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Cloning and characterisation of a bZIP transcription factor from a resurrection grass, *Eragrostis nindensis*

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Submitted in fulfillment of the requirements for the degree of Master of Science in the Department of Molecular & Cell Biology, University of Cape Town.

August 2004

Declaration

The experimental work described in this report was carried out in the Department of Molecular and Cell Biology, University of Cape Town, from February 2002 to April 2004, under the supervision of Assoc. Prof. J.M. Farrant, Dr S.G. Mundree and Prof. J.A. Thomson.

The results presented here are the original, unaided work of the author. Where use has been made of the work of others it is duly acknowledged in the text.

David Brocklehurst
August 2004

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Abstract

The G-box is a plant DNA *cis*-acting element involved in the regulation of gene expression in response to a range of environmental signals including anaerobiosis, dehydration and light as well as by abscisic acid (ABA). Basic leucine zipper (bZIP) transcription factors have been shown to specifically bind and activate transcription from G-boxes in a dimerized form. A 1.5 kb cDNA for a bZIP class transcription factor, designated EnGBF1, was cloned from a desiccation-tolerant grass, *Eragrostis nindensis* by degenerate RT-PCR. EnGBF1 shows greatest homology to GBF1 from maize which is induced during flooding stress as well as high homology to OSBZ8 from rice which is induced by ABA treatment and dehydration. In a yeast transactivation assay, EnGBF1 was able to activate transcription from an ABA-responsive element which contains a G-box core sequence. Western blot analysis of proteins isolated during dehydration indicated that EnGBF1 is present in fully hydrated leaf tissue but declines upon drying and is absent in desiccated tissue. EnGBF1 expression levels were not affected by exogenous application of ABA. A possible role for EnGBF1 as a repressor of α-amylase transcription is speculated on although further studies are required to determine its true biological function during dehydration in *E. nindensis*.

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Abbreviations

ABA	abscisic acid
ABF	ABRE-binding factor
ABRE	ABA-responsive element
ADP	adenosine diphosphate
AREB	ABRE binding protein
bHLH	basic helix-loop-helix
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
bZIP	basic domain leucine zipper
CBF	CRT binding factor
CDPK	Calcium-dependent protein kinase
COR	cold-regulated
CTAB	cetyl-trimethyl-ammonium-bromide
CRT	C-repeat
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DRE	dehydration-responsive element
DREB	DRE-binding factor
GAL4AD	GAL4 activation domain
IP_3	inositol 1,4,5-triphosphate
IPTG	isopropyl-ß-D-thiogalactopyranoside
kb	kilo base pair
kDa	kilodaltons
LEA	late-embryogenesis abundant
MAPK	mitogen-activate protein kinase
MOPS	(*N*-morpholino)propanesulphonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NBT/BCIP	nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate
NR	nitrate reductase

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PA	phosphatidic acid
PEG	polyethylene glycol
PI-PLC	phosphoinositide-specific phospholipase C
PLD	phospholipase D
PRD	proline-rich domain
Py	pyrimidine
RACE	rapid amplification of cDNA ends
ROS	reactive-oxygen species
SD	synthetic defined
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SSC	sodium chloride, sodium citrate
TBE	Tris-borate-EDTA
TBST	Tris-buffered saline Tween
TRP	tryptophan
URA	uracil
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
YPD	yeast extract, peptone, dextrose

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Signal transduction and gene regulation in plant abiotic stress

Abiotic stresses such as low temperature, high salinity and drought adversely affect plant growth and can have severe impacts on the yields of crop plants. Plants respond to the initial stress stimulus through complex networks of signal receptors and transducers leading ultimately to metabolic adjustments and synthesis of protective compounds (Fig.1.1). A thorough understanding of how these signals are relayed is required to breed or engineer plants that are able to respond more rapidly and efficiently to the stress.

1.1 COMPONENTS OF SIGNAL TRANSDUCTION NETWORKS

1.1.1 Stress perception and receptors

The earliest events in abiotic stress signalling are thought to involve receptors sensitive to changes in osmotic or ionic potentials and/or biophysical alterations in cell structures (Xiong et al., 2002a). Although no plant stress receptors have yet been identified which connect to downstream signalling components, mechanisms have been suggested based on characterised bacterial and yeast models.

The perception of cold stress may arise through increases in plasma-membrane rigidity. Őrvar et al. (2000) showed that in the absence of cold stress, chemically-induced membrane rigidification was sufficient to induce the development of freezing tolerance in alfalfa protoplasts. How changes in the status of membrane lipids are translated into a transmissible signal is not well understood. However, in the cyanobacterium *Synechocystis*, a two-component histidine kinase, HIK33, functions as a thermosensor, modulating cold-induced gene expression in response to changes in membrane fluidity (Suzuki et al., 2000). Two-component histidine kinases are also present in plants and are typically composed of a membrane-localised sensory kinase and a response regulator domain that mediates the output (Hwang et al., 2002).

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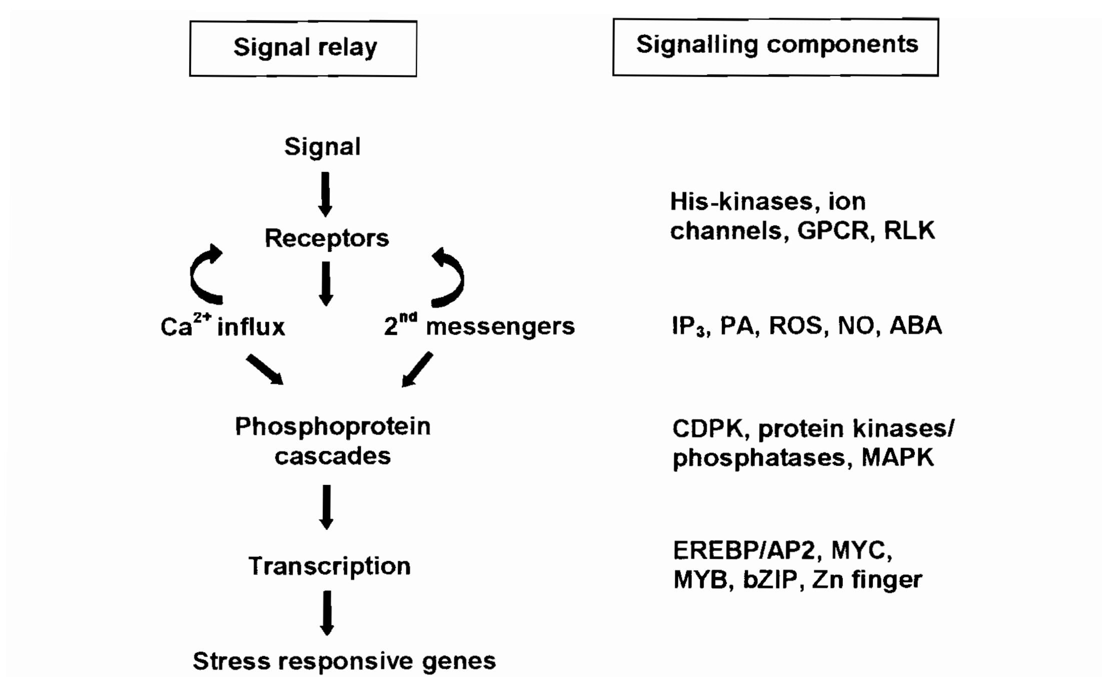

Figure 1.1 Generalized signal transduction pathway in plant abiotic stress responses. Examples of signalling components at each stage in the signal relay are shown on the right. In addition to the receptors discussed in the text, G-protein coupled receptors (GPCR) and receptor-like kinases (RLK) have also been proposed as stress sensors. Second messenger molecules and early Ca^{2+} transients can stimulate further Ca^{2+} release (reversed arrows). Abbreviations are given in the text. Figure adapted from Xiong et al. (2002a).

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The Arabidopsis histidine kinase, AtHK1, has been implicated in osmotic stress signalling. *AtHK1* accumulates mainly in root tissue under conditions of high-salinity and low temperature and is able to complement a deletion of the yeast histidine kinase, *SLN1* (Urao et al., 1999). SLN1 functions as the sensory kinase domain which, together with the response regulator complex YPD-SSK1, activates the HOG MAP kinase (MAPK) cascade for glycerol production (Maeda et al., 1995). Two genes for response regulator-like proteins in Arabidopsis have also been found to be upregulated by cold, salinity and dehydration stress (Urao et al., 1998). The signalling events downstream of AtHK1 activation, possibly a MAPK cascade, remain to be identified.

Additional points of stress perception may include cell shrinkage during osmotic stress resulting in stimulation of mechanosensitive channels for Ca^{2+} release (e.g. Ding and Pickard, 1993), stress-induced protein conformational changes or damage to various cell components from the activity of reactive oxygen species (ROS) (Zhu, 2002). The chloroplast may be another important

sensor of abiotic stresses as variations in light, temperature and water availability impact on the efficiency of the photosynthetic electron transport chain and lead to ROS accumulation (Laloi et al., 2004). Foyer and Noctor (2003) have also suggested that ROS levels may be sensed through the increased activity of key antioxidant components.

Salinity, drought, and to a lesser extent, cold stress cause an increased biosynthesis and accumulation of the stress hormone abscisic acid (ABA) (Xiong et al., 2002a). In contrast to signalling events which are initiated rapidly in reaction to stress injury, ABA-dependent signalling pathways are thought to constitute a slower response leading to long-term adaptive changes (Luan et al., 2002). The mechanism of ABA perception is poorly understood although progress has recently been made in isolating ABA binding proteins. Zhang et al. (2002) isolated a 42-kDa plasmamembrane-associated ABA-binding protein from broad bean epidermal tissue. Treatment of guard-cell protoplasts with an antibody raised against this protein lead to an inhibition of phopholipase D activity, indicating a block in ABA signal transduction (Zhang et al., 2002). Other potential ABA receptors, both plasmamembrane-localised (Razem et al., 2004) and cytosolic (Zhang et al., 2001) have, however, also been described indicating that there may be multiple sites of ABA perception.

1.1.2 Second messengers

Second messengers involved in Ca^{2+} release

Transient increases in cytosolic-free Ca^{2+}, derived either from the apoplastic space or released from intracellular stores, are an early response to perception of cold, drought and salinity stress (Sanders et al., 1999, 2002). Both voltage-gated and second messenger specific channels have been implicated in the release of Ca^{2+} from intracellular stores. The best studied second messenger compound is inositol 1,4,5-triphosphate (IP_3) which is formed by hydrolysis of the membrane lipid, phosphotidyl-inositol-4,5-bisphosphate by phosphoinositide-specific phospholipase C (PI-PLC) (Wang, 2001). IP_3-induced Ca^{2+} release has been demonstrated by microinjection studies in guard cells (Gilroy et al., 1990) and transient increases in IP_3 levels have been reported in response to salinity stress (DeWald et al., 2001) and

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ABA (Sanchez and Chua, 2001). Evidence for the importance of IP_3 to downstream signalling events has come from studies where the endogenous levels of IP_3 were manipulated. When IP_3 was decreased through inhibition of PI-PLC activity, osmotic stress induction of the LEA proteins *RD29A* and *COR47* was inhibited (Takahashi et al., 2001). Conversely, inhibition of IP_3 degradation in the *fry1* mutant lead to enhanced expression of a number of stress responsive genes (Xiong et al., 2001a). *FRY1* encodes an inositol polyphosphate 1-phosphatase and confers increased salinity tolerance when expressed in yeast (Quintero et al., 1996). The involvement of IP_3 in ABA signalling has been demonstrated by Hunt et al. (2003) who produced transgenic tobacco plants with greatly reduced guard cell PI-PLC activity resulting in impaired stomatal closure following ABA treatment.

A number of other compounds have been identified that result in Ca^{2+} release and are thought to be involved in guard cell ABA signalling. These include cyclic ADP-ribose (Leckie et al., 1998), sphingosine-1-phosphate (Ng et al., 2001), inositol hexakisphosphate (Lemtiri-Chlieh et al., 2000) and nicotinic acid adenine dinucleotide (Navazio et al., 2000); all of which accumulate in response to ABA and have been shown to regulate Ca^{2+} levels in microinjection studies.

Other second messengers

The second messenger phosphatidic acid (PA) is generated by the hydrolysis of membrane phospholipids through the activity of phospholipase D (PLD) (Wang, 2002) which is activated by osmotic and dehydration stress (Frank et al., 2000; Munnik et al., 2000). PA has been shown to trigger signalling events leading to stomatal closure in *Vicia faba* (Jacob et al., 1999) and, in rice protoplasts, is required for ABI5-regulated expression of several ABA-inducible genes (Gampala et al., 2002). Sang et al. (2001) found that PLDα–depleted Arabidopsis plants exhibited accelerated transpirational water loss and sensitivity to drought stress. The PLDδ isoform, which is activated by dehydration and salinity (Katagiri et al., 2001), is tightly associated with the plasma-membrane as well as microtubules and has been suggested to function in conveying external signals to cytoskeletal elements (Gardiner et

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al., 2001). It is not clear how PA exerts its effect although Testerink et al. (2004) identified a number of PA-interacting signalling proteins using PA-affinity chromatography. These included 14-3-3 proteins, protein kinases and RCN1 which is the regulatory subunit of a protein phosphatase 2A (Testerink et al., 2004). RCN1 is believed to function as a positive transducer of early ABA signalling events (Kwak et al., 2002).

Hydrogen peroxide (H_2O_2) is produced during normal metabolism via a number of routes and is increased during periods of abiotic stress (Neill et al., 2002a). It is also synthesised in response to stress, mainly through the activity of NADPH oxidase (Kwak et al., 2003) and can be used as a secondary messenger to directly activate signalling components. ABA-induced H_2O_2 production has been reported in maize seedlings (Jiang and Zhang, 2002), in rice roots (Lin and Kao, 2001) and in Arabidopsis guard cells and subsequently results in activation of Ca^{2+} channels and stomatal closure (Pei et al., 2000).

