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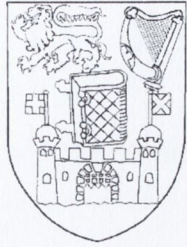
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**The University of Dublin
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**Functional characterisation of *Arabidopsis* genes encoding
members of the AP2/ERF transcription factor family**

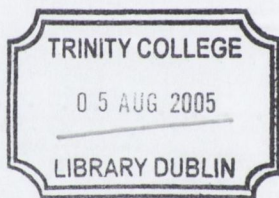
Sinéad Brennan

**A thesis submitted to
The University of Dublin
for the degree of**

Doctor of Philosophy

**Department of Genetics
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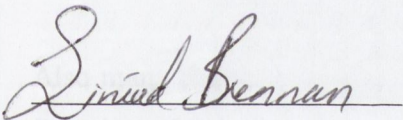


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Sinead Brennan

2005

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Summary

The EU Framework 5 REGIA (Regulatory Gene Initiative in *Arabidopsis*) Consortium, comprising 29 laboratories, was established to investigate transcription factor gene function in *Arabidopsis*. As part of the REGIA project, the present study describes the characterisation, in terms of sequence, expression and function, of two members of the plant-specific AP2/ERF transcription factor family: *AP2.76* and *AP2.72*. Phylogenetic analyses of their predicted amino acid sequences placed *AP2.76* in the DRE/CRT subgroup, members of which bind the DRE/CRT *cis*-acting element and *AP2.72* in the ERF subgroup, members of which bind the GCC-box *cis*-acting promoter element. On the basis of the observed sequences similarities and predicted DNA binding specificities, *AP2.76* was hypothesised to function in an abiotic stress signalling pathway while *AP2.72* was hypothesized to function in a pathogen response pathway.

A putative nuclear localization signal was identified in *AP2.76* and *AP2.72*. Transient expression of an *AP2.76-GFP* translational gene fusion in onion cells resulted in the accumulation of GFP exclusively in the nucleus, consistent with the observation that the *AP2.76* sequence encodes a putative transcriptional regulator. Similar gene fusion experiments in which *AP2.72* was fused with the GFP and the GUS reporter gene revealed accumulation of the fusion protein in both the nucleus and cytoplasm of onion cells, suggesting possible nucleo-cytoplasmic partitioning.

AP2.76 expression was induced by jasmonic acid (JA), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), wounding, dehydration and high-salinity stress in whole seedlings. A time course study revealed that this induction was early and transient. Expression analysis in the abscisic acid-deficient mutants, *aba3-1*, *aba2-4* and *aba1-5* revealed the early dehydration and high salinity-induced expression of *AP2.76* to be abscisic acid-independent. A cDNA microarray analysis further revealed that over-expression of *AP2.76* in transgenic plants induced the expression of several target genes associated with stress and defense responses including *PR3*, *PR4* and *PDF1.2*, but not the genes *rd29A* or *erd10* which contain the DRE/CRT *cis*-acting element in their promoters. A promoter analysis of the *AP2.76* inducible genes suggested both direct and indirect mechanisms by which *AP2.76* might regulate gene expression.

A time course study revealed that *AP2.72* expression was induced differentially by salicylic acid (SA), JA, ABA, ACC, wounding, and high-salinity in whole seedlings.

Expression analysis in *NPR1* mutants revealed the SA-induced expression of *AP2.72* in to be *NPR1*-dependent. Over-expression of *AP2.72* in transgenic plants did not however induce expression of the SAR marker genes *PR1* or *PR5*. Furthermore, when compared with wild-type plants, expression of the JA/ethylene-regulated genes *PR3*, *PR4* and *PDF1.2* was unaltered in *AP2.72* knock-out plants and overexpression lines. It was suggested that *AP2.72* may require co-factor(s) or post-translational modification for activation of its DNA binding function in plants.

Abbreviations

aa	amino acids
ABA	abscisic acid
ABRC	<i>Arabidopsis</i> biological resource centre
ABRE	abscisic acid responsive elements
Ac	accession number
<i>Ac/Ds</i>	<i>activator/dissociation</i>
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	acyl CoA oxidase
amp	ampicillin
AMP	antimicrobial protein/peptide
AP2/ERF	apetala2/ ethylene-responsive element (ERE) binding factors
AREB	ABRE-binding proteins
Avr	Avirulence
<i>AvrPto</i>	<i>Avirulence Pto</i>
<i>BAR</i>	BASTA resistance gene
bHLH	basic helix-loop-helix
BLAST	basic local alignment search tool
bp	base pairs
<i>CAB</i>	<i>chlorophyll A/B binding protein</i>
CaCl ₂	calcium chloride
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
ChIP	chromatine immunoprecipitation
CIAP	calf intestine alkaline phosphatase
cm	centimetre
<i>coi1</i>	<i>coronatine insensitive1</i>
<i>Cor15/47/78</i>	<i>Cold regulated15/47/78</i>
<i>CPR1/5/6</i>	<i>Constitutive expressor1/5/6</i>
CSHL	cold spring harbour laboratory
cv	cultivar
DEPC	diethyl-pyrocarbonate
DIG	digoxigenin-labelled
DNA	deoxyribonucleic acid
INTPs	deoxynucleoside triphosphate
DRE/CRT	dehydration responsive element/C-repeat
<i>DsE</i>	enhancer trap
<i>DsG</i>	gene trap
<i>dSpm</i>	defective <i>Spm</i>
<i>E.Coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
<i>ein2</i>	<i>ethylene insensitive2</i>
<i>En/Spm</i>	<i>enhancer/suppressor</i>
ERD1/10	<i>early response to dehydration</i>
EREBPs	ethylene-responsive element binding proteins
ERFs	ethylene-responsive element (ERE) binding factors
EST	expressed sequence tag

ET	ethylene
EtBr	ethidium bromide
GFP	green fluorescent protein
GUS	β -glucuronidase
h	hour
HCL	hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethansulfonic acid]
HR	hypersensitive response
ISR	induced systemic resistance
JA	jasmonic acid
<i>jar1</i>	<i>jasmonic acid resistant1</i>
<i>JR1/2/3</i>	<i>jasmonic acid-responsive1/2/3</i>
Kb	kilobase
kDa	kilodalton
kn	kanamycin
KOH	potassium hydroxide
L	litre
LB	luria-bertani
LEA	late embryogenesis abundant
M	molar
MASC	multinational <i>Arabidopsis</i> steering committee
mg	milligram
Mg ²⁺	magnesium ion
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger RNA
MS	Murashige and Skoog medium
MS+	MS medium with sucrose
MS-Gamborg	MS medium with hormones and vitamins
MUG	4-methylumbelliferyl β -D- glucuronide
MUN	4-methylumbelliferyl α -N- neuraminic acid
Na ₂ CO ₃	sodium carbonate
NAA	α -naphthalene acetic acid
NaAc	sodium acetate
NaCl	sodium chloride
NAN	neuraminidases
NaOH	sodium hydroxide
NASC	Nottingham <i>Arabidopsis</i> stock centre
NCBI	national centre for biotechnology information
NLS	nuclear localisation signal
NOS	nopaline synthase terminator
<i>NPR1</i>	<i>non expressor of PR1</i>
NPT II	neomycin phosphotransferase gene
NTR	non translated region
°C	degrees Celsius

OD	optical density
OGAs	oligosaccharides
ORC2	<i>octadecanoid-responsive catharanthus AP2</i>
ORF	open reading frame
p.s.i.	pounds per square inch
PCR	polymerase chain reaction
<i>PDF1.2</i>	<i>plant defensin1.2</i>
<i>Pfu</i>	<i>pyrococcus furiosus</i>
pH	hydrogen ion concentration
PLACE	plant <i>cis</i> -acting elements
poly(A)	polyadenylic acid
PR	pathogenesis-related
<i>Pti4/5/6</i>	<i>Pto-interacting 4/5/6</i>
R	resistance
RAP2	related to apetala 2
<i>rd29/22/17</i>	<i>responsive to dehydration 29/22/17</i>
REGIA	regulatory gene initiative in <i>Arabidopsis</i>
RNA	ribonucleic acid
RNAaseA	ribonuclease
rpm	revolutions per minute
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second
ser	serine
SINS	sequence insertion sites
SLAT	sainsbury laboratory <i>Arabidopsis thaliana</i> transposants
smGFP	soluble-modified GFP
SV40	simian virus 40
TBE	tris-borate-EDTA electrophoresis buffer
TE	tris-EDTA buffer
TEV	tobacco etch virus
<i>Thi2.1</i>	<i>Thionin2.1</i>
thr	threonine
<i>Tsi1</i>	<i>Tobacco stress induced1</i>
TSS	transcription start site
tyrosine	tyrosine
uidA	β -glucuronide gene
UTR	untranslated region
UV	ultra-violet
<i>VSP</i>	<i>vegative storage protein</i>
wx	waxy
X-gluc	5-bromo-4-chloro-3-indolyl- β -D-glucuronide
μ g	microgram
μ l	microlitre
μ M	micromolar

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Chapter 1

General Introduction

1.1 Plant Biology

Man is, ultimately, completely dependent on plants. Consequently, acquiring an extensive knowledge about the biology of plants has never been more important than it is today. Research provides insights into natural processes such as disease resistance, responses to environmental stresses and plant metabolism, which in turn provides opportunities to make positive changes in plant species of economic importance. Possibilities include enhancing resistance to diseases caused by insect, bacterial, viral and parasitic pests; increasing tolerance to abiotic stresses, such as low temperature, drought and soil salinity. These goals cannot be accomplished without a thorough understanding of plant biology. Therefore one long-term goal for plant biology following completion of the genome sequence of *Arabidopsis thaliana* (L.) Heynh is to understand every molecular interaction in every cell throughout the plant life cycle (Somerville and Meyerowitz, 1994; Walbot, 2000).

1.2 The model plant *Arabidopsis*

The application of the methods and concepts of molecular genetics, as applied initially to the single-celled model organisms *Escherichia coli* and *Saccharomyces cerevisiae*, has revolutionised plant biology. In particular *Arabidopsis thaliana* (*Arabidopsis*) has emerged as the most widely used and facile experimental organism for studies in a wide range of areas such as metabolism, development, genetics, environmental adaptation and pathogen interactions in plants (Somerville and Meyerowitz, 1994). Although closely related to such economically important crop plants as turnip, cabbage, broccoli, and canola, *Arabidopsis* is not an agriculturally important plant. Despite this, it has been the focus of intense genetic,

biochemical and physiological study for over 40 years (Meinke et al., 1998). *Arabidopsis* was chosen as the key organism for the first plant genome project for a number of reasons such as its diploid genetics, unusually compact genome, short generation time, small size, prolific seed production and the ease with which it can be mutagenized and genetically transformed (Somerville and Meyerowitz, 1994; Meinke et al., 1998; Initiative, 2000).

1.2.1 Biology of *Arabidopsis*

Arabidopsis (thale cress) belongs to the mustard or crucifer family (Cruciferae; Brassicaceae), a widely distributed family of approximately 340 genera and 3350 species (Somerville and Meyerowitz, 1994) with a broad natural distribution throughout Europe, Asia, and North America. Many different ecotypes (accessions) have been collected from natural populations and are available for experimental analysis. The *Arabidopsis* Columbia and Landsberg ecotypes are the accepted standards for genetic and molecular studies. The complete life cycle, involving seed germination, formation of a rosette plant, bolting of the main stem, flowering, and maturation of the first seeds, can be completed in 6 weeks. Mature plants reach 15 to 20 cm in height and often produce several hundred siliques with more than 5000 seeds. Bolting initiates about 3 weeks after planting and the resulting inflorescence forms a linear progression of flowers and siliques for several weeks before the onset of senescence. Flowers are approximately 2 mm long. Seeds are 0.5 mm in length at maturity and are produced in slender fruits known as siliques. Seedlings grow into rosette plants that range from 2 to 10 cm in diameter, depending on growth conditions (Meinke et al., 1998).

1.2.2 The *Arabidopsis* genome

Arabidopsis possesses the smallest known plant genome (125 megabase pairs (Mbp) distributed on 5 chromosomes and encoding an estimated 29,000 annotated genes (AGI, 2000; Meyers et al., 2004), a relatively large number compared to the 18,424 and 13,601 genes predicted to comprise the genomes of *Caenorhabditis elegans* and *Drosophila melanogaster*, respectively (Riechmann and Ratcliffe, 2000).

Arabidopsis genes are typically compact, containing several coding regions (exons) of about 250 base pairs each, interrupted by short non-coding regions (introns). The genes are

closely spaced, approximately one per 4.6 kilobases, indicating that their regulatory regions are also short. In contrast, many animal genes contain dozens of exons, introns that may span several kilobases and regulatory regions of 10 kilobases or larger (Walbot, 2000).

Interestingly, like those of *S. cerevisiae*, *C. elegans* and *D. melanogaster*, the *Arabidopsis* genome contains extensive duplications and a significant amount of genetic redundancy (Riechmann and Ratcliffe, 2000). In *Arabidopsis*, two factors contributed to gene redundancy: polyploidisation (duplication of the entire set of chromosomes) and local gene duplications (copying of individual genes within chromosomes) (AGI, 2000).

Despite the apparent duplication, geneticists have identified thousands of mutations (in *Arabidopsis* and other plant species e.g. maize and tomato) that produce visible defects in the plant but occur in only one of the duplicated genes (Walbot, 2000). Therefore many duplicated genes in these species have unique roles.

The Arabidopsis Genome Initiative assigned functional categories to the complete set of *Arabidopsis* genes. It was estimated that the *Arabidopsis* genome encodes an estimated 1600 transcription factors representing approximately 6% of the estimated 29,000 genes in this species (AGI, 2000; Riechmann and Ratcliffe, 2000). Indeed, regulation of gene expression at the level of transcription influences or controls many of the biological processes in a cell or organism, such as progression through the cell cycle, metabolic and physiological balance, and responses to the environment (Riechmann et al., 2000). Recently, several studies have revealed the crucial role of transcriptional control in plant responses to a range of biotic and abiotic stresses (Singh et al., 2002; Mahalingam et al., 2003; Gutterson and Reuber, 2004).

1.3 Plant responses to biotic and abiotic stress

Biotic and abiotic stress profoundly affects plant growth and development and causes major losses in crop productivity worldwide. Biotic stresses (imposed by other organisms) include the diverse range of enemies, especially microbial pathogens (bacteria, fungi and viruses), but also nematodes and insects, encountered by plants in response to which they have evolved sophisticated and efficient countermeasures. Indeed, despite the continuous presence of many microorganisms in the immediate vicinity of plants, disease occurs relatively rarely. Because plants lack the ability to escape attacking pathogens by moving to a more favourable environment, efficient defense mechanisms are essential for protection from disease. These are

based primarily on pathogen detection systems that activate a battery of local and systemic defense responses (Hammond-Kosack and Jones, 1996; Glazebrook, 2001). Provided activation occurs with sufficient speed, these responses are generally efficient in preventing disease. Consequently there is considerable interest in understanding the signal transduction networks that control the activation of defense responses in plants (Glazebrook, 2001).

Abiotic stresses (imposed from an excess in the physical or chemical environment) include wounding, cold, dehydration, osmotic stress due to high salinity, hypoxia (low oxygen), oxidative stress and heat stress. These are adverse environmental conditions (although the latter three will not be considered in further detail) that limit the geographical distribution of plants and account for significant reductions in the yields of agriculturally important crops. Plant responses to these stresses involve nearly every aspect of plant physiology and metabolism. Consequently, there exists a complex signalling network underlying plant adaptation to these adverse environmental conditions (Zhu, 2001).

The sections that follow discuss the complexity of the biotic and abiotic stress signalling pathways and the transcriptional regulation of target genes that mediate the plant's responses. The role of transcription factors in these processes is discussed in detail in sections 1.8-1.12.

1.4 The complexity of signalling in plant defense responses to pathogens

1.4.1 Gene-for-gene resistance

Gene-for-gene resistance, one of the most effective defense mechanisms in plants, is mediated by Resistance (*R*) genes that function in the detection of specific pathogen races through recognition of pathogen-encoded Avirulence (*Avr*) or elicitor proteins (Martin, 1999; Ellis et al., 2000). *R*-gene-mediated resistance (also termed gene-for-gene resistance) triggers a rapid highly localized defense response that generally includes the programmed death of plant cells that are in contact with the pathogen, a phenomenon called the hypersensitive response (HR) (Hammond-Kosack and Jones, 1996; Glazebrook, 2001).

1.4.2 Systemic acquired resistance and the role of salicylic acid

Activation of the HR triggers an additional systemic resistance response known as systemic acquired resistance (SAR) that confers broad-spectrum resistance to viral, bacterial and fungal pathogens in distant, uninfected plant parts (Ryals et al., 1996). The onset of SAR is accompanied by a local and systemic accumulation of the signal molecule SA (Malamy, 1990; Uknes et al., 1992; Durner, 1997) and the coordinate upregulation of a large set of genes (Ward et al., 1991), including genes that encode the pathogenesis-related (PR) proteins PR1 (encoding a protein of unknown function), PR2 (β -1,3-glucanase) and PR5 (thaumatin-like protein) (Uknes et al., 1992; Durner, 1997) (Figure 1.1). These proteins possess antimicrobial activity and are thought to contribute to the heightened resistance state attained.

Evidence for a key role of SA came from the analysis of transgenic plants expressing the bacterial *nahG* gene, which encodes the enzyme salicylate hydroxylase that inactivates SA by converting it to catechol (Lawton et al., 1995; Ryals et al., 1996). Transgenic *nahG* plants are unable to accumulate SA, and are also incapable of developing SAR, indicating that SA accumulation is required for the expression of SAR (Ryals et al., 1996). Expression of the defense genes *PR1*, *PR2* and *PR5* in response to SA treatment requires a gene called *NPR1* (*non-expressor of PR1*) (Glazebrook et al., 1996). Mutant *npr1* plants accumulate normal levels of SA after pathogen infection but are impaired in their ability to express *PR* genes and to mount a SAR response and display enhanced susceptibility to infection by various pathogens (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). Over-expression of *NPR1* in *Arabidopsis* confers significant resistance to strains of *Pseudomonas syringae* and *Peronospora parasitica* that are normally virulent on wild-type *Arabidopsis* (Cao et al., 1998). The *NPR1* gene encodes a protein with an ankyrin-repeat domain (Cao et al., 1997), a domain known to mediate protein-protein interactions (Bork, 1993). Upon induction of SAR, *NPR1* is translocated to the nucleus (Kinkema et al., 2000), where it interacts with members of the TGA/OBF subclass of basic domain/Leucine zipper (bZIP) transcription factors (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Fan and Dong, 2002) that mediate SA-dependent activation of *PR* genes (Lebel et al., 1998; Niggeweg et al., 2000). A physical interaction between *NPR1* and the TGA-class bZIP transcription factors is essential for binding to the *cis*-acting promoter elements that regulate *PR* gene expression (Despres et al., 2000; Fan and Dong, 2002).

In addition to *npr1*, which is nonresponsive to SAR induction, several *cpr* mutants (for constitutive expressor of PR genes), *cpr1*, *cpr5* and *cpr6* were isolated that have elevated SA levels and show constitutive expression of *PR1*, *PR2* and *PR5* in the absence of SAR induction and enhanced resistance to *P. syringae* and *P. parasitica* (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998). In all cases, *cpr/nahG* plants do not exhibit elevated gene expression or resistance to *P. syringae*, suggesting that the *CPR* genes act upstream from SA as negative regulators of SA signalling (Glazebrook, 1999). In *cpr5/npr1* double mutants, defense gene expression and resistance to *P. syringae* are abolished, confirming that *CPR5* is acting upstream from *NPR1* (Bowling et al., 1997). In *cpr6/npr1* plants, constitutive expression of *PR1*, *PR2* and *PR5* is retained, but resistance to *P. syringae* is lost (Clarke et al., 1998) indicating that a SA-dependent *NPR1*-independent mechanism for activation of *PR1*, *PR2* and *PR5* exists or that the factor responsible for *P. syringae* resistance in *cpr6* plants is not *PR1*, *PR2* or *PR5* (Clarke et al., 1998). Thus *CPR6* may also be a positive regulator of SA signalling upstream from SA (Glazebrook, 1999).

Recently additional genes that act in the SA signalling pathway have been identified. *PAD4* (*phytoalexin deficient4*) seems to act upstream from SA and is thought to be required for signal amplification to activate the SA pathways in response to pathogens that do not elicit a strong defense response (Zhou et al., 1998). Mutations in *EDS5* (*enhanced disease susceptibility5*, also known as *SID1*) and *SID2* (also known as *EDS16*) abolish the increase in SA levels observed in infected plants. In fact, SA levels in these mutants are as low as they are in plants carrying the *nahG* transgene. Hence *EDS5* and *SID2* are suggested to function upstream of SA accumulation (Nawrath and Metraux, 1999). A mutation in *EDS4* causes reduced SA accumulation, so *EDS4* seems to also function in SA signalling (Gupta et al., 2000). Interestingly, although *eds5*, *sid2*, *eds4* and *pad4* mutations (as well as the transgenic *nahG*) reduce SA levels and expression of *PR1*, only *pad4* and *nahG* reduce production of camalexin, a phytoalexin with antimicrobial activity (Zhou et al., 1998; Nawrath and Metraux, 1999; Feys and Parker, 2000; Gupta et al., 2000). It appears, therefore, that low-level SA production does not cause the camalexin defect in these plants, and that *pad4* and *nahG* may affect other signalling pathways in addition to the SA-dependent pathway (Glazebrook, 2001).

1.4.3 Jasmonic acid- and ethylene-dependent defense response(s)

SA is not the only signal involved in induction of *PR* genes and several lines of evidence have shown that some defense responses are activated by signal transduction networks that require JA and ET as signalling molecules and are distinct from the classic SA-mediated SAR pathway (Glazebrook, 1999; Pieterse and van Loon, 1999; Glazebrook, 2001), (Figure 1.1).

JA and ET are plant hormones implicated in many physiological processes. JA is a fatty acid-like hormone derived from linolenic acid and is involved in several aspects of plant biology including pollen and seed development and defense against wounding, ozone, insect pests and microbial pathogens (Creelman and Mullet, 1997; Reymond and Farmer, 1998; Li et al., 2001). *Arabidopsis* mutants that are impaired in JA production (e.g. *fatty acid desaturase (fad)3/fad7/fad8* triple mutants) or perception (e.g. *coronatine insensitive1 (coi1)* and *jasmonic acid resistant1 (jar1)*) exhibit enhanced susceptibility to a variety of pathogens, including the fungal pathogens *Alternaria brassicicola*, *Botrytis cinerea*, and the bacterial pathogen *Erwinia carotovora* (Thomma et al., 1998; Vijayan et al., 1998; Norman-Setterblad et al., 2000; Stintzi et al., 2001). These pathogens employ a common virulence strategy that involves rapidly killing plant cells to obtain nutrients, and thus are often referred to as 'necrotrophs' (Jackson and Taylor, 1996). Several JA-dependent genes that encode pathogenesis-related proteins, including *Plant Defensin1.2 (PDF1.2)*, *Thionin (Thi2.1)*, *Hevin-Like-Protein (PR4)* and *ChitinaseB (PR3)*, are commonly used to monitor JA-dependent defense responses (Reymond and Farmer, 1998).

In *Arabidopsis*, the pattern of altered pathogen responses for the *ethylene insensitive2 (ein2)* ET-signalling mutant generally parallels the patterns observed for the *coi1* and *jar1* JA-signalling mutants. For example, the expression of several JA-signalling defense genes (e.g. *PDF1.2*, *Thi2.1*, *PR3* and *PR4*) also requires *EIN2* (Jackson and Taylor, 1996; Norman-Setterblad et al., 2000). Numerous studies provide evidence for positive interactions between JA and ET signalling pathways. Both JA and ET signalling are required for the expression of the defense-related gene *PDF1.2* in response to infection by *A. brassicicola* (Penninckx et al., 1998) and for the expression of *PDF1.2*, *PR3* and *PR4* in response to treatment with *E. carotovora* (Norman-Setterblad et al., 2000). A microarray experiment that monitored gene expression in response to various defense-related stimuli revealed that JA and ET coordinately regulate many other defense-related genes. In this study, nearly half of the genes

that were induced by ET were also induced by JA treatment (Schenk et al., 2000). The study also indicates that JA and ET independently also regulate separate sets of genes. These observations have led to the development of simple models in which ET and JA are placed together in a single signalling pathway (Kunkel and Brooks, 2002). Crucially, the regulation of *PDF1.2*, *Thi2.1*, *PR3* and *PR4* by JA and ET is clearly distinct from that of the SA-dependent PR genes *PR1*, *PR2* and *PR5* because it does not depend on *NPR1* (Penninckx et al., 1998; Thomma et al., 1999) (Figure 1.1).

1.4.4 Induced systemic resistance

Colonisation of roots by certain rhizosphere bacteria confers a form of disease resistance called induced systemic resistance (ISR). This phenomenon has been studied using *Pseudomonas fluorescens* strain WCS417r to colonise *Arabidopsis* roots (Pieterse et al., 1996). ISR occurs in *nahG* plants, indicating that it is not a SA-dependent phenomenon (Pieterse et al., 1996). Rather, ISR appears to be JA- and ET- dependent. The observation that ET can induce ISR in *jar1* mutants led to the hypothesis that ISR requires a JA signal followed by an ET signal (Pieterse et al., 1998). In addition using the genetic variability of ISR inducibility between *Arabidopsis* accessions, a locus (*ISR1*) was identified that encodes a component of the ET response, which is required for the expression of ISR (Ton et al., 2001). In *Arabidopsis*, WCS417r-mediated ISR is active against the fungal root pathogen *Fusarium oxysporum*, the oomycetous leaf pathogen *Peronospora parasitica* and the bacterial leaf pathogen *Pseudomonas* pv. *tomato* indicating that, like SAR, WCS417r-mediated ISR is effective against different types of pathogens (Pieterse et al., 1996; van Wees et al., 1997; van Wees et al., 2000).

No changes in gene expression associated with ISR have been detected (Pieterse et al., 1998), suggesting that it is different from activation of *PDF1.2*, *Thi2.1*, *PR3* and *PR4* expression by *A. brassicicola* and *B. cinerea* (Penninckx et al., 1998; Thomma et al., 1998; Norman-Setterblad et al., 2000). Several years ago, it was found that ISR requires *NPR1*. This is interesting as it implies that *NPR1* is involved in perception of more than one signal (i.e. SA and the ISR signal), and may respond in one of two ways (by activating *PR1* gene expression or by activating ISR) (Pieterse et al., 1998), (Figure 1.1).

In *Arabidopsis*, SAR and ISR are effective against a broad spectrum of pathogens including *P. syringae* pv. *tomato* (Uknes et al., 1992; Pieterse et al., 1996; Ryals et al., 1996; van Wees et al., 1997). Simultaneous activation of SAR and ISR results in an additive effect on the level of induced protection against this pathogen and in genotypes that are blocked in either SAR or ISR, this additive effect was not evident (van Wees et al., 2000). Moreover, induction of ISR did not affect the expression of the SAR marker gene *PR1* in plants expressing SAR (van Wees et al., 2000). Together, these observations demonstrate that the SAR and the ISR pathway are compatible and that there is no significant crosstalk between them (van Wees et al., 2000).

1.4.5 Crosstalk between signalling pathways in plant defense

There is a growing body of evidence that reports that the JA, SA and ET defense signalling pathways do not function independently. Rather, they are involved in a complex signalling network in which the different pathways influence each other through positive and negative regulatory interactions. Separate signalling pathways may have evolved to allow plants to fine-tune their defense responses, such that they deploy the appropriate combination of defenses against specific pathogens according to their virulence strategies (Kunkel and Brooks, 2002). For example, mutually antagonistic interactions between the JA and SA pathways might further ensure that inappropriate defenses are not activated in response to certain pathogens. As SA can promote programmed plant cell death under certain conditions (O'Donnell et al., 1996; Vanacker et al., 2001), it might be beneficial for the plant to prevent the activation of SA signalling in response to attack by necrogenic pathogens, which utilise cell-death inducing toxins as virulence factors (Kunkel and Brooks, 2002).

1.4.5.1 Crosstalk between the SA and JA signalling pathways

In the network of regulatory interactions during plant resistance responses interactions between JA and SA signalling appear to be complex, and there is evidence for both positive and negative interactions between these pathways. However, the primary mode of interaction appears to be mutual antagonism. Consistent with this, the inhibitory effect of SA on JA-regulated gene expression has been previously reported for the induction of proteinase

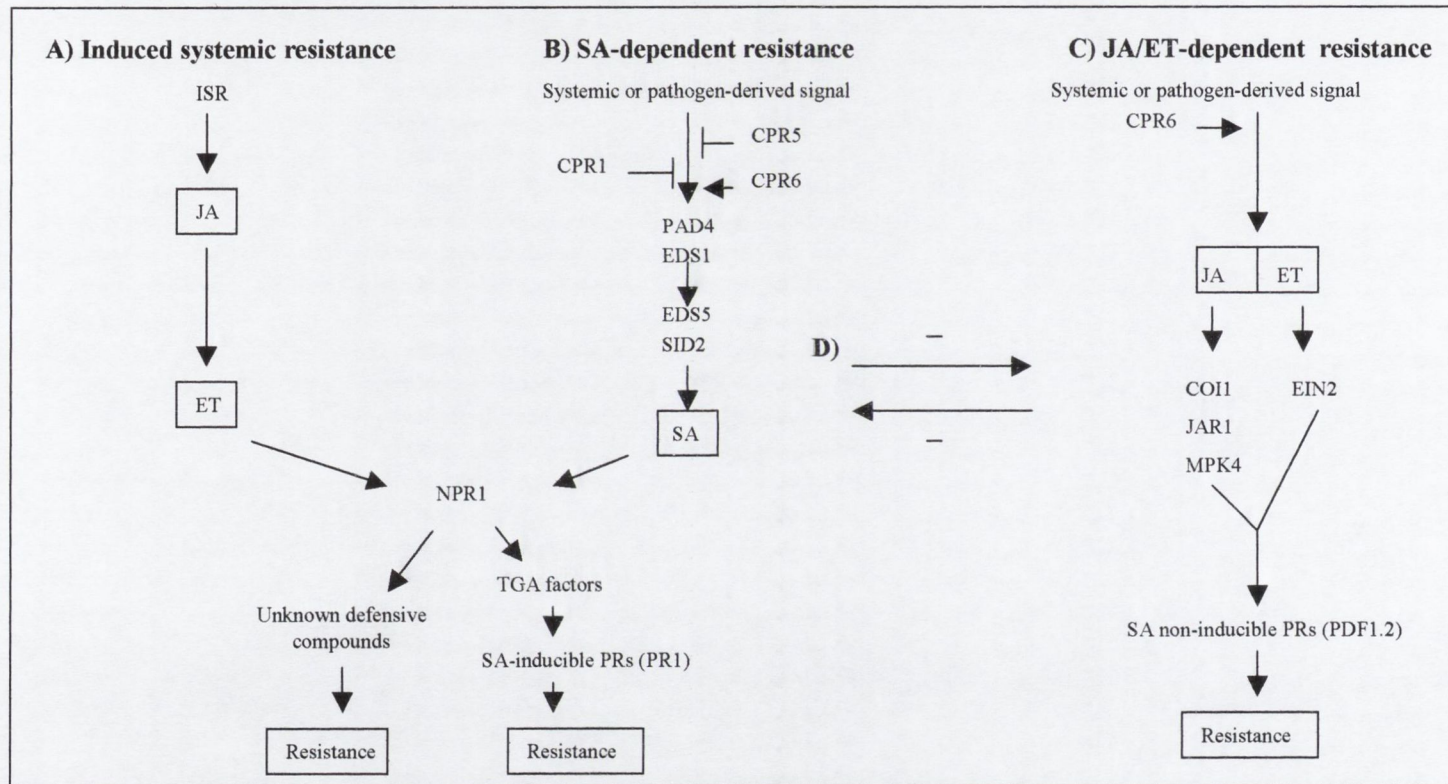


Figure 1.1. A model describing systemic signalling pathways that can be induced in plants by non-pathogenic rhizobacteria or pathogen infection. The illustration is meant to provide a rough outline of the signal transduction networks and the sites of action of various gene products in disease resistance signalling. It does not account for all of the described phenotypes of the various signalling mutants. Boxes containing the word 'resistance' represent the numerous effectors of resistance, whose effectiveness may vary greatly according to the pathogen eliciting the response. There are certainly interconnections among the pathways shown in (A), (B) and (C), but many of these are not sufficiently clear to allow them to be easily drawn. (A) In the rhizobacteria-mediated induced systemic resistance (ISR) pathway, components from the jasmonic acid and ethylene response act in sequence in activating a systemic resistance response, that is dependent on *NPR1*. (B) In SA-dependent signalling, *CPR1*, and *CPR5* tend to repress pathway activation and act upstream of *PAD4*. *CPR6* appears to exert a positive effect upstream of *PAD4*. *PAD4* and *EDS1* promote SA accumulation, as do *SID2* and *EDS5*. Downstream of SA, *NPR1* through interactions with TGA transcription factors mediates the activation of expression of genes such as *PR1*. (C) JA and ET act together to activate expression of genes such as *PDF1.2*. *CPR6* seems to have a positive effect on pathway activation. *MPK4*, *COI1* and *JAR1* are required to transduce the JA signal, while *EIN2* is required to transduce the ET signal. (D) The primary mode of interaction between JA and SA signalling pathways appears to be mutual antagonism. This model does not show the complicated interactions between SA and JA signalling. Signalling pathways A, B, C and D adopted from Glazebrook, 2001 and Pieterse and van Loon., 1999.

inhibitors in wounded tomatoes (Doares et al., 1995b). Several recent genetic studies also provide evidence for an antagonistic effect of SA on JA signalling in *Arabidopsis*. The *eds4* and *pad4* mutants (deficient in SA accumulation) as well as the *npr1* mutant (impaired in its response to SA) exhibit enhanced induction of JA-responsive genes (Penninckx et al., 1996; Clarke et al., 1998; Gupta et al., 2000). In the *cpr6* mutant, which accumulates elevated levels of SA and constitutively expresses both SA- and JA-dependent defenses, reducing the level of SA by crossing in an *eds5* mutation results in a further increase in *PDF1.2* expression (Clarke et al., 2000). New evidence indicates that the subcellular localisation of NPR1 might play a crucial role in modulating the SA-mediated suppression of JA-responsive genes (Spoel et al., 2003).

Several genetic studies provide evidence that JA signalling also negatively regulates the expression of SA-responsive genes (Kunkel and Brooks, 2002). Treatment of tobacco plants with elicitors produced by *E. carotovora* which is known to activate JA signalling in *Arabidopsis* (Norman-Setterblad et al., 2000), inhibited the expression of SA-dependent genes (Vidal et al., 1997). The characterisation of three JA-signalling mutants, *mitogen-activated protein kinase4* (*mpk4*), *suppressor of SA insensitivity2* (*ssi2*) and *coil*, has provided genetic evidence that JA signalling also negatively regulates the expression of SA-mediated defenses in *Arabidopsis* (Petersen et al., 2000; Kachroo et al., 2001; Kloeck et al., 2001). In addition to exhibiting impaired JA signalling, *mpk4* and *ssi2* plants constitutively express SA-mediated defenses and exhibit enhanced resistance to *P. syringae* and *P. parvifolia* (Petersen et al., 2000; Kachroo et al., 2001; Shah et al., 2001). Interestingly, the impairment of JA signalling in these mutants is not due to an inhibitory effect of elevated levels of SA because JA-dependent gene expression was also impaired in *mpk4/nahG* and *ssi2/nahG* plants that do not accumulate high levels of SA (Petersen et al., 2000; Kachroo et al., 2001). Thus constitutive SA signalling in the *mpk4* and *ssi2* mutants is likely due to an antagonistic effect of JA signalling on the SA pathway (Kunkel and Brooks, 2002). The *coil* mutant also exhibits enhanced expression of SA-dependent defenses and enhanced resistance to *P. syringae* (Feys et al., 1994; Kloeck et al., 2001). However, unlike *mpk4* and *ssi2* mutants, *coil* plants do not exhibit constitutive expression of SA-dependent defenses. Rather, the SA-dependent defenses are hyperactivated in response to attack by *P. syringae* (Kloeck et al., 2001). These findings are consistent with the hypothesis that the JA signalling pathway negatively regulates the expression of SA-dependent defenses (Kunkel and Brooks, 2002).

On the other hand there is limited evidence for positive interactions between the JA and SA pathways. A microarray analysis of plants that had been exposed to a variety of defense-inducing treatments has revealed that more than 50 defense-related genes are co-induced by SA and JA (Schenk et al., 2000), suggesting that the two signals co-ordinately regulate these particular genes. Moreover, because both JA and SA contribute to resistance against the fungus *Plectosphaerella cucumerina* (Thomma et al., 2000), suggests that a general antagonism appears to be unlikely.

1.4.5.2 Crosstalk between SA and ET signalling pathways

Evidence that SA and ET may function together to coordinately induce several defense-related genes was obtained in an *Arabidopsis* microarray experiment that monitored gene expression in response to various defense-related stimuli (Schenk et al., 2000). The study identified a large number of genes that were coinduced by both SA and ET treatments. Although the induction of SA-dependent expression of PR genes does not require an intact ET signalling pathway in *Arabidopsis*, exposure to ET has been reported to potentiate the SA-mediated induction of *PR1* in this species (Lawton et al., 1995). However, genetic data from the same study suggest that the ET signalling pathway also negatively affects SA-dependent responses: the basal level of *PR1* mRNA appears to be significantly elevated in *ein2* plants (Lawton et al., 1995). These data, which appear contradictory at first, may reflect the complexity of regulatory cross talk between the SA and ET signalling pathways (Kunkel and Brooks, 2002).

1.5 Molecular responses to abiotic stress

Dehydration, high salinity and low/freezing temperatures have adverse effects on plant growth and seed production. Plants adapt and respond to these stresses through various biochemical and physiological processes, thereby acquiring stress tolerance. Many genes respond to drought, salt and/or cold stress at the transcriptional level, and the products of these genes are crucial for the development of tolerance (Bray et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki et al., 2003). Transcriptome analyses using microarray technology have identified several genes that are induced by abiotic stress (Seki et

al., 2001; Fowler and Thomashow, 2002; Seki et al., 2002; Seki et al., 2003), and these genes have been classified into two major groups (Bray et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2000).

The gene products of the first group include functional proteins that directly protect macromolecules and membranes (e.g. late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins, chaperones and mRNA binding proteins); maintain water movement through membranes (e.g. water-channel proteins and membrane transporters); the enzymes catalysing the biosynthesis of various osmoregulators (e.g. proline, betaine and sugars); proteases for protein turnover (e.g. thiol proteases, Clp protease, and ubiquitin) and detoxification enzymes (e.g. glutathione S-transferase, soluble epoxide hydrolase, catalase, superoxide dismutase and ascorbate peroxidase) (Qiang et al., 2000; Yamaguchi-Shinozaki and Shinozaki, 2001). Some of the stress-inducible genes that encode proteins such as key enzymes for proline biosynthesis and for glycine betaine biosynthesis as well as the detoxification enzyme superoxide dismutase have been over-expressed in transgenic plants to produce a stress-tolerant phenotype of the plants (Kishor et al., 1995; Bohnert and Sheveleva, 1998; Holmstrom et al., 2000).

The second group of gene products function in a signalling or regulatory capacity and includes transcription factors, protein kinases, and the enzymes involved in phospholipid metabolism (Shinozaki and Yamaguchi-Shinozaki, 1997). It is important to analyse the functions of stress-inducible genes not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation. Hundreds of genes are thought to be involved in abiotic stress responses (Shinozaki and Yamaguchi-Shinozaki, 1996; Xiong and Zhu, 2001; Xiong et al., 2002a; Xiong and Zhu, 2002; Zhu, 2002). Several studies have contributed to understanding the regulation of gene expression in response to drought, high salinity and/or cold stress, and in revealing complex gene networks for specificity and crosstalk in abiotic-stress-responsive gene expression.

1.5.1 Regulation of gene expression by dehydration, high-salinity and low-temperature/cold stresses

Promoter analysis of dehydration-, high-salinity- and/or low-temperature-inducible genes has identified at least four independent gene regulatory systems (Shinozaki et al., 2003). Two are ABA-dependent (pathways I and II) and two are ABA-independent (pathways III and IV) (Figure 1.2).

ABA is synthesised *de novo* mainly in response to dehydration and high-salinity stress but not in response to cold stress. Many stress-inducible genes are regulated by the endogenous ABA that accumulates during drought and high-salinity stress (Bray et al., 2000). Recently, genes that are involved in ABA biosynthesis have been identified through genetic and genomics analysis (Seo and Koshiba, 2002; Xiong et al., 2002a). Several of these genes are induced by dehydration and high-salinity but not by cold stress, indicating important roles for ABA in dehydration-stress responses (Seki et al., 2003; Shinozaki et al., 2003). The genes encoding 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key enzyme of ABA biosynthesis, are strongly induced by dehydration stress (Qin and Zeevaart, 1999; Iuchi et al., 2001). Regulatory factors that control the dehydration-inducible expression of the *NCED* gene have not yet been elucidated.

One of the ABA-dependent stress response pathways regulates the expression of a large number of genes via an interaction between promoter *cis*-acting regulatory elements known as abscisic acid-responsive elements (ABREs; AGGTGG/TC) (Figure 1.2, Pathway II) and members of the ABA response element binding (AREB) class of bZIP transcription factors (Choi et al., 2000; Uno et al., 2000). Several AREB proteins such as AREB1 and AREB2, have reduced activity in ABA-deficient *aba2* mutants and in ABA-insensitive mutants, and enhanced activity in the ABA-hypersensitive *eral* mutant, indicating that these transcription factors need ABA for full activation (Uno et al., 2000). Over-expression of *AREB2* causes ABA hypersensitivity, reduced transpiration rate and enhanced drought tolerance of the transgenic plants (Kang et al., 2002).

The induction of the drought-inducible gene *rd22* is mediated by ABA, and this gene requires protein biosynthesis for its ABA-dependent expression (Abe et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 2000). A MYC transcription factor, *RD22BP1* (also known as *AtMYC2*), and a MYB transcription factor, *AtMYB2*, were shown to bind *cis*-elements in the

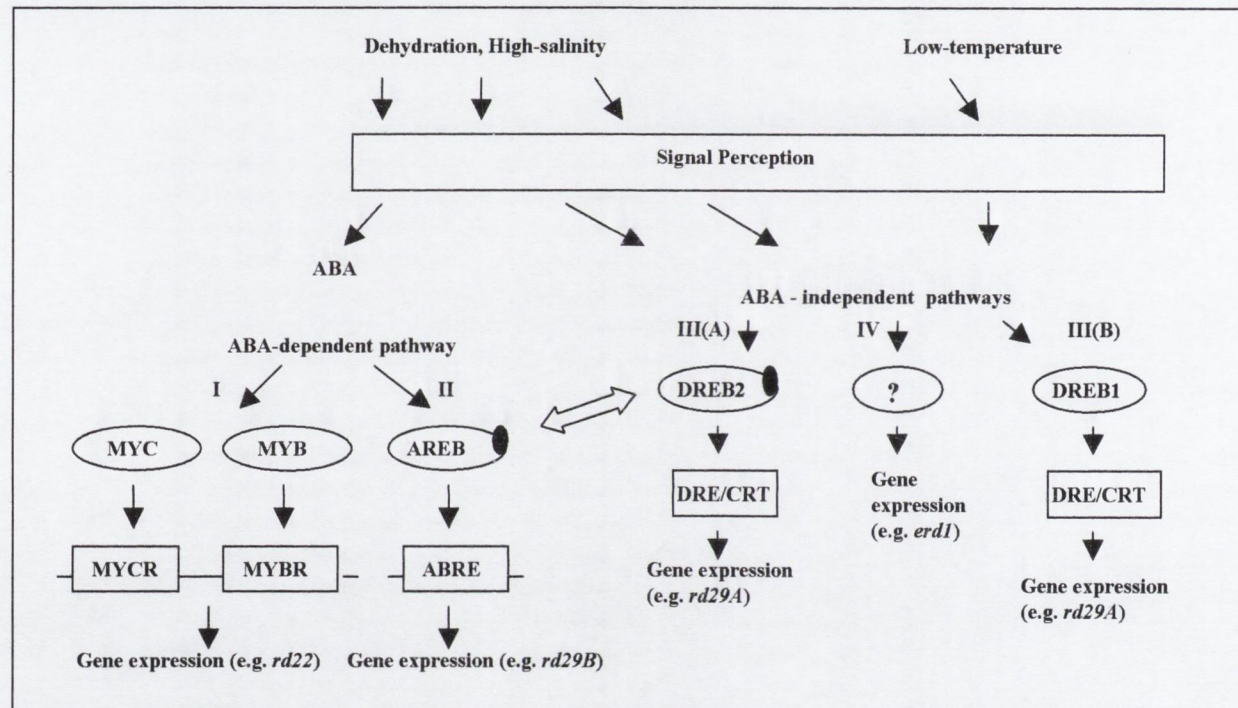


Figure 1.2 A model showing regulatory network of gene expression in response to drought, high salinity and cold stresses. *Cis*-acting elements that are involved in stress-responsive transcription are shown in boxes. Transcription factors that control stress-inducible gene expression are shown in ovals. Small shaded ovals indicate the modification of transcription factors in response to stress signals for their activation, such as phosphorylation. There are at least four signal transduction pathways between initial dehydration/high-salinity stress signal and gene expression. Two are ABA-dependent (I and II) and two are ABA-independent (III and IV). MYB and MYC recognition sites (MYBR and MYCR) are involved in one of the ABA-dependent pathways (I) and ABRE is involved in another (II). In the ABA-independent pathways, DRE/CRT is involved in the regulation of genes not only by dehydration and high-salinity (III(A)), but is also involved in gene regulation in response to low-temperature stress (III(B)). Another ABA-independent pathway is controlled by drought and high-salinity, but not by low-temperature (IV). The open double-headed arrow suggests crosstalk between DREB2 and AREB that is based on DRE/CRT's acting as a coupling element for ABRE. The illustration is meant to provide a rough outline of the signal transduction networks and was adapted from Shinozaki et al., 2003 and Yamaguchi-Shinozaki and Shinozaki 2001.

rd22 promoter and activate *rd22* in a cooperative manner ((Abe et al., 1997):Figure 1.2, Pathway I). These MYC and MYB proteins are synthesised after the accumulation of endogenous ABA, indicating that their role is in a late stage of the stress responses (Abe et al., 1997). Over-expression of both *AtMYC2* and *AtMYB2* not only caused an ABA-hypersensitive phenotype but also improved the osmotic-stress tolerance of the transgenic plants (Abe et al., 2003).

Analysis of the *rd29A* promoter indicate the existence of ABA-independent as well as ABA-dependent signal transduction cascades between the initial signal of water deficit and the expression of specific target genes. The *rd29A* promoter region contains two major *cis*-acting elements, ABRE and the dehydration-responsive element (DRE)/C-repeat (CRT) both of which are involved in stress-inducible gene-expression (Yamaguchi-Shinozaki and Shinozaki, 1994). DRE/CRT and ABRE function in ABA-independent and ABA-dependent gene expression in response to abiotic stress, respectively (Figure 1.2). Transcription factors belonging to the ERF/AP2 (ethylene-responsive element binding factor/apetala 2) subfamily that bind to DRE/CRT were originally isolated as DREB1A/CBF3 (DRE-binding protein/C-repeat-binding factor), DREB1B/CBF1 and DREB1C/CBF2 (Stockinger et al., 1997; Liu et al., 1998). The genes encoding these transcription factors are induced early and transiently in response to low-temperature stress and in turn, activate the expression of target genes (Figure 1.2, Pathway III(B)). Similar transcription factors (DREB2A and DREB2B) are induced by dehydration stress and promote the expression of various genes involved in dehydration and high-salinity tolerance (Liu et al., 1998) (Figure 1.2 Pathway III(A)). The role of these transcription factors in gene expression in response to abiotic stress is described in detail below.

Several drought-inducible genes do not respond to either cold or ABA treatment, suggesting the existence of another ABA-independent pathway in the dehydration and high-salinity response (Figure 1.2, Pathway IV) (Shinozaki et al., 2003). These genes include *ERD1* (*early response to dehydration*), which encodes a chloroplast targeted Clp protease regulatory subunit, ClpD (Nakashima et al., 1997), which is proposed to function in degrading unassembled or mis-folded proteins in the chloroplasts (Peltier et al., 2001). The *ERD1* gene is not only induced by dehydration but also upregulated during natural senescence and dark-induced senescence (Simpson et al., 2003). Promoter studies using the *ERD1* promoter region fused to the luciferase (*LUC*) reporter gene in *Arabidopsis* identified *cis*-acting elements that

are involved not only in ABA-independent stress-responsive gene expression but also in senescence-activated gene expression (Simpson et al., 2003). Cloning of the DNA-binding proteins for these *cis*-elements will reveal this novel regulatory system (Figure 1.2).

1.5.2 Identifying the molecular components of stress signalling pathways

Several signal transduction and stress tolerance mutants have been identified using genetic approaches and biochemical analyses (Browse and Xin, 2001; Knight and Knight, 2001; Xiong et al., 2002a; Zhu, 2002). In a genetic screen using the firefly luciferase reporter gene (*LUC*) under the control of the *rd29A* promoter, many *Arabidopsis* mutants have been isolated that have altered induction of stress-responsive genes by drought, high-salinity, cold and ABA. These mutants exhibited altered expression of the *rd29A::LUC* gene at a high (*hos*), or low (*los*) level in response to various abiotic stresses or ABA treatments (Ishitani et al., 1997). The loci of the mutations, such as *fiery1* (*fry1*) (Xiong et al., 2001b), *hos1* (Lee et al., 2001a), *los1* (Guo et al., 2002), *los2* (Lee et al., 2002), *los5/aba3* (Xiong et al., 2001a), *los6/abal* (Xiong et al., 2002b) have been cloned and have provided new insight into the mechanisms of stress and ABA signal transduction (Xiong et al., 2002a; Zhu, 2002).

For example, *hos1* mutant plants show enhanced cold induction of stress-responsive genes, but salt or ABA induction of these genes was not substantially altered (Ishitani et al., 1997). *HOS1* encodes a novel protein that contains a RING-finger motif and is proposed to function by targeting a positive regulator(s) of CBF/DREB1 expression for degradation, because the expression levels of the CBF/DREB1 genes in *hos1* are higher than those in wild-type plants under cold stress (Lee et al., 2001a).

The *FRY1* gene encodes an inositol polyphosphate 1-phosphatase that is involved in inositol 1,4,5-triphosphate (Ins(1,4,5)P₃) signalling (Xiong et al., 2001b). Mutations in the *FRY1* gene result in elevated levels of Ins(1,4,5)P₃ and enhanced osmotic- and ABA-induction of gene expression (Xiong et al., 2001b). Therefore it was proposed that *FRY1* functions as a negative regulator of ABA and stress signalling and provides genetic evidence that phosphoinositols have an important role in ABA and stress-related signal transduction in plants (Xiong et al., 2001b; Shinozaki et al., 2003).

Characterisation of the *los5* and *los6* mutants has revealed a critical role for ABA in mediating osmotic stress regulation of gene expression (Xiong et al., 2002a). In *los5* and *los6*

mutant plants, the expression of several stress-responsive genes, such as *rd29A/cor48*, *rd17/cor47*, *cor15* and *rd22* was severely reduced or even completely blocked during salt stress and interestingly, *los5* and *los6* plants are defective in dehydration-induced ABA biosynthesis (Xiong et al., 2001a; Xiong et al., 2002b). Exogenous ABA restored the salt-induction of *rd29A::LUC* in *los5* mutant plants to the wild-type level, demonstrating that ABA deficiency was responsible for the defect in osmotic stress regulation of gene expression (Xiong et al., 2001a).

1.6 Molecular responses to wounding

Wounding is a common form of damage that occurs to plants as a result of abiotic stress factors such as wind, rain, hail, and of biotic factors, especially insect feeding. Wounding presents a constant threat to plant survival because it not only physically destroys plant tissue, but also provides a pathway for pathogen invasion (Cheong et al., 2002).

Plants respond to wounding, caused by mechanical stress or by chewing herbivores, with the synthesis of an array of defense proteins that facilitate wound healing and provide protection against further injury or pathogen attacks (Bowles, 1990). The accumulation of these proteins is mainly the result of rapid transcriptional activation of the corresponding genes. These responses occur not only at, or close to, the wound site but also in some cases systemically in distant, non-damaged tissue of wounded plants (Rojo et al., 1999).

The JA-responsive (JR) genes *JR1*, *JR2*, *JR3* and *VSP* which are strongly induced by wounding and by JA, and the wound-responsive (WR) genes *WR3* and *ACO* (encoding acyl CoA oxidase), which are induced only by wounding, have been used as molecular markers of the wound signal transduction pathway(s) in *Arabidopsis* (Titarenko et al., 1997; Rojo et al., 1998). Although further elucidation of the roles of some of these genes in the wound response is required, the rapid and localised pattern of induction of WR genes and the predominantly systemic and slower induction pattern of JR genes (Titarenko et al., 1997) point to the involvement of WR genes in repair and healing processes in the wounded area, whilst JA-responsive genes would fulfil preventive functions in undamaged parts of the plant (Leon et al., 1998). Moreover, studies with these genes have indicated that separate JA-dependent and JA-independent wound signal transduction pathways exist in *Arabidopsis* (Leon et al., 1998; Rojo et al., 1999).

In *Arabidopsis*, cell wall-derived oligosaccharides (OGAs) and the plant hormones JA and ET participate in the signalling network for wound-induced gene expression. These signalling components interact in novel ways to activate distinct responses (Rojo et al., 1999). Oligosaccharides released from plant cell walls upon wounding (Bergey et al., 1999) block JA synthesis, and activate ET production and a set of JA-independent genes (ACO and WR3) in the vicinity of the wound site (Rojo et al., 1999). Wound-induced ET is proposed to suppress JA-induced gene expression in the damaged leaves downstream of JA biosynthesis (Rojo et al., 1999). The relatively short-distance movement of OGA and ET diffusion are thought to prevent suppression of JA-induced gene expression in systemic leaves (Rojo et al., 1999). JR gene (*JR1*, *JR2*, *JR3* and *VSP*) induction in these distant tissues is thought to involve both systemin and JA as the systemic signals (Farmer et al., 1992; Royo et al., 1996; Stratmann, 2003).

While defending against mechanical wounding caused by insect predators plants must also prepare for opportunistic attack by pathogens and consequently have most likely evolved mechanisms that integrate the pathogen and wounding response (Cheong et al., 2002). Evidence is accumulating that the wound response pathway might also play a role in defense against specific pathogens. It has been known for some time that the wound response can be triggered by fungal elicitors. For example, the gene expression profiles observed during an incompatible plant fungal interaction overlap with those derived from wounding (Durrant et al., 2000). Moreover, wounding of young rice plants induces a systemic resistance response that leads to protection against infection by the rice fungus *Magnaporthe grisea* in the absence of *PR* gene expression (Schweizer et al., 1998). In addition, the signalling molecules JA and ET regulate both the wound response genes, and the expression of some basic *PR* genes and defensin (*PDF1.2*) and thionin (*Thi2.1*) genes in *Arabidopsis* (Penninckx et al., 1996; Epple et al., 1997; Thomma et al., 1998).

A cDNA array study designed to identify genes commonly regulated by JA, ET and an avirulent pathogen (Schenk et al., 2000) suggested the existence of a substantial network of regulatory interactions and coordination during pathogen and wounding responses. Another study using a microarray approach focused on transcriptional profiling of genes after pathogen infection and identified genes that are reported to be regulated by wounding (Maleck et al., 2000). Extensive overlap between pathogen- and wound-response genes was further demonstrated by Cheong et al. (2002). However, despite commonalities between the wound

response and pathogen response signalling pathways, several lines of evidence indicate that their respective response pathways are distinct, or even antagonistic and plants respond differently to attack by herbivores, necrotrophs and biotrophs (Traw et al., 2003). For example, proteinase inhibitors are more strongly induced by insect feeding than by biotrophic pathogens (Traw et al., 2003). In addition, the pathogenesis-related proteins PR1, PR2 and PR5 are induced following infection by biotrophic pathogens but not following insect feeding or attack by necrotrophic pathogens (Kunkel and Brooks, 2002). In addition to pathogen resistance, wounding pathways may also interact with other signalling processes.

Several studies show that some genes are induced by both osmotic stress and wounding treatments (Yamaguchi-Shinozaki and Shinozaki, 1994; Reymond et al., 2000). Moreover abscisic acid (ABA) mediates wound induction of some proteinase inhibitor genes (Pena-Cortes et al., 1991; Hildmann et al., 1992) suggesting possible interactions between wounding and other abiotic stress responses. Cheong et al. (2002) also identified many osmotic- and heat shock-regulated genes that were highly responsive to wounding. Wounding not only induces transcription factor DREB1, but also activated many downstream stress genes such as *rd29A/cor78*, *rd17/cor47* and *cor6.6* suggesting that wounding may also activate components of the drought and cold response pathways (Cheong et al., 2002).

1.7 Transcription factors in biotic and abiotic stress responses

Transcription factors are sequence-specific DNA-binding proteins that are capable of activating and/or repressing transcription of multiple target genes. They are often expressed in a tissue-specific, development-stage-specific, or stimulus-dependent manner, and are largely responsible for the selectivity in gene regulation (Zhang, 2003).

Transcription factor genes comprise a substantial fraction of all eukaryotic genomes, and the majority can be grouped into a handful of different, often large gene families according to the type of DNA-binding domain that they encode (Riechmann and Ratcliffe, 2000). Many of the transcription factor families found in *Arabidopsis* are unique to plants (e.g. APETALA2 (AP2), NAC, WRKY, AUXIN RESPONSE FACTOR (ARF)-Aux/IAA and DOF (for DNA-binding with one finger)). Some of the other groups (e.g. MYB, MADS and bZIP), which are not especially numerous in animals or yeast, have been significantly amplified in the plant lineage (Riechmann and Ratcliffe, 2000). Functional redundancy is not uncommon

within these families; therefore the proper characterisation of particular transcription-factor genes often requires their study in the context of a whole family (Riechmann and Ratcliffe, 2000).

As described above the defense and stress responses of plants are regulated by multiple interconnected signalling pathways resulting in significant overlap between the patterns of gene expression that are induced in response to different stresses. A microarray investigation focussing on 402 *Arabidopsis* transcription factor genes revealed a clear overlap between those expressed in response to different stresses and those expressed during senescence (Chen et al., 2002). What had initially appeared to be numerous linear pathways is now emerging as a complex regulatory network of signals that allow plants to respond optimally to their changing environment.

Perception of a pathogen by a plant and exposure to various stresses triggers rapid defence and stress responses via a number of signal transduction pathways. A major target of signal transduction is the cell nucleus where the terminal signals lead to the transcriptional activation of numerous genes and consequently to the *de novo* synthesis of a variety of proteins and antimicrobial compounds (Hammond-Kosack and Jones, 1996; Rushton and Somssich, 1998). Therefore defense and stress gene induction occurs primarily at the level of transcription, and regulating the temporal and spatial expression patterns of specific stress genes is an important part of the plant stress response (Rushton and Somssich, 1998). Plants devote a large proportion of their genome capacity to transcription, with the *Arabidopsis* genome coding in excess of 1600 transcription factors (Riechmann and Ratcliffe, 2000). These transcription factors belong to large gene families, which in some cases are unique to plants. The major transcription factor families that have roles in defense or stress response are ERF/AP2, WRKY, bZIP and MYB (Rushton and Somssich, 1998; Singh, 1998; Riechmann and Ratcliffe, 2000; Singh et al., 2002). These also constitute some of the major transcription factor families encoded by the *Arabidopsis* genome (Riechmann and Ratcliffe, 2000).

1.7.1 WRKY transcription factors

WRKY proteins are a novel family of transcription factors that are unique to plants (Dong et al., 2003) and form a large family with 74 members in *Arabidopsis* (Singh et al., 2002). WRKY proteins bind to the W box sequence element found in the promoters of pathogen-

responsive genes (e.g., parsley *PR1*, potato *PR-10a* and tobacco chitinase *CHN50*) (Despres et al., 1995; Yang et al., 1997; Rushton and Somssich, 1998; Eulgem et al., 2000). W-boxes are present in the promoter of *PR1*, the hallmark gene associated with the induction of SAR in *Arabidopsis* (Maleck et al., 2000). Moreover, the W-box was the only sequence that was common to all the promoters of 26 coordinately-regulated defense genes identified in a microarray analysis that examined changes in gene expression during SAR (Maleck et al., 2000). WRKY proteins are also known to regulate the expression of regulatory genes such as receptor protein kinases (Robatzek and Somssich, 2002) and significantly *NPR1* (Yu et al., 2001) which contains several W-box sequences in its promoter region. Mutation of the W boxes abolished their recognition by WRKY proteins and compromised the ability of NPR1 to complement a *npr1* mutant for disease resistance (Yu et al., 2001). Reverse genetic approaches are providing insights into the function of specific WRKY proteins. For example, over-expression of *AtWRKY18* at moderate levels resulted in enhanced expression of *PR* genes and resistance to *P. syringae* (Chen and Chen, 2002). Additionally, high level over-expression of *AtWRKY18* and *AtWRKY6* lead to growth retardation and other stress-related phenotypes (Chen and Chen, 2002; Robatzek and Somssich, 2002).

1.7.2 bZIP transcription factors

The bZIP transcription factor family comprises 75 members in *Arabidopsis* (Jakoby et al., 2002). One class of bZIP proteins that is linked to stress response comprises the TGA/*octopine synthase (osc)*-element-binding factor (OBF) proteins. These bind to the *activation sequence-1(as-1)/osc* element, which regulates the expression of some stress-responsive genes such as the *PR1* and glutathione S-transferase (*GST*) genes (Ellis et al., 1993; Ulmasov et al., 1994; Lebel et al., 1998). Some TGA family members in *Arabidopsis* have been shown to physically interact with NPR1, a key regulator in the SA defense signalling pathway (Zhang et al., 1999; Despres et al., 2000). Another class of bZIP proteins include G-box binding factors (GBF). The G-box is a ubiquitous *cis*-acting element that functions in concert with neighbouring *cis*-elements to regulate gene expression in many different functional contexts including pathogen attack (Menkens et al., 1995; Kim et al., 2003). Other bZIP proteins have been implicated in signalling in response to UV light and dehydration/high-salinity stresses (Singh et al., 2002). For example, the ABRE-binding factor (AREB) proteins respond at the transcriptional and

post-transcriptional level to drought and high-salinity through an ABA-dependent signal transduction pathway (Choi et al., 2000; Uno et al., 2000).

1.7.3 MYB transcription factors

MYB factors represent a family of proteins that include the conserved MYB DNA-binding domain (Stracke et al., 2001). Different categories of MYB proteins can be identified depending on the number of imperfect repeats of the MYB domain they contain. It is likely that single MYB-domain proteins, a class of expanding importance in plants, bind DNA in a different way than two repeat or three repeat MYB proteins, and these groups are therefore likely to have different functions (Jin and Martin, 1999; Stracke et al., 2001). The two-repeat MYB family (R2R3) is the largest family characterised in plants and there are estimated to be over 100 members in *Arabidopsis* (Jin and Martin, 1999). R2R3-type MYB factors participate in plant responses to environmental factors and in mediating hormone actions (Jin and Martin, 1999). In *Arabidopsis*, AtMYB2 has been found to regulate the *AtADH1* (*ALCOHOL DEHYDROGENASE1*) gene promoter in response to low oxygen (Hoeren et al., 1998), is induced by dehydration and also functions as a transcriptional activator in the dehydration- and ABA-inducible expression of the *rd22* gene (Abe et al., 1997). *AtMYB30* expression is strongly correlated with cell death during the hypersensitive response to pathogen attack or elicitor treatment (Daniel et al., 1999). Elicitor-responsive R2R3-type MYB genes have also been described from *Nicotiana tabacum* (*N. tabacum*) (Sugimoto et al., 2000), and an *AtMYB*-related MYB gene from *Oryza sativa* has been shown to be expressed in response to fungal attack (Lee et al., 2001b).

1.7.4 The importance of combinatorial control in transcriptional regulation

It is becoming clear that a major mechanism underlying eukaryotic transcriptional regulation is combinatorial control. Many genes are regulated by multiple transcriptional activators by virtue of having a specific set of *cis*-acting binding sites in their promoters. At any given time, a distinct set of transcription factors bind to these different sites to give rise to higher-order nucleoprotein complexes that have been called enhanceosomes (Carey, 1998;

Singh, 1998). Specific interactions between the proteins that form an enhanceosome, as well as interactions with components of the general transcriptional machinery, can lead to cooperativity in DNA binding and transcriptional synergy (Carey, 1998). The arrangement of the enhanceosome assembled at a given promoter may change in response to environmental, developmental or other signals. It is possible to form a much larger number of distinct enhanceosomes by using a discrete number of transcription factors in different combinations. A consequence of this is that, through combinatorial interactions, a given transcription factor can play multiple roles and help regulate different genes whose expression is induced by distinct signals (Carey, 1998; Singh, 1998). There is a growing body of evidence that combinatorial control plays an important role in mediating transcriptional regulation in plants.

The regulation of the biosynthesis of a class of maize pigments, the anthocyanins, is one of the best characterised examples of the importance of combinatorial protein interactions in the regulation of transcription in plants. The developmental regulation of anthocyanin expression is the result of combinatorial interactions between two distinct families of plant transcription factors, MYC-like proteins and MYB-like proteins (Mol et al., 1998; Singh, 1998). However, individual family members are not sufficient to induce the anthocyanin biosynthetic genes, but, rather, a member from each family must be coexpressed in a particular tissue for anthocyanin biosynthesis to occur (Roth et al., 1991). In *Arabidopsis*, the MYC-like rd22BP1 protein interacts with the MYB-like protein ATMYB2 to *trans*-activate dehydration-responsive genes under drought conditions (Abe et al., 1997). An interaction between a MYC protein and an ERF protein is involved in transcriptional regulation of the rice *Wx* gene (Zhu et al., 2003).

1.8 The AP2/ERF transcription factor family

The AP2/ERF (APETALA2/Ethylene-responsive element (ERE) binding factors) family of plant-specific transcription factors comprises 145 distinct members, whose distinguishing characteristic is that they contain either one or two AP2/ERF domains (Riechmann and Meyerowitz, 1998; Sakuma et al., 2002). The AP2 domain was first recognised as a repeated motif within the *Arabidopsis* AP2 protein (Jofuku et al., 1994). Shortly afterwards, four DNA-binding proteins from tobacco were identified that interact with a sequence that is essential for the responsiveness of some promoters to ET, and were

originally designated as 'ethylene responsive element binding proteins' (EREBPs) (Ohme-Takagi and Shinshi, 1995) but were later renamed ERE binding factors (ERFs) (Suzuki et al., 1998). The DNA-binding domain of EREBP-2 was mapped to a region that was common to all four proteins (Ohme-Takagi and Shinshi, 1995), and that was found to be closely related to the AP2 domain (Weigel, 1995). Since then, an increasing number of ERFs from various plants have been reported which are divergent except for the highly conserved DNA binding domain, the so called AP2/ERF domain, of 58 or 59 amino acids (Hao et al., 2002).

AP2/ERF genes play a variety of roles throughout the plant life cycle: from being key regulators of various developmental processes to acting as components of the mechanisms used by plants to respond to various types of biotic and abiotic stress (Riechmann and Meyerowitz, 1998). Different AP2/ERF proteins carry out a diverse range of functions throughout the plant life cycle. This diversity is illustrated by AP2 and the EREBPs themselves. *AP2* participates in the control of several processes during flower development, among them specification of organ and meristem identity, and ovule and seed development (Bowman et al., 1989; Kunst et al., 1989; Bowman et al., 1991; Shannon and Meeks-Wagner, 1993; Okamuro et al., 1997). In contrast, EREBP proteins bind to a *cis*-regulatory sequence known as the GCC-box widely conserved in the promoters of *PR* genes, activated in response to pathogen attack (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997).

The AP2/ERF family can be divided into three subfamilies (Figure 1.3) based on the number of their AP2/ERF DNA binding domains (Sakuma et al., 2002). The first subfamily includes genes that have 2 AP2/ERF domains (AP2 subfamily) and are exclusively involved in plant development (Kunst et al., 1989; Okamuro et al., 1997). The second subfamily includes genes that contain 2 different DNA-binding domains, AP2/ERF and B3 (RAV subfamily) (Kagaya et al., 1999). The B3 DNA-binding domain is conserved in VP1/ABI3 homologues (Suzuki et al., 1997). The third subfamily includes genes that have only 1 AP2/ERF DNA-binding domain (the ERF/AP2 subfamily) and are involved in the plants adaptation to biotic and abiotic stresses (Sakuma et al., 2002).

Genes from the ERF/AP2 subfamily can be further classified into 2 subgroups on the basis of similarity of the amino acid sequences of the DNA-binding domain and the DNA element to which they bind; the DRE/CRT subgroup and the ERF subgroup (Sakuma et al., 2002). Several members of the DRE/CRT subgroup bind to the dehydration-responsive element (DRE), also named C-repeat element (CRT) (DRE/CRT element) and regulate

The AP2/ERF transcription factor family

The AP2 subfamily

- Members contain 1 AP2/ERF domain
- Members involved in plant development

The RAV subfamily

- Members contain 2 different DNA binding domains (the AP2/ERF and B3)

The ERF/AP2 subfamily

DRE/CRT subgroup

ERF subgroup

- Members contain 1 AP2/ERF domain
- Members involved in adaptation to biotic and abiotic stress

Figure 1.3 The AP2/ERF transcription factor family

The AP2/ERF family can be divided into three subfamilies based on the number of AP2/ERF DNA binding domains. The first subfamily includes genes that have 2 AP2/ERF domains (AP2 subfamily). The second subfamily includes genes that contain 2 different DNA-binding domains, AP2/ERF and B3 (RAV subfamily). The third subfamily includes genes that have only 1 AP2/ERF DNA-binding domain (ERF/AP2 subfamily). The ERF/AP2 subfamily can be further classified into two subgroups; the DRE.CRT subgroup and the ERF subgroup.

dehydration-, high-salinity- and cold-induced target gene expression (Baker et al., 1994; Stockinger et al., 1997; Liu et al., 1998). The DRE/CRT *cis*-acting element (consensus CCGAC) is shared by many cold-responsive or dehydration-induced genes (Yamaguchi-Shinozaki and Shinozaki, 1993; Baker et al., 1994).

Members of the ERF subgroup are known to bind the GCC box (Park et al., 2001; Gu et al., 2002), a *cis*-acting element with the consensus GCCGCC, that is found in the promoter regions of many ET-regulated pathogenesis-related (PR) genes (Ohme-Takagi and Shinshi, 1995; Penninckx et al., 1996).

1.9 DNA binding specificity of the ERF/AP2 subfamily

Arabidopsis ethylene-responsive element binding factor 1 (AtERF1) is so far the only protein in this superfamily whose AP2/ERF domain structure has been determined (Allen et al., 1998). The AP2/ERF domain of AtERF1 consists of a 3-stranded anti-parallel β -sheet and an α -helix packed approximately parallel to the β -sheet. Unlike other DNA-binding proteins that interact with DNA through amino acids of an α -helix, the AP2/ERF domain of AtERF1 makes contact specifically with the GCC-box-containing double helix in the major groove through seven amino acids consisting of mainly arginines and tryptophans in the β -sheet (Allen et al., 1998). This indicates that the β -sheet in the AP2/ERF domain has important roles in the formation of the domain-GCC-box complex.

On the other hand, it was recently reported that different amino acids are conserved in 2 positions of the β -sheet in the AP2/ERF domains between the DRE/CRT and ERF subgroups. In these two positions, the valine at position 14 (V14) and the glutamic acid at position 19 (E19) are conserved in the DRE/CRT subgroup and are important for binding to the DRE/CRT *cis*-element. In contrast, in the ERF proteins alanine (A14) and aspartic acid (D19) occupy the corresponding positions in their AP2/ERF domains; these replacements are thought to be important for binding to the GCC-box *cis*-element (Sakuma et al., 2002).

The core sequence of the DRE/CRT *cis*-element is the 5 bp CCGAC sequence of which the 2nd C, 3rd G and 5th C (CCGAC) are essential for the highly specific interactions with the DRE/CRT subgroup of proteins (Sakuma et al., 2002). The core sequence of the GCC-box is the 6 bp GCCGCC sequence of which the 1st G, 4th G and 6th C (GCCGCC) are

involved in the highly specific interactions with the ERF subgroup (Hao et al., 1998). These two core sequences closely resemble each other, and a common core sequence is CCGNC. In this common core sequence, the 3rd G and 5th C were essential for the highly specific interactions with both the DRE/CRT and ERF proteins. However, these 2 subfamilies of DNA-binding proteins strictly recognise different nucleotides, the 2nd C of the DRE/CRT-box sequence by the DRE/CRT subgroup of proteins and the 1st G of the GCC-box by the ERF subgroup of proteins (Sakuma et al., 2002). Moreover DRE/CRT- and ERF- subfamilies of proteins generally function in clearly different signal transduction pathways of dehydration- and cold-stress response and pathogen defense response, respectively (Riechmann and Meyerowitz, 1998).

Several examples of ERF/AP2 proteins have emerged that do not strictly fit this criterion for DNA binding specificity and classification. Some ERF/AP2 transcription factors such as *Tsi1* (for *Tobacco stress-induced gene1*), a member of the ERF subgroup could bind specifically to both the GCC-box and the DRE/CRT-box sequences (Park et al., 2001). *Tsi1*, was induced not only by ET and SA but also by high-salinity and the over-expression of *Tsi1* improved tolerance to salt and pathogens (Park et al., 2001). However, only *PR* genes were found to be over-expressed in the transformants and it is still unclear whether *Tsi1* can regulate gene expression through the DRE/CRT-box *cis*-acting element. In addition a GCC-like element that directs JA and elicitor-responsive expression (JERE; AGCCGCC) has been identified in the periwinkle secondary metabolite biosynthetic gene *Str* (*Strictosidine synthase*) that interacts with a JA- and elicitor-inducible ERF/AP2 domain transcription factor, *ORC2* (octadecanoid-derivative responsive *Catharanthus* AP2) (Menke et al., 1999). *ORC2* showed strong homology to the tobacco EREBP1 (Ohme-Takagi and Shinshi, 1995) and tomato Pti4 (Zhou et al., 1997) proteins indicating that *ORC2* is a member of the ERF subgroup of proteins. Recently, another similar GCC-like element called box S (AGCCACC) has been identified that directs expression in response to fungal elicitors (Kirsch et al., 2000).

1.10 The role of the ERF subgroup in regulating defense responses

ERFs (ethylene(ET)-responsive factors), induced by pathogen infection, bind the GCC-box element that is present in the promoters of ET-regulated *PR* genes such as *PR3*, *PR4* and *PDF1.2* (Park et al., 2001; Gu et al., 2002). ERFs have been identified in plant species

from phylogenetically distinct taxa. Several genes have been characterised in *Arabidopsis* (ERF1, AtEBP, and AtERFs (Buttner and Singh, 1997; Solano et al., 1998; Fujimoto et al., 2000; Ohta et al., 2000)), tomato (Pti4, 5 and 6 (Zhou et al., 1997)), soybean (GmEREBP (Mazarei et al., 2002)) and tobacco (Ohme-Takagi and Shinshi, 1995)).

Tobacco EREBP-1, -2, -3 and -4 proteins were identified owing to their specific binding to the GCC-box (Ohme-Takagi and Shinshi, 1995). All four *EREBP* genes are ubiquitously expressed, and their mRNA levels are substantially increased by ET (Ohme-Takagi and Shinshi, 1995). The tomato ERF Pti4 was isolated, along with two other ERFs, Pti5 and Pti6, from a yeast two-hybrid screen by virtue of its interaction with the Pto kinase (Zhou et al., 1997). Pto specifies gene-for-gene resistance against *P. syringae* pv *tomato* strains that express the avirulence protein AvrPto or AvrPtoB (Gu et al., 2002). Pti4, Pti5 and Pti6 were shown to bind specifically to the GCC-box *cis*-element and to regulate the expression of a wide array of PR genes that contain the GCC-box in their promoters (Gu et al., 2002). The expression of *Pti4/5/6* is enhanced in response to infection by *P. syringae* pv *tomato* and by treatment with different signalling molecules (Thara et al., 1999; Gu et al., 2000). Phosphorylation of Pti4 by the Pto kinase enhances Pti4 binding capacity for the GCC-box (Gu et al., 2000). Expression of *Pti4* is induced in tomato leaves upon inoculation with *Pseudomonas* strains, by wounding, and by exposure to ET, SA, or JA (Thara et al., 1999; