CGGCGGCCCG AGCTACGAGT TTGCCTTCAA
531  CTCGGTCAAC TTCTCCGACC GGGTCCTGCG GATCGAGATC GTCGCCGGGG
581  ACGACGCGCC GGGGGCCAAG GGCGCCGCCG GCGAGGGCTG CTCCTCCATC
631  GCCGACTGGG CGCGCCATCG CAAGCGCCGC AGGGAGGATC TCCGTCGGGA
681  GAAAGGCGGG GAAGAATATG GAAAGTATAT GTTCGAACCA TCAAATGTCA
731  AAATTGAAGC AGAAGAGCAT GATACCTATG AGGAAACTGG TGAGGAGCCT
781  GTAGCTATGA TAGAAGAATC TCCACCTGAT ATTGGACAAG ATGGGGAGGA
831  TGGAGAAAAC AGTGACTCAT CCTGGAATAT GGAGTGTAAT CAGGTTTTGA
881  GAGTGAAATC TATTTATATC AGCTCTGCAA TTCTAGCTGC AAAAAGTCCC
931  TTTTTTTACA AGCTTTTCTC CAATGGCATG AAAGAATCCG ATCAGAGGCA
981  TGCTACTCTT AGAATAACTA CTTCAGAGGA AAGTGCCCTT ATGGAGCTTT
1031 TAAGCTTTAT TTACAGTGGG AAGCTGACAA CAAATGAGCC AACCCTTCTG
1081 CTTGATATCT TGATGATTTC TGACAAATTT GAAGTTGTTT CTTGCATGAG
1131 GCACTGCAGT CAATTGCTAA GAAGCTTACC TATGACCACA GAATCTGCAC
```

Clone 4 (continued)

```
1181 TTCTCTATCT AGATCTACCT TCCAGCATTT CAATGGCTGC AGCAGTTCAG
1231 CCACTGACTG ATGCTGCCAA GGAATTCCTC GCCAATAAAT ACAAGGATTT
1281 GACCAAGTTT CAGGATGAAG TGATGAACAT TCCCCTTGCT GGGATTGAAG
1331 CCATTTTATG TAGTAATGAC CTTCAGGTGG CATCAGAGGA TGCAGTCTAT
1381 GACTTTGTGA TCAAGTGGGC TCGTGCTCAA TGCCCAAGAA CGGAAGAAAG
1431 ACGTGAAATC TTGGGTACTC GCTTACTGCC GCTCGTTCGG TTCTCTCATA
1481 TGACCTGCAG GAAGTTGCGG AAGGTCCTTG CGTGCAGTGA TCTGGATCAT
1531 GAGCAAGCAT CTAAAAGTGT CACTGATGCA CTCCTGTACA AAGCTGATGC
1581 ACCACATCGA CAGCGCGCCC TTGCTGCAGA TGTGTTGACC TGTAGGAAAT
1631 ATACCGAACG AGCTTACAAA TATCGTCCGC TTAAGGTGGT GGAATTTGAT
1681 CAACCATATC CTCAGTGCAT AGCATACTTG GATCTGAAGC GTGAGGAGTG
1731 TAGCCGACTT TTCCCATCCG GGCGGATTTA CTCGCAAGCA TTCCATCTTG
1781 CTGGACAGGG ATTCTTCCTC TCAGCACATT GCAACATGGA TCAGCAAAGT
1831 GCTTTCCACT GCTTTGGTCT CTTCTTAGGG ATGCAAGAGA AAGGCTCAAC
1881 AAGTGTTACC GTGGACTATG AGTTTGCTGC AAGGACAAGG CCGTCGGGCG
1931 ATTTTGTCAG CAAGTATAAG GGCTACTACA CCTTCACTGG TGGAAAGGCA
1981 GTTGGCTACC GGAATCTCTT TGCAATTCCC TGGCCGTCGT TTATGGCTGA
2031 TGACAGCCTC TTCTTCATTG ATGGAGTACT ACATCTGAGA GCGGAACTGA
2081 CCATAAAGCA ATCATAGATA GTGGAAAATC TTGTGTGGTT CAAACATGTT
2131 TTTCTTGAGC GACAAAATAG ACACTTTTGA AACGAGAAGC AAAATCCATG
2181 AACTGTGAGA ACTCAAAAGC AACATCTTAT GAACTGTGAA GGTGTAGTTG
2231 TGCCACATGA GTTTTTCTTA ATGTGTAGCG CNNCATGAGT TAAAT
```

Clone 5 – similar to SKIP nuclear coactivator

```
1    GGCTAGGGTC CAAGATCCTC GCGCTCACCG TCGACGCGCA CGGCAGCGTC
51   GCCTTCGACG CCGTCGTCAA GCAGGGCGAG AACGCCAAGA AGATCGTCTA
101  CTCCAAGCAC AGCGACATCG TGCCCAAGAT CGCCACGGCC GACTCGGAAG
151  CCGTCGAGGA CGAGGAATAC GAGAAGTTGG TCGAGGAGAC CACAGAGCGG
201  ACCGTAGCCG CCCTGCAGAA GATCGTCAAC GTTCGCCTCT CTGCTGCCCA
251  GCCCAAAAAC GTCCCGACGC ATGATTCGGA GTCCAAGTTC ATCAAGTATA
301  AGCCGTCGCA GCAGTCGGCA GCGTTCAATT CGGGTGCCAA GGAGAGGATT
351  ATTAGGATGT CGGAAATGGC TTCAGATCCT CTTGACCCAC CAAAGTTCAA
401  GCATAAGCGG GTGCCCCGTG CATCTGGGTC GCCGCCTGTG CCGGTTATGC
451  ACTCGCCACC ACGGCCCGTC ACTGTGAAGG ACCAGCAGGA TTGGAAGATC
501  CCACCTTGCA TTTCAAATTG GAAGAATCCA AAGGGTTACA CAATCCCACT
551  CGACAAGAGG TTGGCTGCTG ATGGGAGGGG GCTGCAGGAG GTTCAGATTA
```

Clone 5 (continued)

```
601  ATGATAACTT TGCAAAGCTT TCAGAAGCAT TGTATGTTGC AGAGCAGAAG
651  GCGAGGGAAG CAGTGCAGAT GCGCTCCAAG GTGCAGAGGG AGCTAATGCT
701  AAAGGAGAAG GAGAGGAAGG AGCAAGAGCT GAGGGCACTT GCACAGAAGG
751  CCCGCATGGA AAGGTCTGGT GCTCCACCTC CATCCACGGG TATGCCTGTC
801  GGAGGTGGGA GGGACAGAGA GCGGGAGAGG GTTGATGATG GTGATGCAGA
851  TATGGATTTG GAGCAGCCGC GTGAGCAGCG CAGGGAGACT AGAGAAGAGA
901  GGGAGGCGAG GATTGAGCGT GACAGGATCC GTGAGGAGCG GAGGCGTGAG
951  AGGGAGAGGG AGAGGAGGCT GGAGGCAAAG GATGCTGCAA TGGGTAAAAA
1001 GAGTAAGCTC ACTAGAGACA GGGACCGTGA CGTCGGTGAG AAGATGGCCC
1051 TGGGTATGGC AAATACTGGT TCGAAGACCG GGGAAGTCAT GTATGACCAG
1101 AGGCTCTTTA ACCAGGACAA GGGAATGGAC TCTGGTTTTG CTGCTGATGA
1151 TCAGTACAAT GTTTATTCGA AGGGCCTCTT CACAGCACAG TCTAGCATGT
1201 CAAGTCTTTA CAGGCCCAAG AAGGATGGTG ATTCTGAAGT GTATGGTGGT
1251 GATGCAGACG AACAGCTGGA GAAGGTTATG AAGACAGAGA GGTTCAAGCC
1301 TGACAAAGCA TTTACTGGTG CTCCAGAGAG GGCTGGCAAG AGAGATAGAC
1351 CCGTGGAGTT TGATAAGCAA GAGGAGGCTG ATCCATTCNG TCTTGACCAG
1401 TTCTTGACTG AGGTGAAGAA GGGAAAGAAA GCTGTGGACA AGATTGGTGG
1451 CGGAGGAACT ATGAAGGCAA GTGGTGGATC CTCTAGGGAT GATTACGAGG
1501 GCGGAGGATC TGGGAGGTCT CGCATTAACT TTGAAAGAGG AGGACGTTGA
1551 GGTATTTGTT GTGCACGTTT ATCTTCTGCA TTCTCATGTT CTCAGTGCAT
1601 TTTCAAATAT CATCCTGAAG GATTTCTTTG GAGAATATGA TCCTTCTTAC
1651 ACAAGGAAGA ACTCAAATAT CAACACTGAA GGATTTCTAT GGAGAATATG
1701 ATCTTTCTTA GGTAAGGATG ATCCTGATGG TTTTAGTTGC TGTGATCTGG
1751 CTGCTTGTTA TATTTCAACC ATCGTGTTAA ACTGTGTACC TACTTATTAG
1801 ATCCTACAAT GCTTGTATGA TGTGTATCTT TGTGACAAGA TCCATGAACT
1851 TTGTCGTTAC ACATGTTACA TAGGCTTA
```

Clone 6a – barley chitinase

```
1                                   CCGGACTA  CTGCGGCGAT  GGATGCCAGA
29   GCCAGTGCAC  CGGCTGCGGC  GGCGGCAGCA  CGCCCGTCAC  GCCCACCCCA
79   TCGGGCGGCG  GCGGCGTGTC  CTCCATCGTC  TCACGCGCCC  TCTTCGACCG
129  CATGCTGCTG  CACCGCAACG  ACGGCGCCTG  CCAGGCCAAG  GGCTTCTACA
179  CCTACGACGC  CTTCGTCGCC  GCCGCATCCG  CCTTCCGGGG  CTTCGGCACC
229  ACCGGCGGCA  CCGACACCCG  GAAGCGCGAG  GTGGCCGCCT  TCCTGGCCCA
279  GACCTCCCAC  GAGACCACCG  GCGGGTGGGC  GACGGCACCG  GACGGAGCTT
329  TCGCCTGGGG  CTACTGCTTC  AAGCAGGAGC  GTGGCGCCAC  CTCCAACTAC
```

Clone 6a (continued)

```
379 TGCACTCCGA  GCGCGCAGTG  GCCGTGCGCC  CCAGGGAAGA  GCTACTACGG
429 CCGTGGGCCC  GATCCAGCTC  TCCCACAACT  ACAACTACGG  CCTGCGGCCG
```

Clone 6b – barley chitinase

```
1                     GCCGT  GGTTGCCATG  GCGGCCACCA  TGGCCGTCGC
36  CGAGCAGTGC  GGCTCGCAGG  CCGGCGGGGC  GACCTGCCCC  AACTGCCTCT
86  GCTGCAGCCG  CTTCGGTTGG  TGCGGCTCCA  CCCCGGACTA  CTGCGGCGAT
136 GGATGCCAGA  GCCAGTGCAC  CGGCTGCGGC  GGCGGCAGCA  CGCCCGTCAC
186 GCCCACCCCA  TCGGGCGGCG  GCGGCGTGTC  CTCCATCGTC  TCACGCGCCC
336 TCTTCGACCG  CATGCTGCTG  CACCGCAACG  ACGGCGCCTG  CCAGGCCAAG
386 GGCTTCTACA  CCTACGACGC  CTTCGTCGCC  GCCGCATCCG  CCTTCCGGGG
436 CTTCGGCACC  ACCGGCGGCA  CCGACACCCG  GAAGCGCGAG  GTGGCCGCCT
486 TCCTGGCCCA  GACCTCCCAC  GAGACCACCG  GCGGGTGGGC  GACGGCACCG
536 GACGGAGCTT  TCGCCTGGGG  CTACTGCTTC  AAGCAGGAGC  GTGGCGCCAC
```

Clone 7a – similar to rice thaumatin

```
1                                                 G  ACGCGGCGAC
12  CTTCACGGTG  ATCAACAAGT  GCCAGTACAC  GGTGTGGGCG  GCGGCGGTGC
62  CGGCCGGCGG  GGGGCAGAAG  CTGGACGCGG  GGCAGACGTG  GAGCATCAAC
112 GTGCCGGCCG  GCACGACGAG  CGGGCGCGTG  TGGGCGCGCA  CGGGGTGCAG
162 CTTCGACGGC  GCCGGCAACG  GGCGGTGCCA  GACGGGGGAC  TGCGGCGGGA
212 AGCTGCGGTG  CACGCAGTAC  GGGCAGGCGC  CCAACACGCT  GGCGGAGTTC
262 GGGCTCAACA  AGTACATGGG  GCAGGACTTC  TTCGACATCT  CCCTGATCGA
312 CGGGTACAAC  GTGCCCATGT  CGTTCGTCCC  CGCCCCCGGC  TCCACCGGGT
362 GCCCCAAGGG  CGGGCCGCGG  TGCCCGAAGG  TGATCACGCC  GGCGTGCCCC
412 AACGAGCTGC  GGGCGGCGGG  AGGGTGCAAC  AACGCGTGCA  CGGTGTTCAA
```

Clone 7b – similar to rice thaumatin

```
1                                       C  GGTGGAGCCG  GGGGCGGGGA
22  CGAACGACAT  GGGCACGTTG  TACCCGTCGA  TCAGGGAGAT  GTCGAAGAAG
72  TCCTGCCCCA  TGTACTTGTT  GAGCCCGAAC  TCCGCCAGCG  TGTTGGGCGC
122 CTGCCCGTAC  TGCGTGCACC  GCAGCTTCCC  GCCGCAGTCC  CCCGTCTGGC
172 ACCGCCCGTT  GCCGGCGCCG  TCGAAGCTGC  ACCCCGTGCG  CGCCCACACG
222 CGCCCGCTCG  TCGTGCCGGC  CGGCACGTTG  ATGCTCCACG  TCTGCCCCGC
272 GTCCAGCTTC  TGCCCCCCGC  CGGCCGGCAC  CGCCGCCGCC  CACACCGTGT
```

Clone 7b (continued)

```
322 ACTGGCACTT  GTTGATCACC  GTGAAGGTCG  CCGCGTCCGC  TGTAGCGGTG
372 ACCAAGACGA  GGAGGAGGAT  TGGGAGCAGG  ACGGAAGAAG  TGGGGAGGGA
422 TGCCCTC
```

Clone 8 – barley glycine rich protein

```
1                                                   CTCTCGTA  CACACTTTAT
19  TTACCAGAGA  CCATATTTAC  CAGCAACTCT  GCAACTTACT  ATAAGTGTAT
69  CTGTGCAGAG  CAATGTCACG  TAGAGTTGTA  CTACCTTGTA  CGCACGCACG
119 TATGGCTGCA  TCTCGAGCGC  TCAGTTGCGG  ACCTCCGCGC  GGTACATGGG
169 CTCAGGGACC  TCGTCCGCGC  GCGCACAGCA  GCGGCAGCCA  TTGTACCCGT
219 GACCCGCAGC  AGCCCCAGTG  ACATCCACTG  CCGCCTCCGC  CGTGTCCGGG
269 GTAGCCACCA  CCGCCGCCTC  CGCCGTGTCC  GGGGTAGCCG  CCACCGCCTT
319 TACCACCGTG  TCCCGGGTAG  CCGCCGCCAC  CTCCTCCACC  GTGACCTGGG
369 TAGCCGCCGC  T
```

Clone 9 – similar to wheat pathogen secretory protein

```
1                       GCCTT  CACCAGCGCC  AACGGCATCC  ACCTCAGCGC
36  CCAGTACGTC  GCCAGCATCT  CCGGCGACGT  CAAGAAGGAG  GTGACCGGCG
86  TGCTGTACCA  CGAGGCGACG  CACGTGTGGC  AGTGGAACGG  GCAGGGCAAG
136 GCGAACGGCG  GGCTCATCGA  GGGGATCGCC  GACTACGTGC  GGCTCAAGGC
186 CGGGTTCGCG  CCGGGGCACT  GGGTGAAGCC  GGGGCAGGGC  GACCGGTGGG
236 ATCAGGCTCG  TGCCGAATTC  TGCAGATATC  CATCACACTG  GCGGCGCTCG
286 AGCATGCATC  TAGAGGGCCG  CATCATGTAA  TTAGTTATGT  CACGCTTACA
336 TTCACGCCCT  CCCCCCACAT  CCGCTCTAAC  CGAAAAGGAA  GGAGTTAGAC
386 AACCTGAAGT  CTAGGTCCCT  ATTTATTTTT  TTATAGTTAT  GTTAGTATTA
436 AGAACGGTAT  TTTATATTTC  AAATTTTTCT  TTTTTTTCTT  GTACAGACCG
```

Clone 10 – similar to cotton callose synthase

```
1                                   GAAG  ATGGGTATTC  TGCACTTGAA
25  TATATTGACA  CTCAAGGGTA  TCAGTTATCT  CCTGATGCAC  GGGCCCAGGC
75  AGATCTAAAA  TTTACATACG  TTGTTTCATG  TCAAATATAT  GGACAGCAGA
125 AGCAAAGGAA  GGCTCCAGAG  GCTGCGGATA  TTGCTCTTCT  AATGCAAAGA
175 AATGAGGCCC  TTCGGGTTGC  TTTCATACAT  GAAGAGGATG  GTGTATCGAG
225 TGATGGGCTA  GCTATAAAAG  AATATCATTC  CAAGCTCGTG  AAGGCTGATA
275 TTCACGGAAA  AGATCAAGAA  ATATACTCCA  TTAAACTGCC  TGGGAACCCC
```

Clone 10 (continued)

```
325 AAGCTAGGTG  AGGGGAAACC  TGAAAACCAA  AATCATGCTA  TAATTTTTAC
375 TCGTGGAGAT  GCAATACAGA  CGATCGATAT  GAATCAGGAT  AACTACTTGG
425 AGGAGGCAAT  GAAAGTGAGG  AATTTACTTG  AAGAATTTCG  TGGTAACCAT
```

Clone 11 – similar to sorghum PEP carboxylase

```
1                         G GATACTCAGA CTCTGGCAAG GATGCTGGGC
32  GTCTTTCTGC  AGCATGGCAA  ATGTATAAAG  CACAGGAAGA  GCTCATAAAG
82  GTTGCAAAGC  AGCATGGAGT  AAAGTTGACA  ATGTTTCATG  GAAGAGGTGG
132 AACGGTTGGC  AGAGGAGGGG  GTCCCAGTCA  TCTTGCCATA  TTATCTCAAC
182 CACCAGACAC  GATACATGGA  TCACTCCGTG  TAACAGTTCA  AGGCGAGGTC
232 ATAGAGCACT  CGTTTGGAGA  GGAACACTTG  TGCTTTAGAA  CTCTGCAGCG
282 CTTCACTGCA  GCTACTCTTG  AGCATGGAAT  GCATCCCCCA  ATTCCACCCA
332 AGCCAGAATG  GCGTGCTCTA  ATGGATGAGA  TGGCTGTTGT  GGCAACAAAA
382 GAGTATCGAT  CAATAGTCTT  CCAAGAACCA  CGCTTTGTTG  AATACTTCCG
432 CTCGGCAACA  CCGGAGACTG  AATATGGTCG  GATGAATATT  GGCAGCCCGG
```

Clone 12 – similar to rice metallothionein

```
1                         GAGGGACACA CTGGTCGAAC GCTACACGCA
31  GGGCATACAG  AACTAACGAA  CACGACGGCG  GTTTTATTTT  ATTCAGCGCG
81  CGCGCATGCA  GCGGCTACTG  ATGATACGAT  GGCGATGGTG  ATGGTGCTTG
131 GTTGGTGTTG  CTTGTCCTCC  TCTCATCCTC  GTGCTCGCCT  CTGCCGGCCG
181 GCCTCCCGGG  CGCCGGCTCA  CTTGCAGCTG  CAGCAGGAGC  AGCCGCACGC
231 GGTGCCGCAC  TTGCAGGTGG  CGCAGTCGCA  GCCGTCGGTC  TCCCCGCCGG
281 CCATCTCGAA  CCCGCCGGAG  CTCCCCTTGT  GGGAGGCGGT  GGGCATGACC
331 ATGGCGGCCG  CGCCGGCGGT  GGCCTCAACG  TCGGGGAACA  TCTTGCACCC
381 GCCGCAGCCG  GTGCCGCACT  GGCAGCCGGC  GCCGCACCCG  CACTTGCCGC
431 CGCAGCAAGA  CATGCTCCTT  TCCTTGGTGG  GCGGTGGCGA  GGGAAGGAAC
481 TCGTGCCGAA  TCTGCAGATA
```

Clone 13 – similar to maize cystathione gamma synthase

```
1                                   CTG ACTTTAGGTG CTGACATTGT
24  TGTTCATTCT  GCAACGAAGT  ATATTGCTGG  GCACAATGAT  GTTATTGGAG
74  GCTGTATCAG  TGGCAGGGGC  GAGCTAGTCT  CCAAGGTCCG  CATTTATCAC
124 CATGTAGTTG  GTGGTGTTCT  AAACCCGAAT  GCTTCCTATC  TGATCCTCCG
```

Clone 13 (continued)

```
174 GGGCATGAAA  ACACTGCATC  TCCGTGTTCA  ATGCCAGAAT  AATACTGCTC
224 TACGGATGGC  CCAGTTTTTA  GAGGAACATC  CAAAGATTGC  ACGTGTATAC
274 TATCCTGGCT  TGCCAAGTCA  CCCAGAACAT  CACATTGCCA  AGAGCCAAAT
324 GACCGGCTTT  GGTGGTGTTG  TCAGTTTTGA
```

Clone 14 – similar to sweet potato purple acid phosphatase

```
1                           TTGCAGTA  TACGTTCTAC  AAATATACTT
29  CGGGATACAT  TCATCATTGT  ACAATCAAGA  AGCTGGAGTT  TGACACAAAG
79  TATTACTATG  CTGTTGGAAC  TGAAGAGACG  CTGAGGAAGT  TTTGGTTCAG
129 GACCCCTCCA  AAAAGTGGCC  CAGATGTTCC  ATATACATTT  GGTCTGATAG
179 GTGATCTCGG  CCAGAGTTTC  GACTCGAATG  TCACGCTCGC  TCATTACGAG
229 TCC
```

Clone 15 – similar to Arabidopsis monooxygenase

```
1                                       CGACGCGTTC  CACCCGATGA
21  CGCCGGAGCT  CGGCCAGGGC  GGCTGCGCGG  CGCTCGAGGA  TGGCGTCGTC
71  CTCGCCCGGT  GCCTCGGCGT  GGCCTTCGCC  GCCGGCGGGC  ACGGGAGCGC
121 CGAGGCCGCC  CTGGCGAAGT  ACGCCGAGGA  GCGGAGGTGG  CGCGCCATCC
171 GGCTGGTCAC  GGCCGCGTAC  GTCGTCGGCT  TCGTGCAGCA  GAGCAGCAAC
221 CCG
```

Clone 16 – barley α-amylase

```
1                                   GCGGA  CGTGGGCGGC  GACGGCAAGC
26  CCAACTACGA  CCAGGACGCG  CACCGGCAGA  ATCTGGTGAA  CTGGGTGGAC
76  AAGGTGGGCG  GCGCGGCCTC  GGCAGGCATG  GTGTTCGACT  TCACGACCAA
126 AGGGATACTG  AACGCTGCCG  TGGAGGGCGA  GCTGTGGAGG  CTGATCGACC
176 CGCAGGGGAA  GGCCCCCGGC  GTGATGGGAT  GGTGGCCGGC  CAAGGCCGTC
226 ACCTTCGTCG  ACAACCACGA  TACAGGCTCC  ACGCAGGCCA  TGTGGCCATT
276 CCCCTCCGAC  AAGGTCATGC  AGGGCTACGC  GTACATCCTC  ACCCACCCCG
326 GCATCCCATG  CATCTTCTA
```

Clone 17 – similar to soybean dihydroxyflavonal reductase

```
1                                                GCCGTCTT  CCACGCCGCC
19  GCGTCCGTCG  AGCCGTGGCT  CCCCGACCCC  TCCGTCTTCA  CCGCCGTCAA
69  TGTGAGAGGG  CTTGAGAATG  TGTTAAAAGC  GGCCAAGACA  ACACCGACAG
119 TAAAGAAGAT  AATATATACA  TCGTCCTTCT  TCGCGCTTGG  CCCAACGGAC
169 GGTTATGTCG  CAGATGAGAC  GCAGATGCAC  AAGGGGAAAA  CGTTTTGCAC
219 GGAGTATGAG  AAATCAAAGG  TTCTTGCGGA  TAGAATAGCA  CTGCAGGCAG
269 CAGCAGACGG  GGTGCCAATA  ACCATTGTCT  GTCCAGGAGT  CATCTATGGT
319 CCTGGAAAAC  TTACAACTGG  AAACCTTGTT  TCCCGCATTT  TAATTGAAAG
369 GTTTAACGGC  CGTCTACCTG  GCTACATTGG  AGATGGGTAT  GATAGAGAAT
419 CATTTTGCCA  TGTCGACGAT  GTTGTTAGTG  GGCACATAGC  AGCTATGGAG
469 AAGGGCAGGG  TTGGGGAAAG  ATATCTGCTC  ACCGGCGAAA  CTCGTGCCGA
```