Although no ROS-specific receptors have been described, direct activation of transcription factors (Tron et al., 2002) and MAP kinases by ROS have been reported. Studies have shown that H_2O_2 can directly stimulate the activity of MAP kinase cascades at different points. ANP1, an Arabidopsis MAPK kinase kinase (MAPKKK), is activated by H_2O_2 and thought to initiate the cascade which results in AtMPK3 and AtMPK6 activation (Kovtun et al., 2000). The activity of AtMPK3 is enhanced *in vitro* by AtNDPK2 (nucleotide diphosphate kinase 2) which is also strongly induced by H_2O_2 (Moon et al., 2003). H_2O_2 also inactivates a protein tyrosine phosphatase, AtPTP1, which itself inactivates AtMPK6 (Gupta and Luan, 2003).

Nitric oxide (NO) is another highly reactive molecule regulating ABA-induced stomatal closure (Desikan et al., 2004; García-Matta and Lamattina, 2003). The involvement of NO as an essential signalling molecule in ABA-induced stomatal closure has been shown in *Pisum sativum* (Neill et al., 2002b), *Vicia faba* (García-Mata and Lamattina, 2002) and in Arabidopsis where nitrate reductase (NR) has been shown to be the main enzyme responsible for NO

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synthesis (Desikan et al., 2002). The analysis of NR-deficient Arabidopsis *nia* mutants may shed light on the downstream signalling components with which NO interacts as these have not yet been identified (Desikan et al., 2002, 2004).

1.1.3 Phosphoprotein cascades

Ca^{2+}-dependent protein kinases (CDPKs) and the SOS3 family of Ca^{2+} sensors are thought to be involved in further transduction of stress signals from Ca^{2+} transients. CDPKs are serine/threonine protein kinases with C-terminal calmodulin domains for direct binding of Ca^{2+} (Ludwig et al., 2004). Their involvement in abiotic stress signalling is suggested by studies where induction or activation in response to drought, cold and salinity has been shown in a number of species (Urao et al., 1994; Tähtiharju et al.,1997; Saijo et al., 2000). Additionally, transient expression of Arabidopsis CDPK1 in a maize protoplast system was found to induce expression from a stress-responsive promoter (Sheen, 1996).

It is not known how CDPKs interact with downstream signalling components although a CDPK-interacting protein, CSP1, from the common ice plant has been shown to function as a two-component pseudo-response regulator which could activate transcription directly (Patharkar and Cushman, 2000). Unlike bacterial and Arabidopsis response regulators, pseudo-response regulators lack the absolutely conserved D-D-K motif required for phosphorylation by typical two-component histidine kinases (Makino et al., 2000). CDPKs may therefore play the role of histidine kinases in the regulation of some plant two-component systems (Patharkar and Cushman, 2000).

The Arabidopsis SOS3/SOS2 protein kinase complex is a well characterized model in osmotic stress signalling. SOS3 is most closely related to yeast calcineurin, possessing three EF-hand motifs for Ca^{2+} binding and is membrane-localised (Liu and Zhu, 1998). SOS3 senses the salt-induced Ca^{2+} signal and interacts with SOS2, a serine/threonine protein kinase, leading to its de-repression and activation of SOS1, a plasma-membrane Na^{+}/H^{+}

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antiporter (Shi et al., 2000; Qiu et al., 2002). Activated SOS2 has also been shown to be an enhancer of vacuolar Na^+/H^+ antiporter activity (Qiu et al., 2004).

Similar signalling modules featuring SOS3-like Ca^{2+} binding proteins (SCaBPs) and SOS2-like protein kinases (PKS) have been suggested to function in response to ABA, drought and cold stress (Gong et al., 2004). SCaBP5/CBL1 is induced by drought, cold and wounding in Arabidopsis (Kudla et al., 1999) and interacts specifically with PKS3 *in vivo* which, in turn, interacts with the type 2C protein phosphatases ABI1 and ABI2 (Guo et al, 2002). The ABI protein phosphatases act as negative regulators of ABA-responses (Merlot et al., 2001). Thus this trimeric complex may function in the feedback regulation of ABA-induced Ca^{2+} influx (Guo et al, 2002).

1.1.4 MAP kinase cascades

MAPK cascades are highly conserved signalling modules in eukaryotes and consist of three interlinked kinase components, a MAPKKK, a MAPKK and a terminal MAPK along which a signal is relayed by sequential phosphorylation. In Arabidopsis, a complete MAPK cascade which may function in abiotic stress has been proposed based on yeast two-hybrid studies and mutant complementation (Ichimura et al., 1998). A MAPKKK, MEKK1 which is expressed in response to high salinity, phosphorylates the MAPKKs, MKK1/2 which in turn are able to activate AtMPK4, a cold and drought-inducible MAPK (Ichimura et al., 1998, 2000; Huang et al., 2000; Matsuoka et al., 2002). However, AtMPK4 also functions as a negative regulator of pathogen responses and although *mpk4* mutants show increased pathogen resistance, no changes in gene expression were detected upon exposure to abiotic stresses (Petersen et al., 2000) suggesting that other terminal MAPKs could be involved.

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Another MAPK pathway, mentioned in 1.1.2, appears to function in response to oxidative stress in Arabidopsis and involves an H_2O_2-activated MAPKKK, ANP1, and the MAPKs AtMPK3 and AtMPK6 (Kovtun et al., 2000). Interestingly, transgenic tobacco plants expressing a constitutively active form

of an ANP1 orthologue from tobacco were more tolerant to cold, heat and salinity stresses (Kovtun et al., 2000).

MAP kinase phosphatases (MKP) may play an important role as negative regulators of MAPK cascades. Ulm et al. (2002) showed that Arabidopsis MKP1 interacts with the stress-activated MAPKs, MPK3, 4 and 6 in yeast two-hybrid assays and that *mkp1* mutant plants were more tolerant of salinity stress.

1.1.5 Protein modification, scaffolding and adaptor proteins

Modifying the activity of signalling components, as well as ensuring their correct spatial orientation in the cell, are essential to the precision and specificity of signal transduction. While phosphorylation is the most important post-translational modification affecting signalling others such as methylation, acetylation, glycosylation, myristoylation and isoprenylation may also be also play important roles (Xiong and Zhu, 2001). A requirement for farnesylation in the ABA signal transduction pathway is seen in the *era1* mutant of Arabidopsis which displays ABA hypersensitivity (Allen et al., 2002). *ERA1* encodes a subunit of a farnesyl-transferase and functions to negatively regulate ABA responses upstream of Ca^{2+} signals. A possible target for ERA1 is ROP10, a small G protein which negatively regulates Ca^{2+} channel activation in response to ABA and requires farnesylation for insertion into the plasma-membrane (Zheng et al., 2002).

The regulated degradation of proteins may be another way of controlling the activity of signalling components. The *HOS1* locus of Arabidopsis encodes a putative E3 ubiquitin ligase and *hos1* mutant plants display a sustained induction of CBF2 and CBF3 transcription factors and their downstream target genes during cold stress (Lee et al., 2001). HOS1 therefore functions to fine tune the level of CBF expression by targeting a positive regulator of this pathway for degradation (Lee et al., 2001). Another protein degradation pathway involving tagging by small ubiquitin-like modifier (SUMO) has been implicated in regulating stress-induced signal transduction. In response to ABA, transgenic Arabidopsis plants over-expressing AtSUMO1/2 showed

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increased expression of the stress-responsive genes *AtPLC1* (*phospholipase C1*) and *RD29A* compared to wild-type and also showed an attenuation of ABA-mediated growth inhibition (Lois et al., 2003).

Proteins that tether signalling components into functioning units or aid in their subcellular localization are important in controlling signalling specificity as well as the rate of signal transduction (Xiong and Zhu, 2001). This may be particularly important where signalling components have dual roles in different pathways such as the MAPKK, SIMKK from alfalfa which functions both in salinity stress and pathogen signalling (Cardinale et al., 2002). A similar situation exists in yeast where the MAPKKK, STE11 functions both in pheromone and osmotic stress signalling but can be complexed to pheromone-specific components by the anchor protein, STE5 or to osmotic stress components by the intrinsic anchor function of a MAPKK, PBS2 (Whitmarsh and Davis, 1998; Jonak et al., 2002). Similar anchoring proteins have, however, not yet been described in plants.

14-3-3 proteins, so named from their chromatography and electrophoresis profiles, are an important class of adapter proteins which function at multiple levels in signal transduction pathways (Ferl, 2004; Comparot et al., 2003). They bind conserved phosphoserine peptide motifs (Yaffe et al., 1997) via a highly conserved binding groove and can cause an alteration in target protein activity or localization, or facilitate interactions with other proteins (Roberts, 2003). 14-3-3 proteins are required for activation of the plasma membrane H^+-ATPase during osmotic and cold stress (Babakov et al, 2000; Kerkeb et al., 2002; Korthout and de Boer, 1994) and cause inhibition of nitrate reductase activity (Bachmann et al., 1996). Binding of 14-3-3 proteins in both cases is dependent on prior phosphorylation of the target proteins, suggesting that phosphorylation serves as a tag for 14-3-3 association which subsequently brings the signal transduction event to completion (Sehnke et al., 2002; Ferl, 2004).

The activity of various signalling components can be modulated by 14-3-3 protein binding. These include the Arabidopsis CDPKs, CPK1 (Camoni et al.,

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1998) and CDPK2 (Cotelle et al., 2000) as well as components of G-protein signalling pathways (Niu et al., 2002). 14-3-3 proteins have also been shown to bind the general transcription factor TFIIB and the TATA box binding protein (Pan et al., 1999) as well as more specific transcription factors such as maize GBF1, a bZIP transcription factor (de Vetten et al., 1992; de Vetten and Ferl, 1995) and the ABA-responsive transcription factors VP1 and EmBP1 (Schultz et al., 1998).

1.2 TRANSCRIPTIONAL REGULATION

1.2.1 *cis-* and *trans*-acting elements in abiotic stress

A number of *cis-* and *trans-* acting factors have been described that function in the transcriptional response to abiotic stress (Fig.1.2). The best defined of these are the DRE/CRT motif (dehydration-responsive element/C-repeat), A/GCCGAC (Sakuma et al., 2002), and the ABRE (ABA responsive element) motif, PyACGTGGC (Busk and Pagés, 1998). Both elements have been found in the promoters of genes induced by ABA, cold, dehydration and high salinity stress.

The DRE/CRT motif was first defined in the promoter of the *RD29A* gene (Yamaguchi-Shinozaki and Shinozaki, 1994) and is bound by transcription factors of the AP2/EREBP family in ABA-independent signalling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000). These are DREB1A, B and C which are rapidly induced by low temperature. (Liu et al., 1998) and are identical to the independently isolated CBF3, 1 and 2 respectively (Stockinger et al., 1997; Gilmour et al., 1998). DREB2A and B are induced by drought stress (Liu et al., 1998) and resemble DREB1 proteins only in the AP2 domain which consists of a DNA-binding region and an amphipathic α-helix (Kizis et al., 2001). When over-expressed in Arabidopsis, CBF1/DREB1B and CBF3/DREB1A conferred freezing, drought and salinity tolerance (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000; Liu et al., 1998).

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An upstream regulator of *CBF3/DREB1A* expression has recently been identified and is called *ICE1* (Chinnusamy et al., 2003). *ICE1* encodes a MYC-type basic helix-loop-helix (bHLH) transcription factor and is constitutively expressed but induces *CBF3/DREB1A* expression only when exposed to low temperature stress. This indicates a requirement for phosphorylation of ICE1 or the presence of a MYB partner transcription factor to activate *CBF* expression (Chinnusamy et al., 2003).

Haake et al. (2002) cloned a DREB1 homologue, CBF4 from Arabidopsis, which is induced by drought stress and ABA but not by cold. Transgenic Arabidopsis plants over-expressing CBF4 were, however, more tolerant to cold as well as drought stress and showed activation of cold-regulated (*COR*) genes (Haake et al., 2002). CBF4 therefore confers ABA-responsiveness to genes with DRE/CRT promoter elements, possibly as a result of divergent evolution in its own promoter region which shows little sequence identity to that of other *CBF* genes (Haake et al., 2002). However, ABA-induced expression of the other DREB1/CBF proteins has also recently been shown by Knight et al. (2004) indicating that these proteins may also be recruited in ABA-dependent signalling pathways.

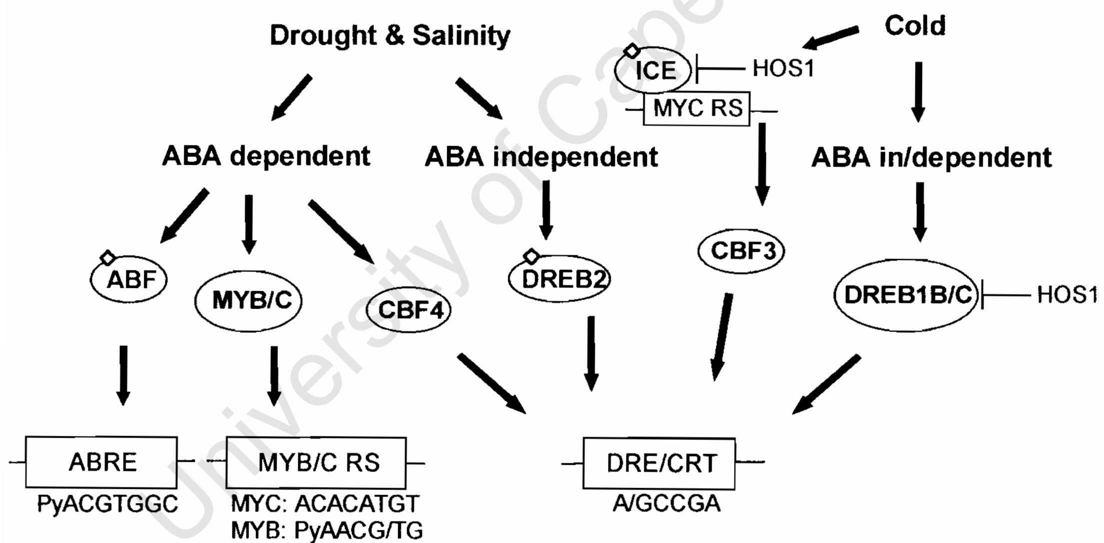

Figure 1.2 Simplified model for the regulation of cold-, drought- and salinity-responsive gene expression. Transcription factors are shown in ovals and are tagged with a diamond where modification or a partner protein is required for activation. *cis*-acting elements appear in boxes with the conserved recognition sequences (RS) below. *DREB1/CBF* gene expression is modulated by both ABA-dependent and independent pathways (Knight et al., 2004). HOS1 functions as a negative regulator of ICE and DREB1/CBF (Lee et al., 2001). Figure adapted from Haake et al. (2002) and Shinozaki et al. (2003).

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ABREs were first identified in the promoters of the ABA inducible genes *Em* from wheat (Marcotte et al., 1989) and *rab16* (Mundy et al., 1990) from rice. Both G-box promoter elements (CACGTG) and ABREs share the same ACGT core sequence and are bound by the basic domain leucine-zipper (bZIP) class of transcription factors (Sibèril et al., 2001). A family of ABA-induced bZIPs, the ABFs/AREBs, are expressed in vegetative tissues in response to cold, drought and salinity (Choi et al., 2000; Uno et al., 2000). Constitutive expression of ABF3 and ABF4 in Arabidopsis leads to enhanced drought tolerance and increased expression of a number of ABA and stress-responsive genes (Kang et al., 2002).

In the absence of ABREs, ABA-induced gene expression can also be mediated by MYC (bHLH) and MYB class transcription factors. Abe et al. (1997) defined functional MYC (ACACATGT) and MYB (PyAAC[G/T]G) elements in the *rd22* promoter and cloned cDNAs for the proteins which were shown to bind these elements and were themselves induced by drought and ABA treatment. Transgenic plants over-expressing AtMYC2 and/or AtMYB2 were more sensitive to ABA and more tolerant of osmotic stress (Abe et al., 2003). Two MYB-related genes, *cpm7* and *cpm10*, have also been cloned from the desiccation-tolerant plant, *Craterostigma plantagineum,* and are upregulated by ABA and dehydration (Iturriga et al., 1996).

1.2.2 Post-transcriptional regulation in ABA responses

Proteins involved in pre-mRNA processing and nuclear export have been shown to play important roles in regulating ABA-induced gene expression (Kuhn and Schroeder, 2003). Mechanisms for controlling the mRNA maturation of specific ABA-induced genes are displayed by the ABH1 protein, the large subunit of an mRNA-cap-binding complex (Hugouvieux et al., 2001) and by SAD1, an Sm-like small nuclear ribonucleoprotein (Xiong et al., 2001b). *abh1* mutant Arabidopsis plants show ABA-hypersensitive stomatal closure and cytosolic Ca^{2+} increases in guard cells (Hugouvieux et al., 2001). Microarray analysis found only 31 genes out of a possible 8000 with altered mRNA levels relative to the wild-type. Amongst the down-regulated genes was *AtPP2C*, which functions as a negative-regulator of ABA-signalling

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(Sheen, 1998). ABH1 may therefore function as a modulator of early ABA signalling events through selective interaction with mRNAs of key signalling components (Hugouvieux et al., 2001). Similarly, the *sad1* mutation, which also causes ABA-hypersensitivity, is probably defective in mRNA processing as Sm-like proteins are known to participate in the splicing, export and degradation of mRNA (He and Parker, 2000; Xiong et al., 2001b).

Another protein functioning in RNA processing is AKIP1 from *Vicia faba* which has two motifs for single-stranded RNA binding (Li et al., 2002). When phosphorylated by an ABA-activated protein kinase (AAPK), AKIP was shown to bind a dehydrin mRNA and be translocated to nuclear speckles which are associated with functions of the spliceosome (Li et al., 2002).

Post-transcriptional regulation of mRNA abundance is displayed by the Arabidopsis HYL1 protein which binds double stranded RNA and may be involved in determining mRNA half-life through a microRNA-mediated process (Lu and Fedoroff, 2000; Vazquez et al., 2004). *hyl1* mutant plants are ABA-hypersensitive and show increased levels of AtMPK3 activity (Lu et al., 2002) suggesting that HYL1 negatively regulates the abundance of mRNAs involved in ABA signalling.

While it is not yet clear how important these proteins are in modulating the expression of ABA-induced genes, they do represent several novel mechanisms for fine-tuning the plant's response to abiotic stress at the post-transcriptional level.

1.2.3 Stress-regulated genes

The targets of signal transduction pathways include gene products which function directly in the amelioration of the stress and those which are involved in downstream signalling events (Shinozaki and Yamaguchi-Shinozaki, 1997). Proteins which are thought to contribute to stress tolerance include enzymes for synthesis of osmolytes (sugars, amino acids and polyols), antioxidants for ROS detoxification, proteases and protease inhibitors, as well as ion

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transporters, water channel proteins (aquaporins) and ATPases for ion homeostasis (Ramanjulu and Bartels, 2002; Xiong and Zhu, 2002).

Microarray studies in Arabidopsis and rice have shown that there is considerable overlap in the transcriptional response to drought, cold and salinity (Seki et al., 2002; Rabbani et al., 2003). In both species however, the response to drought and salinity stress is more similar than to cold and salinity stress. Additionally, in both rice and Arabidopsis, more genes are induced in response to drought stress than the other two. In Arabidopsis five times as many genes as cold stress and three times as many as for salinity stress are induced by drought (Seki et al., 2002). While the stress-induced genes identified in these studies generally confirm the already-described adaptations used by plants to adapt to abiotic stress, the majority have no described function. This implies that a better understanding of stress tolerance mechanisms may be gained by characterising the outputs of signal transduction pathways.

1.3 PROSPECTS FOR IMPROVING PLANT STRESS TOLERANCE

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Components of signalling networks are attractive targets for stress-tolerance improvement applications as they have the potential to activate multiple downstream effector genes resulting in an enhancement of the plant's own stress response. While stress-tolerant species may express proteins with unique protective or repair functions, the difference in gene expression between stress-tolerant and sensitive species may also involve differences in gene regulation (Bartels and Salamini, 2001). Ouellet et al. (1998) found that the freezing tolerance of different wheat cultivars was correlated with the transcript levels of a cold-induced gene *wcs120* and that while *wcs120* homologues are present in the genomes of cold-sensitive rice and maize, they are not expressed. Similarly, genes which contribute to the desiccation tolerance of seeds in various species may also be expressed in vegetative tissues of resurrection plants during drying (e.g. Mowla et al., 2002).

Improved stress tolerance through the over-expression, down-regulation or mutation of a range of signalling components has been demonstrated in Arabidopsis and a few agronomically important species (Table 1.1). The constitutive induction of stress-responses may be detrimental to plant growth and thus the use of stress inducible promoters or signalling proteins that require a stress-induced signal such as Ca^{2+} or phosphorylation for activation are preferable. Identifying the rate-limiting steps in signal transduction pathways will help to identify more targets for engineering stress tolerance.

Table 1.1 Transgenic attempts to improve abiotic stress tolerance using signalling/ regulatory proteins

Gene*	Product	Phenotype and species**	Notes	Reference
ABI3	transcription factor (VP1-like)	F	Faster acclimation, basal tolerance unchanged	Tamminen et al. (2001)
CBF1/DREB1B	transcription factor (AP2/EREBP)	F	Multiple *COR* genes expressed	Jaglo-Ottosen et al. (1998)
..	..	F (*B. napus*)	Tested by electolyte leakage assay only	Jaglo et al. (2001)
..	..	D C O (tomato)	Increased catalase and proline levels	Hsieh et al. (2002a, 2002b)
..	..	D C S (tomato)	Improved yield with stress-inducible promoter	Lee et al. (2003a)
CBF3/DREB1A	transcription factor (AP2/EREBP)	F	Increased proline and total soluble sugars	Gilmour et al. (2000)
..	..	D F	Severe dwarf phenotype	Liu et al. (1998)
..	..	D S F	*rd29a* promoter prevented dwarfing	Kasuga et al. (1999)
OsDREB1A	transcription factor (AP2/EREBP)	D C S	Rice *DREB1A* homologue induces *AtDREB1A* targets	Dubouzet et al. (2003)
CBF4	transcription factor (AP2/EREBP)	D C	Multiple *COR* genes expressed	Haake et al. (2002)
CBF2/DREB1C (null mutant)	transcription factor (AP2/EREBP)	D S F	Increased *CBF1* and *CBF3* expression	Novillo et al. (2004)
Tsi1	transcription factor (AP2/EREBP)	S (tobacco)	Also shows pathogen resistance	Park et al. (2001)
CpMYB10	transcription factor (MYB)	D S	gene from *Craterostigma plantagineum*	Villalobos et al. (2004)
OsMyb4	transcription factor (MYB)	C F	Constitutive expression of cold-induced genes	Vannini et al. (2004)
AtMYC and AtMYB2	transcription factors (bHLH and MYB)	S	Enhanced expression of ABA-inducible genes	Abe et al. (2003)

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ICE1	transcription factor (bHLH)	F	Increased CBF3 and CBF3-target genes during cold	Chinnusamy et al. (2003)
ABF3/4	transcription factor (bZIP)	D	Reduced transpiration rate	Kang et al. (2002)
Alfin 1	transcription factor (zinc finger)	S (alfalfa)	Controls expression of cell wall protein MsPRP2	Winicov and Bastola (1999)
SCOF-1	transcription factor (zinc finger)	C (tobacco)	Enhances ABRE-binding activity of SGBF1	Kim et al. (2001)
ZPT2-3	transcription factor (zing finger)	D (petunia)	Functions as repressor	Sugano et al. (2003)
STO	zinc-finger protein	S	Binds a salinity-induced MYB transcription factor	Nagaoka and Takano (2003)
OSISAP1	zinc-finger protein	D C S (tobacco)	All tests done at germination or on seedlings	Mukhopadhyay et al. (2004)
NPK1	MAPKKK	C S (tobacco)	Also heat-shock tolerant (48°C 45 min)	Kovtun et al. (2000)
NPK1	MAPKKK	F (maize)	Higher total soluble sugars	Shou et al. (2004)
OsMAPK5	MAPK	DCS (rice)	Pathogen resistance when *OsMAPK5* suppressed	Xiong and Yang (2003)
mkp1 (null mutant)	MAPK phosphatase	S	MKP1 interacts with MPK3/4/6	Ulm et al. (2002)
SOS2 (active mutant)	protein kinase	S	Increased SOS1 activation	Guo et al. (2004)
AtNDPK2	protein kinase	C S O	Increased activation of AtMPK3/6-like proteins	Moon et al. (2003)
AtGSK1	GSK3/shaggy-like protein kinase	S	Plants accumulate anthocyanins	Piao et al. (2001)
OsCDPK7	calcium-dependent protein kinase	D C S (rice)	Enhanced *LEA* expression only during stress	Saijo et al. (2000)
AtCaMBP25 (antisense)	calmodulin-binding protein	Os	NaCl and mannitol tolerance	Perruc et al. (2004)
CBL1 and *cbl1* (mutant)	calcineurin B-like protein	D S F	Only *cbl1* plants were F but not D or S	Cheong et al. (2003)
PLDδ	phospholipase D	F	Cold acclimation period still required	Li et al. (2004)
PLDα	phospholipase D	D	Water loss assay on detached leaves	Sang et al. (2001)
NtC7	receptor-like protein	Os	Mannitol tolerant but not NaCl tolerant	Tamura et al. (2003)
gcr1 (null mutant)	G-protein coupled receptor	D	GCR1 negatively regulates GPA1 (Gα subunit)	Pandey and Assmann (2004)
fry2/cpl1 (null mutant)	dsRNA-binding phosphatase	S	Increased expression of DRE/CRT-class genes	Xiong et al. (2002b)
abh1 (null mutant)	mRNA cap-binding protein	D	Decreased expression of *AtPP2C*	Hugouvieux et al. (2001)

*Constitutive over-expression unless indicated **Plants are Arabidopsis unless indicated
Abbreviations: F, freezing tolerant; D, drought tolerant; S, salinity tolerant; O, oxidative-stress tolerant; Os, osmotic-stress tolerant.

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1.4 OUTLINE OF INVESTIGATION

1.4.1 *Eragrostis nindensis*, a desiccation-tolerant grass

Desiccation-tolerant or 'resurrection' plants are capable of tolerating severe water loss, retaining less than 5% of their total water content , and then revive completely and resume normal metabolism within hours of watering (Gaff, 1971). *Eragrostis nindensis* Ficalho & Hiern is one of only a few desiccation-tolerant grasses and is distributed across a broad region of southern Africa, growing in shallow soils (Fig. 1.3). Upon dehydration, *E. nindensis* undergoes a controlled shutdown of physiological activity, losing chlorophyll and dismantling thylakoid membranes (Vander Willigen et al., 2001a). Cell wall folding of mesophyll cells is also observed and is thought to reduce physical damage on drying (Vander Willigen et al., 2001b). A number of protective measures described in other resurrection plants (Bernacchia and Furini, 2004) are put in place and include the accumulation of anthocyanins and the osmoprotectant, sucrose (Vander Willigen et al., 2001b). In addition, the accumulation of numerous small vacuoles in bundle sheath cells filled with proline, sucrose and proteins is thought to contribute to the mechanical stabilisation of dehydrated tissue (Vander Willigen et al., 2004).

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Figure 1.3 *Eragrostis nindensis*, a desiccation-tolerant grass. (a) *E. nindensis* plants (foreground) growing on an inselberg (granitic outcrop) in Gamsberg, South Africa. (b) Desiccated (left) and hydrated (right) *E. nindensis* plants.

1.4.2 Aims of this study

Resurrection plants are valuable model systems for studying mechanisms of abiotic stress tolerance. The signal transduction pathways which lead to the co-ordinated expression of proteins with protective or repair functions are of particular interest. It is not known if, and to what extent, resurrection plants and desiccation-sensitive plants use the same *cis-* and *trans-* acting factors in abiotic stress responses or whether novel elements unique to resurrection plants are more important. This study aims to address this question using *E. nindensis* as a model desiccation-tolerant plant and focuses specifically on bZIP transcription factors involved in the regulation of ABA-inducible genes. As no genomic or cDNA libraries are available for this plant, a degenerate RT-PCR approach is taken with the aim of cloning a candidate bZIP transcription factor and characterising its possible role in the acquisition of desiccation-tolerance.

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2.1 Plant material and treatments

Mature *E. nindensis* plants were collected from an inselberg in the Gamsberg region of South Africa, potted in soil from the site and maintained in a greenhouse with no supplementary lighting or temperature control. All plants were allowed to acclimatise to greenhouse conditions for at least one month prior to any experimentation.

For dehydration/ rehydration treatment, plants were moved to a growth room (constant 25 °C, 50% humidity, 16/8 h light/dark cycle, 350-400 $\mu mol.m^{-2}.s^{-1}$ light intensity) and allowed to acclimate for at least one week. Plants were allowed to dehydrate by withholding water for appoximately two weeks, during which leaf samples from a number of plants were taken at the same time each morning. Only the inner, desiccation-tolerant, leaves from each tiller were sampled from. Approximately 1-2 cm from leaf mid-sections were kept aside for RWC determination and the remainder frozen in liquid nitrogen and stored at –80 °C. Plants were maintained in the dry state for at least one week prior to rewatering and sampling as above.

For ABA treatment, leaves of fully-hydrated plants were sprayed with 0.1 mM or 2.5 mM solutions of (±)-*cis,trans*-ABA (Sigma) containing 0.02% (v/v) Tween-20 as a wetting agent and leaf samples taken over a period of 4 hours under laboratory conditions. To test the effects of diurnal rhythms on gene expression, leaf samples were taken from greenhouse plants every 6 h over a 24 h period and to examine the effect of light-deprivation, plants were kept in a light-proof cupboard and sampled under low-light after 12, 24 and 48 h.

2.2 Relative water content determination

Relative water content was determined gravimetrically using the equation RWC= [(fresh weight – dry weight)/ dry weight)] / [(full turgor weight – dry weight)/ dry weight)] where fresh weight refers to the weight measured immediately after excision and dry weight to that after oven drying at 70 °C for 48 h. Mean moisture content at full turgor was calculated from hydrated plants covered with plastic bags overnight. At each sampling time, duplicate

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measurements were performed, each consisting of 10-15 leaf sections weighed simultaneously and RWC was calculated from the average of the two.

2.3 Nucleic acid and protein isolation

Genomic DNA was isolated according to the method of Doyle and Doyle (1987) with modifications. Leaf tissue (0.1g per 0.5 ml extraction buffer) was ground to a fine powder in liquid nitrogen and added to prewarmed extraction buffer (2% [w/v] CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA) and incubated at 65 °C for 2 h with intermittent mixing. Tissue debris was pelleted by centrifugation at 12000 x *g* for 5 min. Supernatants were transferred to fresh tubes and extracted with an equal volume of chloroform: isoamyl alcohol (24:1), mixed by gentle inversion for 2 min and centifuged as above for 1 min. DNA was precipitated from the aqueous phase by addition of 1/10 volume of 3M sodium acetate, pH 5.5 and two volumes of ice-cold ethanol followed by centrifugation as above for 2 min. The DNA was washed twice in 70% (v/v) ethanol, dissolved in water and quantified by spectrophotometry.

RNA and proteins were isolated simultaneously using Trizol reagent (Invitrogen, 1 ml per 0.1 g tissue) according to the manufacturer's instructions. RNA was dissolved in DEPC-treated water and quantified by spectrophotometry or stored in isopropanol at –80 °C. Proteins, dissolved in 0.1% (w/v) SDS, were quantified by the Bradford (1976) method using the Biorad Dye Reagent in the microassay procedure described by the manufacturer.

2.4 cDNA synthesis

cDNA was synthesised from 2 µg total RNA isolated from ABA-treated leaf tissue. Each reaction contained 0.5 mM each dNTP, 1 µM oligo-dT_{18} primer, 10 units RNase inhibitor (Promega), 4 units Omniscript reverse transcriptase (Qiagen) and 2 µl of the supplied 10X buffer in a final volume of 20 µl. Reactions were incubated at 37 °C for 90 min.

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2.5 Degenerate primer design

Protein sequences from a broad range of plant bZIPs thought to be responsive to ABA or environmental signals were obtained from the NCBI database (http://ww.ncbi.nlm.nih.gov) and multiple alignments made using DNAssist software (ClustalW algorithm). Highly conserved regions were reverse translated and two degenerate forward primers (Pab1F: 5'-GCTTTG-GGTC[A/T/G/C]ATGAA[C/T]ATGG-3' and Pab2F: 5'-CGCATGCAGGCATA-[C/T]TA[C/T]GG-3') and a reverse primer, targeting the basic domain (Pab1R: 5'-CTTGA[C/T]C[T/G][C/T/G]C[T/G][T/A]GCIGA[C/T]TC-3'), were designed. Another degenerate forward primer, Pab3F (5'-GTCATGG[C/G]I-[C/A]A[C/T/A/G]GA[C/T]GA[A/G]GC-3'), was used to isolate the 5' end of a cDNA fragment obtained in preliminary experiments. Degeneracy was reduced by taking account of any codon bias observed in bZIP nucleotide sequences from monocot species as well as the use of the inosine (I) base in primer sequences. All primers were synthesized in the Dept. of Molecular and Cell Biology Oligonucleotide Synthesis Unit, UCT.

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2.6 RT-PCR

PCR reactions were made up in MiliQ water to a final volume of 25 or 50 µl and typically contained 0.3 mM each dNTP, 2.5 mM $MgCl_2$, 1.5 µM each of forward and reverse primers, 1-2 units *Taq* DNA polymerase (SuperTherm), an appropriate amount of the supplied 10X reaction buffer and 2-5 µl of template cDNA. Reaction yields were optimised by varying the amount of enzyme, cDNA and the inclusion of DMSO (2-4% v/v) or BSA (0.1 - 0.4 µg/µl).

As the melting temperature of degenerate primers spanned a broad range, a touch-down PCR program (Don et al., 1991) was used in initial experiments as follows: 95 ºC (3 min), 20 x [95 ºC (30 s), 60 ºC (30 s) decreasing by 0.5 ºC per cycle to 50 ºC, 72 ºC (45 s)], 10 x [95 ºC (30 s), 50 ºC (30 s), 72 ºC (45 s)] and 72 ºC (10 min). Touch-down PCR favours the accumulation of specific amplification products over non-specific products at higher annealing temperatures. In subsequent rounds of PCR, specific products are in greater abundance and are preferentially amplified at the lower annealing temperature (Don et al., 1991).

2.7 5' and 3' RACE

First-strand cDNA was prepared from total RNA isolated from ABA-treated leaf tissue and subsequently amplified by long-distance PCR using the Creator SMART cDNA Library Construction Kit (Clontech) according to the manufacturer's protocol. The resulting double-stranded cDNAs, preferentially enriched for full length transcripts, carried the SMART oligonucleotide sequence at both ends. The SMART RACE cDNA amplification kit (Clontech) was then used together with gene specific primers, EnGSP1 (5'-CGCTCGA-CCCTCGGCACTGTAATC-3') and EnGSP2 (5'-ACGAGCGTGGAGCCAG-CCAAGTC-3') for 5' and 3' RACE respectively, in touch-down PCR programs as recommended.

2.8 Cloning and analysis of PCR products

PCR products were isolated from agarose gels (gel purified) using the High Pure PCR Product Purification kit (Roche), cloned into the pGEM-T Easy vector (Promega) using the manufacturer's protocol and used to transform competent *E. coli* JM109 cells by heatshock. Cells were subsequently plated onto LB agar plates containing 100 µg/ml ampicillin, 10 µg/ml IPTG and 5 µg/ml X-Gal and incubated overnight at 37 ºC. White colonies were selected and screened for inserts by colony PCR with the M13R and M13F primer or a gene specific primer as appropriate. Colonies containing inserts were grown overnight in LB containing 50 µg/ml ampicillin and plasmid DNA was extracted using the High Pure Plasmid Isolation kit (Roche). Inserts were sequenced using a DYEnamic ET Dye terminator Cycle sequencing kit and a MegaBACE 500 sequencer (Molecular Dynamics).

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2.9 Southern blot analysis

Genomic DNA (30-40 µg) was digested overnight at 37 ºC with 40 units of *Eco*R1, *Hin*dIII, *Pst*I or a combination of *Eco*R1 and *Hin*dIII in a final volume of 200 µl containing 2.5 mM spermidine, precipitated as described in 2.3 and redissolved in 20 µl water. DNA was separated on a TBE-buffered 0.8% agarose gel run at 20V for 14 h and transferred to a positively charged nylon membrane (GeneScreen Plus, NEN Life Sciences) using a rapid, downward alkaline capillary transfer method (Koetsier et al., 1993).

A 680-bp probe was synthesised with the PCR DIG Probe Synthesis Kit (Roche) using Pab1R and Pab2F primers and a 1:6 ratio of DIG-dUTP:dTTP. The template used was pGEM-T Easy:EnGBF1 plasmid DNA. Success of the labelling reaction was evaluated by agarose gel electro-phoresis, labelled probe migrating slower than the unlabeled control.

Hybridisation buffers and conditions were according to Engler-Blum et al. (1993) with modifications. Membranes were prehybridised in 20 ml of DIG Easy-Hyb buffer (Roche) for 2 h. The pre-hybridisation buffer was removed and 10 ml of hybridisation buffer containing 20 µl of denatured probe was added. Hybridisation was performed overnight at 37 °C with gentle shaking. Membranes were washed twice in 2x SSC, 0.1% (w/v) SDS at room temperature for 5 min and twice in 0.1x SSC, 0.1% (w/v) SDS at 65 °C for 15 min each. Membranes were then washed in maleic acid buffer (0.1M maleic acid, 3M NaCl, 0.3% Tween-20 [v/v] pH 8) for 3 min followed by 1 h of shaking in blocking solution (0.5% [w/v] Roche blocking reagent, 0.1M maleic acid, 3M NaCl) before addition of Anti-DIG-AP Fab fragments (Roche) at 1:15000 dilution. Blots were incubated for 30 min at room temperature with gentle shaking, washed four times for 10 min each in maleic acid buffer (as above). Blots were incubated with detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for 5 min and placed between plastic sheets. Excess liquid was removed and 2 ml of CDP-Star solution (1:100 dilution of CDP-Star reagent [Roche] in detection buffer) were applied directly to membranes. Blots were incubated for 5 min and exposed to X-ray film (Kodak BioMax ML) for various times.

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2.10 Northern blot analysis

Total RNA isolated from leaf tissue at various stages of dehydration was separated by electrophoresis though 1.2% (w/v) agarose gels containing 2% (v/v) formaldehyde in 1x MOPS buffer (20 mM MOPS, 0.5 mM sodium acetate, 0.1 mM EDTA, pH 7.0) for 4 h at 50V. RNA was then transferred to Hybond XL (Amersham) for radioactive detection, or GeneScreen Plus (NEN Life Sciences), for non-radioactive detection, by downward capillary transfer using 50 mM NaOH as transfer buffer (Ingelbrecht et al., 1998).

For non-radioactive detection, pGEM-T Easy:EnGBF1 plasmid DNA (1 µg) was linearized by *Sal*1 digestion, purified by phenol-chloroform extraction, and used as template to produce a DIG-labelled RNA probe by *in vitro* transcription using the DIG RNA labeling kit (Roche) according to the supplied protocol. The probe was quantified by comparison to a DIG-labeled control RNA and diluted to a concentration of 0.1 µg /ml hybridisation buffer (DIG Easy-Hyb, Roche). Hybridisation, washing and detection procedures were performed as described in 2.9 except that a hybridisation temperature of 42 °C was used.

For radioactive detection, template DNA was prepared by PCR, gel purified and labeled with [α-^{32}P]dCTP (Amersham) by random priming using the Megaprime DNA labelling kit (Amersham) according to the recommended protocol. Unincorporated label was removed using SigmaSpin Post-Reaction Purification columns (Sigma) and labelling efficiency was estimated by liquid scintilation counting. Membranes were prehybridised for 1 h in Church and Gilbert buffer (0.25 M Na_2HPO_4 [pH 7.2], 1 mM EDTA, 7% [w/v] SDS, 1% [w/v] BSA), denatured probe was then added and hybridisation performed overnight at 68 °C. Membranes were washed twice in 2x SSC, 0.1% (w/v) SDS at room temperature for 5 min and in 0.5x SSC, 0.1% (w/v) SDS at 68 °C for 10 min or until background counts were low. Signals were detected by autoradiography (HyperFilm, Amersham).

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2.11 Bacterial expression and purification of EnGBF1

EnGBF1 (ORF and 100 bp of 3' UTR) was excised from pGEM-T Easy:EnGBF1 by digestion with *Eco*R1. An *E. coli* expression vector, pProEx-HTc (GibcoBRL, Fig. 2.1), was linearized by digestion with the same enzyme. Digestion products were separated by agarose gel electrophoresis and gel purified. Ligation reactions were performed using an approximate vector:insert ratio of 1:1 in a final volume of 20 µl containing 2 units T4 DNA ligase (Promega) and 2 µl 10x ligase buffer and incubated overnight at 14 °C. *E. coli* DH5α was transformed with 4 µl of ligation mix by heatshock and selected on LB agar plates containing 100 µg/ml ampicillin. Transformants were screened

for correct orientation of the insert by colony PCR using M13R and EnGSP1 primers.

Expression was induced in 10 ml *E. coli* cultures (LB media, 100 µg/ml ampicillin) grown to density of 0.7 A_{600nm} by addition of IPTG to a final concentration of 0.6 mM. Samples were taken 3 h after induction and analysed by 12% SDS-PAGE using the BioRad minigel system. Purification of the histidine-tagged proteins was performed using the MagneHis Protein Purification kit (Promega) under denaturing conditions.

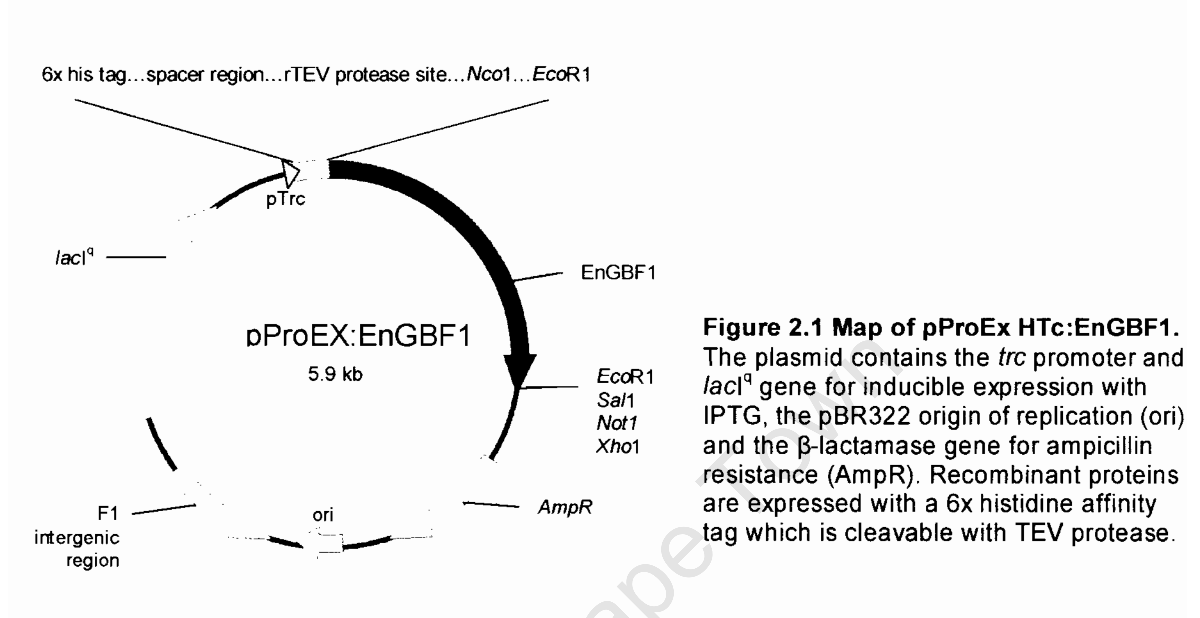

Figure 2.1 Map of pProEx HTc:EnGBF1. The plasmid contains the *trc* promoter and *lacI*^{q} gene for inducible expression with IPTG, the pBR322 origin of replication (ori) and the β-lactamase gene for ampicillin resistance (AmpR). Recombinant proteins are expressed with a 6x histidine affinity tag which is cleavable with TEV protease.

2.12 Yeast transactivation assay

EnGBF1 was excised from pProEX:EnGBF1 by digestion with *Eco*R1 and *Sal*1 for insertion into a GAL4 activation-domain (GAL4AD) fusion vector, pPC86 (Fig. 2.2), (a gift of R. Reed, Wang and Reed, 1993), which was linearized by *Bgl*II digestion. 5' overhangs of DNA fragments were filled in by treatment with Klenow fragment and gel purified. Ligations were performed as described except that incubations were at room temperture. *E. coli* transformants were screened for correct orientation of the insert by colony PCR using a GAL4AD-specific primer (5'-CGTTTGGAATCACTACAGGG-3') and EnGSP1. The vector-insert junction region was sequenced to confirm in-frame insertion with the GAL4 domain. pYC7:ABRE (Fig. 2.2), a *lacZ* reporter plasmid (a gift of S-Y. Kim, Choi et al., 2000) containing a trimer of the Em1A

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ABRE element (5'- GGACACGTGGCG-3') from the wheat *Em* promoter (Marcotte et al., 1989) as target sequence, was used in conjunction with pPC86: EnGBF1.

Saccharomyces cerevisiae strain YM4271 (Clontech, genotype: *MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-Δ512, gal80-Δ538, ade5::hisG*) was simultaneously co-transformed with pPC86:EnGBF1 and pYC7:ABRE by a lithium acetate-mediated transformation procedure (Ito et al., 1983) as modified by Elbe et al. (1992). Briefly, a yeast colony (±3 mm diameter) was scraped off a YPD plate and resuspended in 0.5 ml PLATE solution (40% [w/v] PEG 4000, 0.1 M lithium acetate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) to which 100 µg salmon sperm DNA and 1 µg of each plasmid DNA was added. The mixture was vortexed and incubated overnight at room temperature. Yeast cells were pelleted by brief centrifugation, resuspended in sterile water, plated on SD-TRP-URA media and incubated at 30 °C for 3-5 days. Control yeast strains carrying empty pYC7 or pPC86 plasmids were prepared as above.

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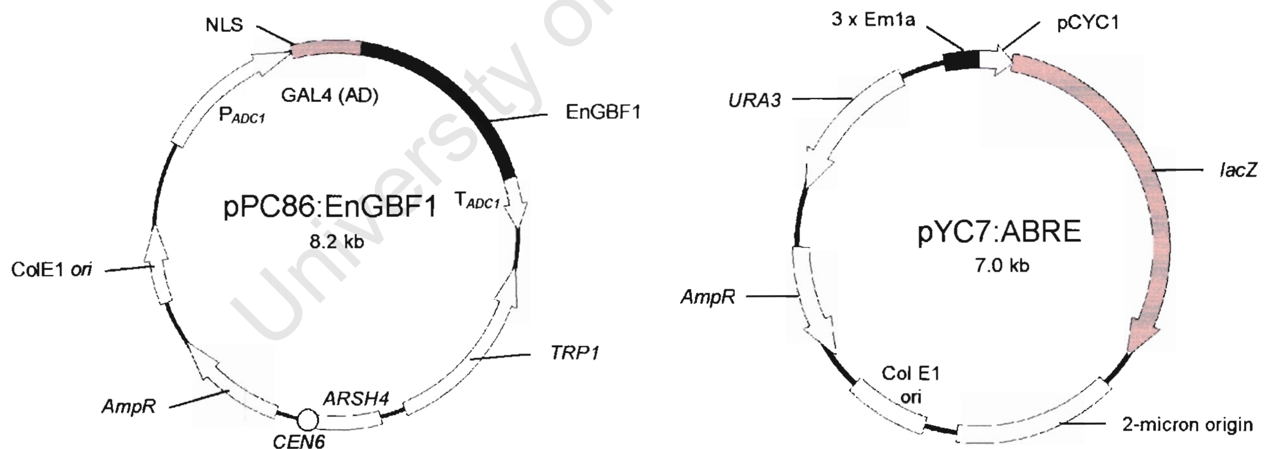

Figure 2.2 Maps of yeast vectors used in transactivation assay. (*Left*) pPC86:EnGBF1 encodes the GAL4 activation domain (AD) expressed from a strong constitutive promoter (P_{ADC1}), fused to the inserted gene (EnGBF1), and followed by the yeast *ADC1* terminator (T_{ADC1}). The vector also carries a yeast centromere (*CEN6*), a yeast replication origin (*ARSH4*), a bacterial replication origin (ColE1 *ori*) and confers ampicillin resistance in *E. coli* (*AmpR*) and tryptophan prototrophy in yeast (*TRP1*). (*Right*) pYC7:ABRE carries the *CYC1* [*CYC1*(p/1)] promoter lacking upstream activation elements fused to *E. coli lacZ*. Target elements (3x Em1a) are inserted upstream of the *CYC1* promoter. The vector also carries a 2-micron yeast replication origin and a uracil prototrophic marker (*URA3*).

β-galactosidase activity was detected by performing colony-lift filter assays. Yeast colonies were transferred to filter paper, frozen in liquid nitrogen and allowed to thaw. Filters were then placed face-up onto filter papers presoaked in Z buffer/X-Gal solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 30 mg/ml X-Gal) and incubated at 30 °C, checking periodically for appearance of blue colonies.

2.13 Western blotting and immuno-detection

Proteins (5-10 μg) were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Osmonics) using a Mini Trans-Blot Cell (Biorad). To confirm equal loading and transfer of proteins, membranes were stained briefly in Ponceau S stain (0.5% [w/v] Ponceau S in 1% [v/v] acetic acid) and destained by washing in water. Membranes were blocked in a solution containing 5% (w/v) non-fat dry milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05% [v/v] Tween-20, pH 7.5) at room temperature for 2 h then incubated in the same buffer containing anti-ZmGBF1 polyclonal antiserum at 1:2000 dilution (a gift of R. Ferl, de Vetten and Ferl, 1995) overnight at 4 °C. After incubation, membranes were washed 3x 5 min in TBST then incubated for 1 h in 5% (w/v) non-fat dry milk in TBST containing 1:2000 dilution of either goat anti-mouse horseradish peroxidase (HRP) or goat anti-mouse alkaline phosphatase (AP) conjugated IgG (both Sigma) and finally washed 3x 5 min in TBST.

For chemiluminescent detection of HRP activity, equal volumes of detection reagent A (2.5 mM Luminol [Sigma], 100 mM Tris-HCl, pH 8.5) and reagent B (400 mM *p*-coumaric acid, 5.4 mM H_2O_2, 100 mM Tris-HCl, pH 8.5) were mixed. Membranes were then incubated in this solution for 1 min. Excess liquid was drained off; the blots were wrapped in cling film and exposed to X-ray film (Kodak BioMax ML). AP activity was detected chromogenically using NBT/BCIP tablets (Roche) according to the manufacturer's instructions.

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3.1 Conserved motifs identified in bZIP subfamilies

In order to target bZIP proteins that are specifically involved in ABA- and/or abiotic stress responses, degenerate primers were designed against highly conserved regions identified in two chosen subfamilies of bZIPs.

The first subfamily (Fig 3.1) includes proteins with high sequence similarity to OsBZ8, which is thought to function in ABA-induced gene expression (Nakagawa et al., 1996), and fall into a larger subfamily of bZIPs possessing an N-terminal proline rich domain (PRD). Meier and Gruissem (1994) categorized bZIPs with an N-terminal PRD into three subfamilies: A, B, and C, based on the presence of a number of short conserved motifs. According to this classification scheme, OsBZ8 and related sequences are members of subfamily C, defined

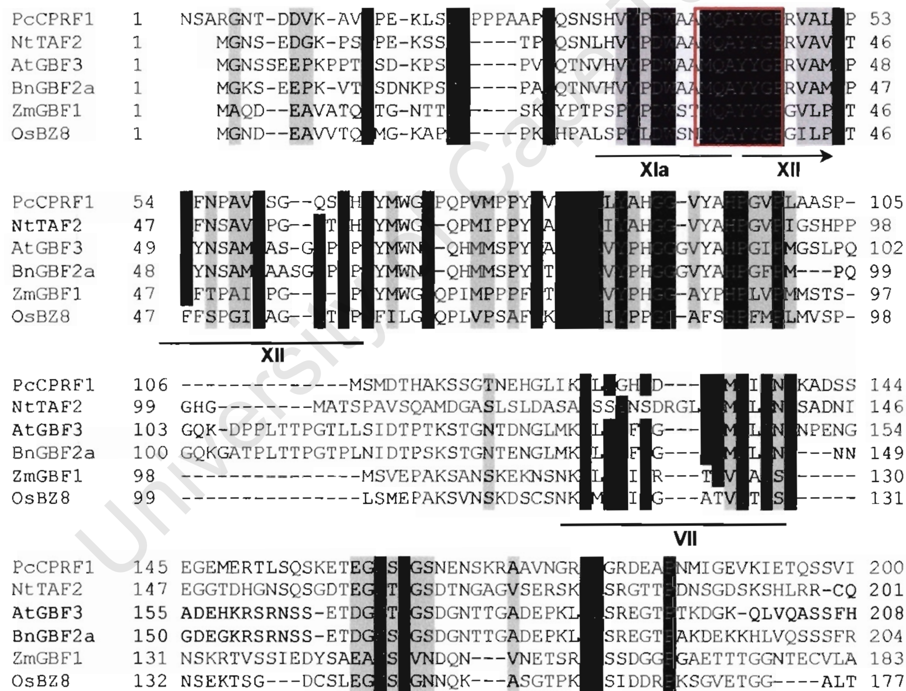

Figure 3.1 Sequence alignment of bZIP proteins with an N-terminal proline-rich domain. Sequences with high similarity to OsBZ8 were aligned and a conserved region was identified for degenerate primer design (Pab2F, red box). Conserved motifs discussed in the text are underlined. Only sequence regions upstream of the basic domain are shown. Identical residues are shaded in black and similar residues in grey. GenBank accession numbers are X58575 (*Petroselinum crispum* CPRF1), Z48602 (*Nicotiana tabacum* TAF2), U51850 (*Arabidopsis thaliana* GBF3), X83920 (*Brassica napus* BnGBF2a), U10270 (*Zea mays* GBF1) and U42208 (*Oryza sativa* BZ8).

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by the presence of motif VII. Using a larger database of bZIP sequences, Sibèril et al. (2001) were able to identify several other motifs unique to each group. Two of these, motifs XIa and XII, were found to be highly conserved in sequences with homology to OsBZ8 and presented an ideal region for primer design, likely being positioned near the 5' end of the cDNA (Fig. 3.1).

The second subfamily (Fig 3.2) has been referred to as ABI5-like due to similarity with Arabidopsis ABI5 which functions in bringing about ABA-dependent growth arrest of embryos under conditions of water deficit (Lopez-Molina et al., 2001). The ABI5-like group lack the PRD and comprise a subfamily of bZIPs having three unique conserved N-terminal motifs and a leucine zipper composed of four rather than the usual six Leu residues (Kim and Thomas, 1998; Casaretto and Ho, 2003). The functional significance of the conserved motifs are not known although Kagaya et al. (2002) have shown that for TRAB1, ABA-induced phosphorylation of residues in the second of these motifs is essential for activating gene expression from ABRE-

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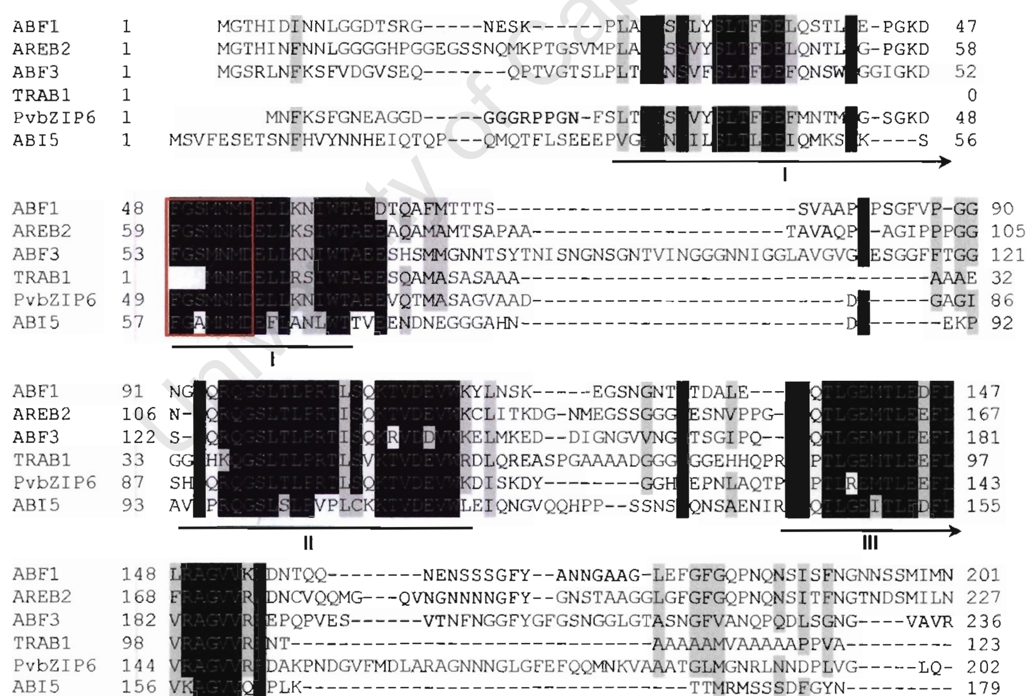

Figure 3.2 Sequence alignment of ABI5-like bZIP proteins. Three unique conserved motifs appear in the N-terminal half (underlined). The sequence chosen for degenerate primer design is in motif 1 (Pab1F, red box). Only sequence regions upstream of the basic domain are shown. GenBank accession numbers are AF093545 (ABF1), AF093547 (AREB2), AF093546 (ABF3), AB023288 (TRAB1), AF369792 (*Phaseolus vulgaris* bZIP6), AF334206 (ABI5).

containing promoters. Also included in the group are the Arabidopsis ABFs (Choi et al., 2000) and AREBs (Uno et al., 2000) which have proven *in vivo* roles in ABA-induced gene expression (Kang et al., 2002).

The basic domain of bZIP proteins (Fig. 3.3) is the most highly conserved and best studied region, functioning in sequence-specific DNA binding as well as cytoplasm-to-nucleus translocation through a bipartite nuclear localisation signal, NLS B (Varagona et al., 1992). Composition of the basic domain and the hinge region, which separates the basic domain and leucine zipper, determines the degree of preference for either G-box (CACGTG) or C-box (GACGTC) binding (Niu et al., 1999).

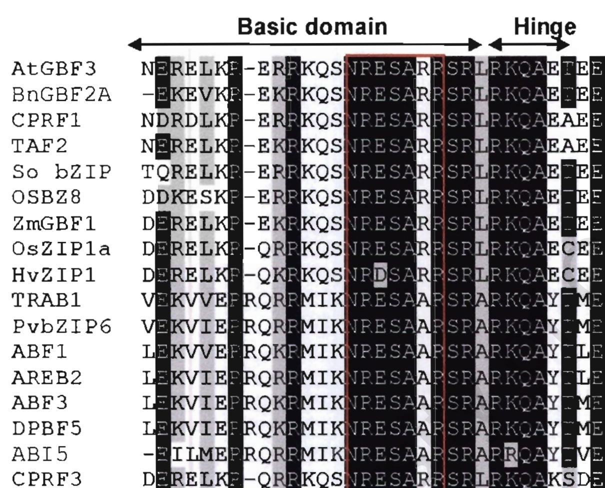

Figure 3.3 Sequence alignment of basic domain and hinge regions from G-box binding bZIP proteins. A highly conserved region, present in both subfamilies of bZIPs was selected for degenerate primer design (red box). GenBank accession numbers for additional sequences are AJ223624 (*Spinacea oleracea* bZIP), U04295 (*O. sativa* ZIP1a), AY150677 (*Hordeum vulgare* ZIP1) and AF334210 (*A. thaliana* DPBF5).

3.2 Cloning of *EnGBF1* by degenerate RT-PCR and 3' RACE

Initial experiments using an oligo-dT$_{18}$ primer together with degenerate forward primers yielded only broad smearing patterns or products of only a few hundred base pairs. The combination of the basic-domain specific reverse primer, Pab1R, with the degenerate forward primer, Pab2F (Fig. 3.1), resulted in the amplification of a 680-bp product (Fig. 3.4a) with high sequence similarity to a number of plants bZIPs, most notably to GBF1 from maize which is induced by flooding stress (de Vetten and Ferl, 1995) and OsBZ8 (Nakagawa et al., 1996). No products were obtained using Pab1R with the

other degenerate forward primer, Pab1F (Fig. 3.2), although optimisation of the PCR conditions was not attempted. The 680-bp fragment was used in further experimentation.

3' RACE experiments yielded two bands in the expected size range of 1-1.5 kb based (Fig. 3.4b) on the length of sequences with similarity to the 680-bp fragment. Both PCR products were sequenced and found to be identical except for an additional 100 bp preceding the poly-A tail in the larger PCR product. The 5' end sequences overlapped with that of the 680 bp fragment already obtained, possibly indicating that alternatively spliced transcripts were being amplified. Sequence analysis revealed that the PCR products consisted of 831 bp of coding region, followed by a stop codon and a putative 3'UTR of 392 bp in the larger product and 288 bp in the smaller product. An online analysis tool, UTRscan (Pesole and Liuni, 1999), was used to search for the presence of any characterised functional elements in the 3'UTR sequence but none were found.

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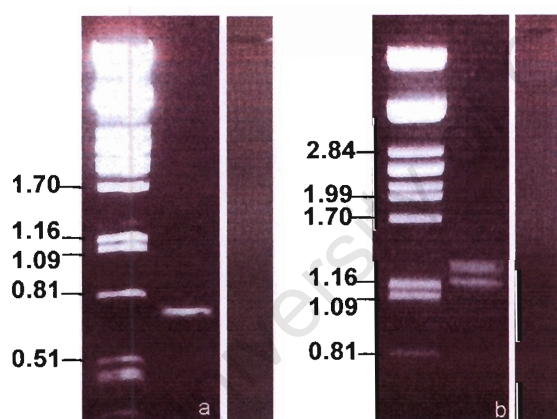

Figure 3.4 Cloning of EnGBF1 middle and 3' end fragments. (a) 680-bp PCR product obtained using the Pab2F and Pab1R degenerate primer pair in RT-PCR (b) 1.2- and 1.3-kb PCR products obtained by 3' RACE. Molecular weight marker is *Pst*I-cut λ DNA with sizes indicated in kb. Control reactions lacking template DNA are shown alongside each experiment.

Attempts to isolate the 5' end by 5' RACE were not successful despite extensive optimisation and modification of the recommended protocols. However, the two bZIPs most similar to the obtained sequence, ZmGBF1 and OsBZ8, displayed a short, conserved amino acid sequence (M[A/G][Q/N]DEA) at their N-termini which was used to design a degenerate forward primer, Pab3F. PCR with this primer and a reverse primer specific to a region of the 3'UTR shared by both 3' RACE products yielded multiple bands over a broad

size range (not shown). A PCR product of the expected size (1.2 kb) was isolated and found to be identical in sequence to fragments already obtained with an additional 100 bp of sequence at the 5' end. This putatively full length cDNA was designated *Eragrostis nindensis* G-box binding factor 1 (EnGBF1).

3.3 Sequence analysis of ***EnGBF1***

The longest open reading frame encoded a protein of 371 amino acids with a predicted molecular weight of 39.8 kDa. Examination of the deduced EnGBF1 amino-acid sequence revealed structural features typical of plant bZIP proteins (Fig. 3.5). PROSITE analysis (Bairoch, 1992) detected the highly conserved basic domain and the proline-rich region (28% proline from amino-acid residues 27 to 102) which has been shown to function as a transcriptional repressor in some GBFs and an activator in others (Sibéril et al., 2001). A leucine zipper motif consisting of six leucine residues arranged as heptad repeats is also present. This arrangement of leucine residues results in the formation of an amphipathic α helix which functions as a dimerization domain (Sibéril et al., 2001).

A similarity search using the NCBI BLAST facility (Altschul et al., 1997) indicated that EnGBF1 shows similarity to several proteins belonging to the subfamily C of bZIPs possessing an N-terminal PRD (Fig. 3.6). The signature motif VII, which functions as a nuclear localisation signal (Meier and Gruissem, 1994), is present as are other motifs described by Sibéril et al. (2001) which are also unique to subfamily C (Fig. 3.6). These include motifs XIa, XII, XVIIIc (poorly conserved), XIX and XX whose functional relevance is not known. Motif IIIc is a putative cytoplasmic retention signal composed of a high proportion of acidic residues (Sibéril et al., 2001) and is present in other subfamily C members but is very poorly conserved in EnGBF1.

The basic domain and hinge regions of this group show sequence identity over their entire length indicating similar DNA sequence binding specificity. The highest sequence similarity (64% identity) is to GBF1 from maize which is induced by hypoxia and is thought to be involved in the expression of an alcohol dehydrogenase gene (de Vetten and Ferl, 1995). The second highest

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similarity (47% identity) is shown to OsBZ8 which is induced by dehydration and can bind G-box and ABRE elements in *in vitro* assays (Nakagawa et al., 1996). Parsley CPRF1 (37% homology) is induced by UV light and binds G-box elements in the chalcone synthase promoter (Feldbrugge et al., 1994).

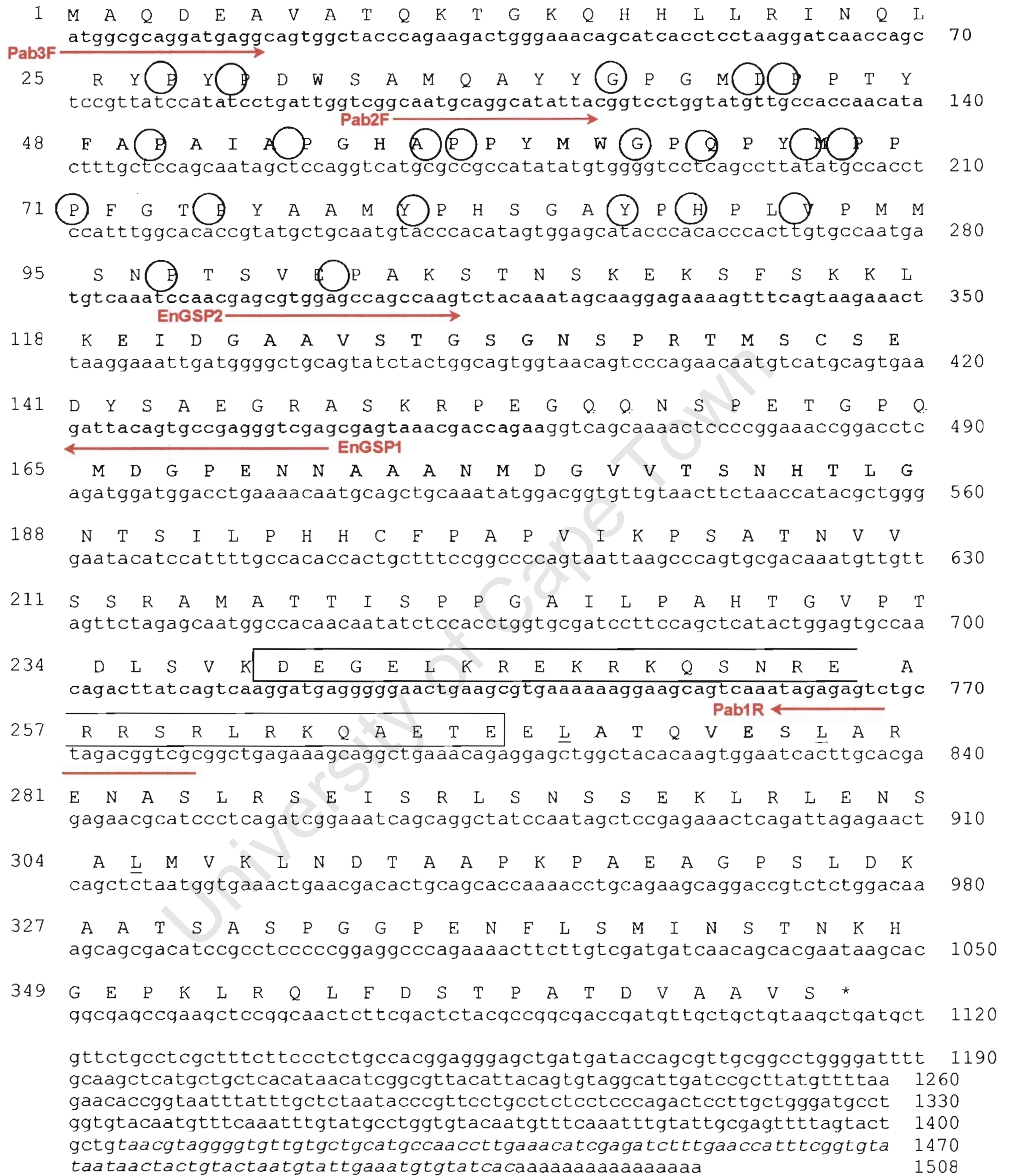

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Figure 3.5 Nucleotide and deduced amino acid sequence of EnGBF1. Proline residues within the N-terminal domain are circled and the basic region of the bZIP domain is boxed. The six leucine residues comprising the leucine zipper region are underlined. The nucleotide sequence present only in the larger of the two 3'RACE products is shown in italics. Primer positions are shown in red. The full sequence was compiled by joining overlapping sequences from individual clones of the 5' end, middle and 3' end fragments.

```
NtTAF2    1       MGNSEDGKPS---KPEKSSSP----TPDQSNLHVYF-DWAAMQAYYGPRVAVPTYENSAVAP 54
CrGBF1    1       MGSSEETKSS---KPEKSSSP----APEQSNVHVYP-DWAAMQAYYGPRVAVPPYFSSAVAS 54
PcCPRF1   1   NSARGNTDDVKAV---KPEKLSSPPPPAAPDQSNSHVYF-DWAAMQAYYGPRVALPPYFNPAVAS 61
So bZIP   1       MGSSDDVKSP---KSEKTSPP----ATEHNGVHMYP-DWAAMQAYYGPRVALPPYFNSAVAP 54
BnGBF2a   1       MGSNEEGKTTQSDKPAQVQAPPP--PPEQSNVHVYHHDWAAMQAYYGPRVAITPQYYNSNGH 60
ZmGBF1    1       MAQDEAVATQ---KTGNTTSP----SKDYPTPSPYP-DWSTMQAYYGPGVLPPTYFTPAIAP 54
EnGBF1    1       MAQDEAVATQ---KTGKQHHL----LRINQLRYPYP-DWSAMQAYYGPGMLPPTYFAPAIAP 54
OSBZ8     1       MGNDEAVVTQ---KMGKAPSP----PKDHPALSPYL-DWSNMQAYYGPGILPPTFFSPGIAA 54
                                                  XIa ——————— XII ——————→

NtTAF2    55  GHTPHPYMWGP-QPMIPPYGAPYAAIYAHGGVYAHPGVPIGSHPPGHGMATSPAVSQAMDGASLS 118
CrGBF1    55  GHPPHPYMWGPPQPMMPPYGTPYAAIYAHGGVYTHPGVPLGSHANAHAGATSPGATEAIAASPLS 119
PcCPRF1   62  GQSPHPYMWGPPQPVMPPYGVPYAALYAHGGVYAHPGVPL-------------------AASPMS 107
So bZIP   55  GHPPPPYMWGPPQPMVPPYGAPYAAIYAPGGVYAHPAIPLASQAHGQEVAP-PAVSEPLVATPLS 118
BnGBF2a   61  AAPPPPYIWGSPSPMMAPYGTPYPPFCPPGGVYAHPALQMGSQPQGPASQATP-----VVATPLN 120
ZmGBF1    55  GH-PPPYMWGPQPIMPPPFGTPYAAVYPHGGAYPHPLVPM-------------------MSTSMS 99
EnGBF1    55  GH-APPYMWGPQPYMPPPFGTPYAAMYPHSGAYPHPLVPM-------------------MSNPTS 99
OSBZ8     55  GHTPPPFILGPQPLVPSAFGKPYAAIYPPGGAFSHPFMPL-------------------MVSPLS 100
              ————————

NtTAF2    119 LDASAK-SSENSDRGLL----------AMSLGNGSADNIEGGTDHGNSQSGDTEGSTDGSD--TNG 171
CrGBF1    120 IDTPTK-SSANGSQGLMNKLRGFDGLAMSIGNGNTDSADGGTDHGISQSGDTEGSSDGSNGTTSK 183
PcCPRF1   108 MDTHAK-SSGTNEHGLIKKLKGHDDLAMSIGNGKADSSEGEMERTLSQSKETEGSSDGSN--ENS 169
So bZIP   119 MDTPAK-SPKNADKGFIKKLKGFNGLAMSIGNGNTEKTEGDVENRLSQSAESEGSSNGSDG-NYT 181
BnGBF2a   121 LEAHPANSSGNTDQGFMTKLKEFDGLAMSISNNKSGSGEHSSEPKNSQSSENDDSSNGSD--GNT 183
ZmGBF1    100 VEPAKS--ANSKEKNSNKKLKEIDRTAVSAGSGNSKRT-----VSSIEDYSAEASSDVN-----D 152
EnGBF1    100 VEPAKS--TNSKEKSFSKKLKEIDGAAVSTGSGNSPRT-----MSCSEDYSAEGRASKRP--EGQ 155
OSBZ8     101 MEPAKS--VNSKDSCSNKKMKEIDGATVSTGSGNSE--------KTSGDCSLEGSSDGN-----N 150
                             VII ——————————————                IIIc ——————

NtTAF2    172 AGVSERSKKPSRGTTPDNSGDSKSH-LRRCQPTGEVN-N-DSEKTIVVVR--PGKVEEKVMGTVL 231
CrGBF1    184 AG--QKNKKPSREGTPANDRERKS--LTPSSPSAAVNTNGSSEKAMRASKV-PAAATEKVMGAVL 243
PcCPRF1   170 KRAAVNGRKPGRDEAPNMIGEVKIETQSSVIPSPRAK----SEKLLGITVA-TPMVAGKVVGTVV 229
So bZIP   182 EDADDNKRKPSREGTPISVEAEKTE-IKAVLP-PP------ADKVLGIPVTSANAVAGQVMGTVV 238
BnGBF2a   184 TGGEQSRKKPSREGSPNNDG--K--------P----------SSQIVPLLR--D--ESEKHAVTMG 225
ZmGBF1    153 QNVNETSRKPSSDGGPGAETTTGGNTECVLAP----------NHTLGNATILP---QHCFSAPVI 204
EnGBF1    156 QNSPETGPQMD--G-P--ENNAAANMDGVVTS----------NHTLGNTSILP---HHCFPAPVI 202
OSBZ8     151 QKASGTPKKPSIDDRP----KSGVETGGALTP----------NDRPSEQAALP---NLCIPVTAI 198
                XVIIIc ——————

NtTAF2    232 SPSMTTTLEMPNPASTHLKASPTNVSQLSPALPNEAWLQNERELKREKRKQSNRESARRSRLRKQ 296
CrGBF1    244 SPNMTTASELPNPSAANAKTSPAKVSQSCSSLPGETWLQNERELKRERRKQSNRESARRSRLRKQ 308
PcCPRF1   230 SPSMTSSLELKDSPKEHAVNSPAGGQQPSTMMPNDSWLHNDRDLKRERRKQSNRESARRSRLRKQ 294
So bZIP   239 SPGVATTLELPNPG-VTVKGNSSPVSSPNGVVSSEAWLQTQRELKRERRKQSNRESARRSRLRKQ 302
BnGBF2a   226 TPVMPTAMDFP---------------QPFPGAPHEVWN--EKEVKREKRKQSNRESARRSRLRKQ 273
ZmGBF1    205 KPSATNVANSPAIGTALSP-PPGVMVPVHNAVPSDLSVKDERELKREKRKQSNRESARRSRLRKQ 268
EnGBF1    203 KPSATNVVSSPAMATTISP-P-GAILPAHTGVPTDLSVKDEGELKREKRKQSNRESARRSRLRKQ 265
OSBZ8     199 KPDVSTASDFPVIATPVT---------------EVPTKDDKESKRERRKQSNRESARRSRLRKQ 247
                                                      DNA BD ——————————————→

NtTAF2    297 AEAEELAIRVQSLTGENMTLKSEINKLMENSEKLKLENAALMEKLNNEQLSPTEEVSLG--KIDD 359
CrGBF1    309 AETEELAKKVQTLTAENMTLRSEINKLTENSEHLPHES-ALLDKLKNARVMQAGEMN----KYDE 368
PcCPRF1   295 AEAEELAIKVDSLTAENMALKAEINRLTLTAEKLTNDNSRLLEVMKNAQAERAADVGLG--NNNE 357
So bZIP   303 AETEELARRVESLSAENMALKSEVNLLVENSQKLRLENAALTGKLKNLQSGQGDENGLA--NVDT 365
BnGBF2a   274 AETEELSVKVDALVAENMTLRSKLGQLNDESEKLRLENQALLDQLK-AQATGKTENLIS--GVDK 335
ZmGBF1    269 AETEELATQVESLAAENTSLRSEIGRLTESSEKLRLENSALMVKLKDTAEPSPSKA------AAS 327
EnGBF1    266 AETEELATQVESLARENASLRSEISRLSNSSEKLRLENSALMVKLNDTAAPKPAEAGPSLDKAAT 330
OSBZ8     248 AETEELARKVELLTAENTSLRREISRLTESSKKLRLENSALMEKLTETGPDEAQEVPP----VKT 308
              ———————

NtTAF2    360 KRVQPVGTANLLARVNNSGSLNRANEES--EVYENNS-SGAKLHQLLDSSPRTDAVAAG 41538%
CrGBF1    369 LHRQPTGTADLLARVNNSGSTDKSNEEGGGDVFENRN-SGTKLHQLLDASPRADAVAAG 42635%
PcCPRF1   358 KKASTLSTANLLSRVDNAGSGDRDEGES--DVYEKTTKSGAKLHQLLDANPRTDAVAAG 41437%
So bZIP   366 KRNPSDSTENLLSRVNNSSSVGRNSDEG-AEMFGKSAKSGAKFHQLLD-NRRGDAVAAR 42236%
BnGBF2a   336 NNS---VSGTVVVRMRNSNS                                        35235%
ZmGBF1    328 PSSPRASAENFLSMIDSANA-PSVSRHT----EHGG----PRLRQLLDSSSATDVAAVS 37764%
EnGBF1    331 SASP-GGPENFLSMINSTN-------------KHGE----PKLRQLFLSTPATDVAAVS 371 -
OSBZ8     309 KAQQARGVENFLSMIDKTGT-PRSSGHM----DHAIA--TPKLRQLLGSGLATDAVAAR 36047%
                  XIX ——————                    XX ————————————————
```

Figure 3.6 Comparison of the deduced amino acid sequence of EnGBF1 with related proteins. Several conserved sequence motifs unique to subfamily C of bZIPs possessing an N-terminal PRD are underlined. EnGBF1 shows sequence variation in the regions corresponding to motifs IIIc and XVIIIc. The DNA binding domain (DNA BD) is also underlined. Percentages after sequences indicate percent identity to EnGBF1. The GenBank accession number of *Catharanthus roseus* GBF1 is AF084971.

3.4 Southern blot analysis

Southern blot analysis suggested that EnGBF1 is likely to be a single copy gene. Three *Pst*1 sites are present in the cDNA sequence, with 570 bp between the first and second sites and 15 bp between the second and third sites. In accordance with this, two hybridising *Pst*1 fragments of approximately 800 and 570 bp were detected. The third expected fragment was outside of the region covered by the probe but would most likely have been too small to detect. No *Eco*R1 or *Hin*dIII restriction sites are present in the EnGBF1 clone and single hybridising fragments of approximately 14 and 13 kb respectively were detected for each digest. A single 7 kb fragment from the *Eco*R1/*Hin*dIII double digest was also detected. Faint bands that hybridised to the EnGBF1 probe were observed from the *Eco*R1 and double digests indicating that distantly related genes may be present in the *E. nindensis* genome.

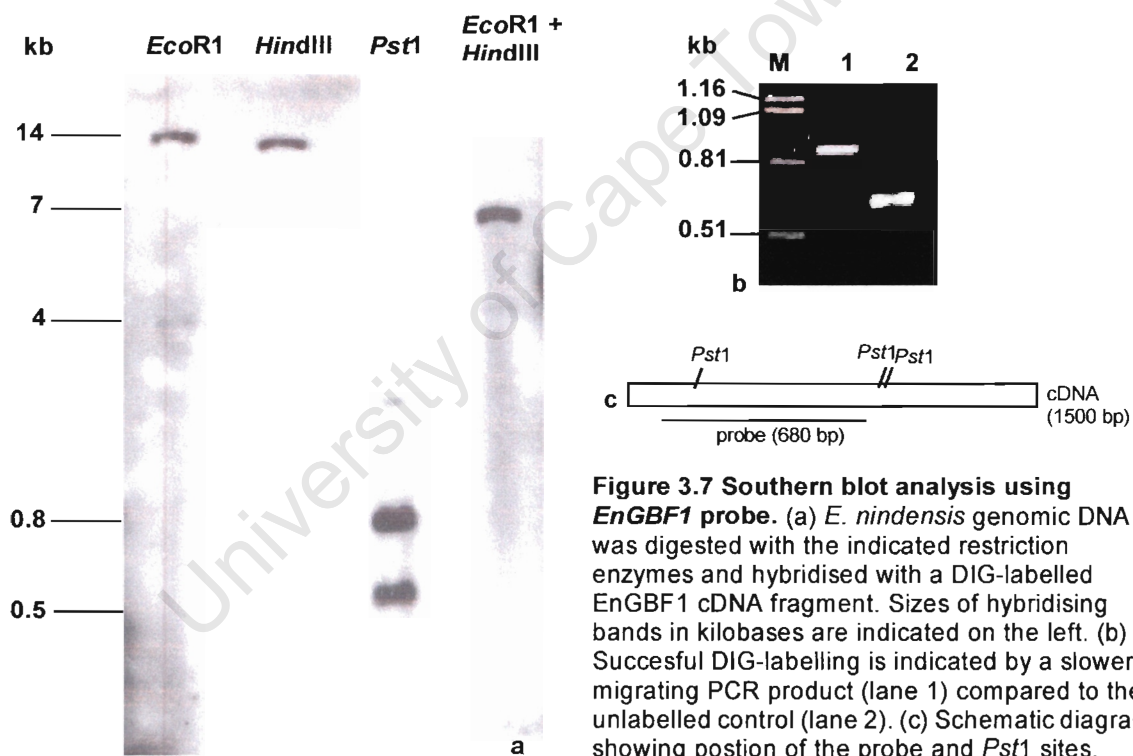

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Figure 3.7 Southern blot analysis using *EnGBF1* probe. (a) *E. nindensis* genomic DNA was digested with the indicated restriction enzymes and hybridised with a DIG-labelled EnGBF1 cDNA fragment. Sizes of hybridising bands in kilobases are indicated on the left. (b) Succesful DIG-labelling is indicated by a slower-migrating PCR product (lane 1) compared to the unlabelled control (lane 2). (c) Schematic diagram showing postion of the probe and *Pst*1 sites.

Considerable effort was invested in obtaining acceptable results from Southern blotting using the non-radioactive DIG-detection system (Roche). No hybridisation, except to the positive control, was detected in initial experiments using the manufacturer's protocol even after low stringency

washing. Feint bands were observed after a revised protocol was employed (Engler-Blum et al., 1993), using high salt buffers in the antibody detection steps. The signal-to-noise ratio was however too low to permit exposure times longer than a few minutes. Best results (Fig. 3.7) were obtained after increasing the amount of DNA to 30-40 µg, lowering the antibody concentration to 1: 15 000 and using a positively-charged nylon membrane from a different manufacturer. Highly charged membranes are known to cause background problems in the DIG system (DIG System manual, Roche) and choice of membrane may therefore be an important factor in optimising detection sensitivity.

3.5 Bacterial expression and purification of EnGBF1

Bacterial expression of EnGBF1 was performed to verify the coding integrity of the cDNA prior to experimentation in yeast as well as to provide a resource for future work (gel shift assays and antibody generation). EnGBF1 expression was inducible by IPTG but formed insoluble inclusion bodies which necessitated the use of denaturing conditions during purification. The migration of the his-tagged protein in SDS-PAGE corresponded to a mass of greater than 50 kDa (Fig. 3.8). While the vector contributed approximately 5 kDa to the size of the protein including the histidine tag, the value estimated by SDS-PAGE was still significantly higher than the predicted 40 kDa. Such discrepancies have been reported for AtGBF1 (Klimczak et al., 1992) and ZmGBF1 (de Vetten and Ferl, 1995) and are thought to be due to the high proline content.

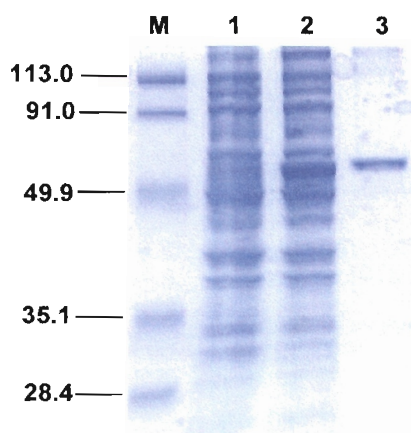

Figure 3.8 Expression and purification of recombinant EnGBF1 from *E. coli*. Crude bacterial extracts, uninduced (lane 1), induced (lane 2) and affinity-purified EnGBF1 (lane 3) were analysed by 12% SDS-PAGE and visualised by Coomassie Blue staining. Lane M contains molecular mass standards (Biorad) with sizes indicated in kDa.

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3.6 Yeast transactivation assay

The yeast transactivation assay, first described by Wang and Reed (1993), makes use of the fact that many transcription factors are composed of independent DNA binding and activation domains. In the current work, a translational fusion was made of the yeast GAL4 activation domain (GAL4AD) and EnGBF1 which contributes the DNA binding function. The ability of EnGBF1 to bind an ABRE-target element was then assayed by qualitative assessment of β-galactosidase reporter activity.

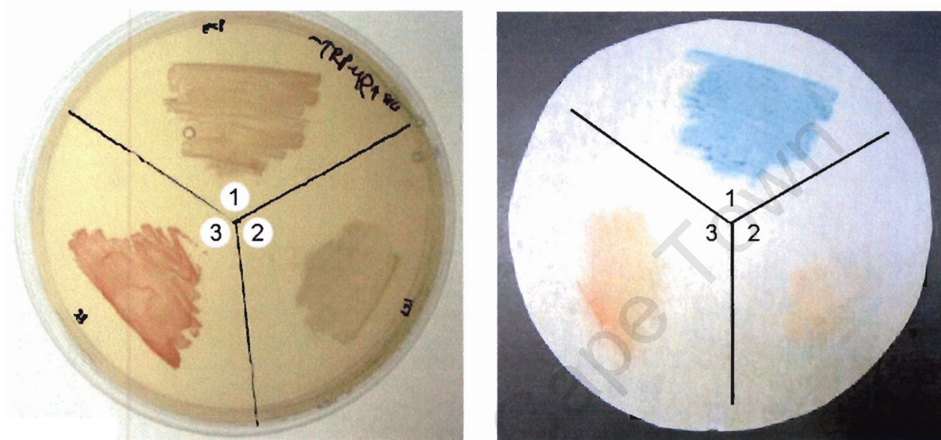

Figure 3.9 Transcriptional activation function of EnGBF1. (a) Yeast transformants plated on SD-TRP-URA selection media transformed with the following plasmid pairs: (1) pPC86:EnGBF1 + pYC7:ABRE, (2) pPC86:EnGBF1 + empty pYC7 (3) empty pPC86 + pYC7:ABRE. (b) Results of β-galactosidase filter assay from corresponding plate.

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Only transformed yeast colonies carrying the ABRE-lacZ reporter (pYC7:ABRE) and the EnGBF1 expression construct (pPC86:EnGBF1) were found to turn blue within 30 min of incubation with X-Gal substrate (Fig. 3.9). Yeast transformants lacking the ABRE-target element or not expressing the GAL4AD-EnGBF1 fusion protein displayed only low, background levels of β-galactosidase activity after prolonged incubation. Given that the ABRE-element and the G-box share identical core sequences, these results show only that EnGBF1 is a functional G-box binding factor. Xiong and Zhu (2001) have commented that ABRE-binding factors and G-box binding factors may be functionally interchangeable *in vitro* and that partner proteins such as adapters may be required for conferring signal specificity in the plant cell.

3.7 Expression of EnGBF1 during de/rehydration

Antibodies raised against ZmGBF1 were obtained from Robert Ferl (University of Florida, Gainesville) and were found to cross-react with recombinant EnGBF1 from *E. coli* (not shown). Western blot analysis was used in subsequent experiments to determine expression levels of EnGBF1 (Fig. 3.10). A single band of the predicted size (± 47 kDa) was detected in proteins extracted from fully hydrated leaf tissue (100% RWC) as well as at the first sampling point after dehydration had commenced (65% RWC). This band was not observed at more advanced stages of dehydration or in desiccated tissue. Upon rehydration, a similar expression pattern was found with the band being absent in dry tissue and reappearing only once a relative water content of 76% was reached.

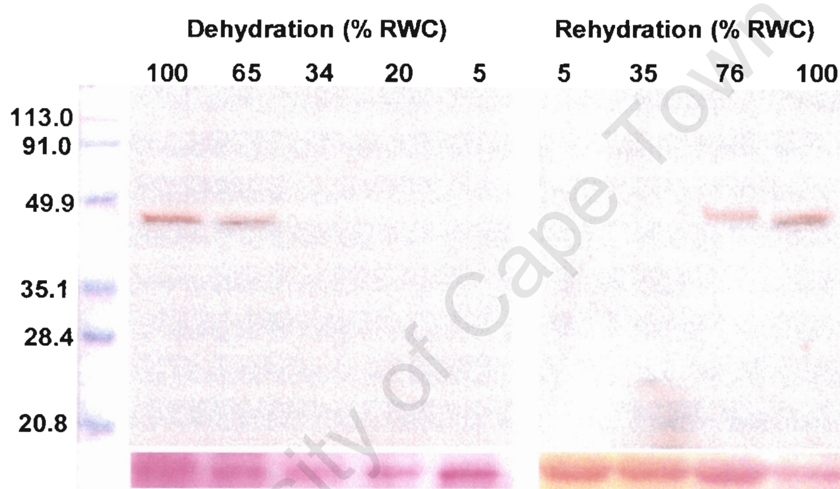

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Figure 3.10 Western blot analysis of proteins (10 µg) isolated from leaf tissue during dehydration and rehydration using anti-ZmGBF1 antibodies. Immunodetection was performed using chemiluminesence. Molecular mass standards (Biorad) with sizes in kilodaltons are shown on the left. Sections of corresponding Ponceau S stained membranes appear below to show equal loading.

Speculating on the possible *in vivo* function of EnGBF1, two possibilities can be envisaged given that it is expressed in fully hydrated but not dry tissue. It may either be expressed normally under unstressed conditions and be down regulated as part of the programmed metabolic shutdown during dehydration Alternatively, it may function as a transcriptional repressor of specific desiccation-regulated genes. Its down-regulation might then facilitate their expression only during dehydration. Examples of bZIP proteins acting as

repressors have been reported and include parsley CPRF1 (Feldbrugge et al., 1994), ROM2 from *Phaseolus vulgaris* which is active as a repressor in the late stages of seed maturation (Chern et al., 1996) and EEL from Arabidopsis which acts antagonistically to ABI5 in activating *LEA* gene expression (Bensmihen et al., 2002).

Attempts to determine the mRNA levels of EnGBF1 by Northern blot analysis were not successful. Both radioactive and non-radioactive methods were attempted and, while the positive control could be detected, no hybridisation to native targets was apparent. As with many transcription factors, this may be the result of low levels of target mRNA, requiring the isolation of polyA RNA for detectable levels of the transcript to be present.

3.8 Effect of ABA-treatment on EnGBF1 expression

To determine whether expression of EnGBF1 was possibly under control of an ABA-dependent signalling pathway, proteins were extracted from leaf tissue after exogenous application of ABA and western blot analysis was performed. Initial experiments using a 0.1 mM ABA solution revealed a constant level of EnGBF1 expression up to the last sampling point at 6 h post application (not shown). To confirm these results, the concentration of ABA was increased to 2.5 mM as used by Neale et al. (2000) on another desiccation-tolerant grass, *Sporobolus stapfianus*. Again, no significant change in the level of expression compared to the water-treated control was observed (Fig. 3.11). These results suggest that expression of EnGBF1 is not affected by ABA but requires another dehydration-induced signal for the observed down-regulation to occur.

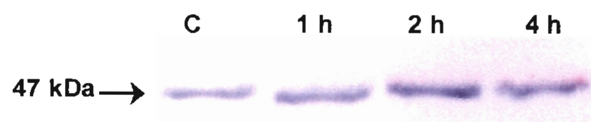

Figure 3.11 Effect of exogenous ABA application on expression of EnGBF1. Proteins (7 µg) were extracted from leaf tissue treated with 2.5 mM ABA after 1, 2 and 4h or after 1h for the water control (C) and analysed by western-blot analysis. Immunodetection was performed using NBT/BCIP.

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3.9 Effect of light/dark on EnGBF1 expression

OsBZ8 shares a significant degree of similarity to EnGBF1 and recent work on the former has suggested that it is involved in the sugar-induced repression of an α-amylase gene in rice embryos (Lee et al., 2003b). This was in contrast to earlier work by Nakagawa et al. (1996) which proposed that OsBZ8 was involved in regulation of ABA-inducible *LEA* genes such as *Osem* and *Rab16A* although no direct causal relationship was shown. OsBZ8 mRNA was strongly induced by ABA in the same study although significant levels were also detected in uninduced conditions.

The possibility that EnGBF1 could similarly repress expression of an α-amylase gene in unstressed conditions but release repression upon dehydration is an intriguing one. Sucrose is accumulated in almost all resurrection plants is response to drying and serves as an osmoprotectant (Ghasempour et al., 1998; Scott, 2002). Carbohydrate storage reserves in source and sink tissues are likely to be fully utilised for conversion to sucrose during this period and in support of this, starch was found to be completely depleted in leaves of *Ramonda nathaliae*s during dehydration (Müller et al., 1997). The regulatory aspects of starch metabolism during dehydration-induced sucrose accumulation have not been explored.

As a preliminary investigation into whether the expression of EnGBF1 was at all related to α-amylase activity, western blot analysis was performed at times when the plant was thought to be actively degrading starch i.e. at night and after prolonged periods of light deprivation. No variation in the expression levels of EnGBF1 was found in samples taken every 6 h over a 24 h day/night period (Fig. 3.12). Expression of EnGBF1 was also found to be consistent in samples taken at the same time of day over 48 h in the dark (not shown). Iodine staining of leaf tissue confirmed that plants had been fully destarched by this treatment.

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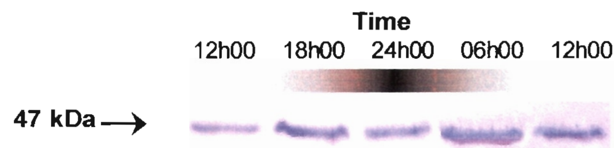

Figure 3.12 Expression levels of EnGBF1 during day and night. Proteins (5 µg) were extracted from leaf tissue sampled at noon and every 6 h thereafter over a 24 h period and analysed by western-blot analysis. Day/night transition is indicated by the shaded panel. Immuno-detection was performed using NBT/BCIP.

These results suggest that EnGBF1 is not involved in the transcription of α-amylase genes. However, α-amylase genes constitute a multigene family in the genomes of many plants with different isoforms displaying divergent promoter regions and patterns of expression (reviewed by Mitsui and Itoh, 1997). In rice seedlings for example, α-amylase isoforms A and B are hormonally regulated and repressed by anoxia while isoforms G and H are anoxia-induced and activated by low sugar levels (Hwang et al., 1999). If EnGBF1 does indeed regulate expression of an α-amylase isoform it may be one that is activated specifically by dehydration and not subject to repression as sucrose levels increase.

It is interesting to note that the expression of the two bZIP proteins most closely related to EnGBF1, OsBZ8 and ZmGBF1, might both be modulated by altered sugar status. Expression of OsBZ8 was found to be repressed during sucrose starvation (Lee et al., 2003) and ZmGBF1 was induced during hypoxic stress (de Vetten and Ferl, 1995). Loreti et al. (2003) have speculated that some anoxia-modulated genes might in fact be expressed as a result of an anoxia-induced altered sugar status, suggesting that ZmGBF1 could be subject to regulation by a sugar-sensing pathway. While it may seem counter-intuitive that EnGBF1 could be regulated by a sugar-deprivation signal, such a signal might be derived from the reduced supply of sugars to storage reserves occurring as photosynthesis is shutdown in the early stages of dehydration (Vander Willigen et al., 2001).

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More work is required to elucidate the role, if any, played by EnGBF1 in acquisition of desiccation tolerance in *E. nindensis*. This includes determining the regulatory sequences bound by EnGBF1 and identification of genes downstream of these sequences. This could be achieved by using the chromatin immunoprecipitation assay which involves purification of *in vivo* protein-DNA complexes (Wang et al, 2002). Studies testing the effects of other abiotic stresses such as high light, cold and salinty on EnGBF1 expression may also provide useful insights.

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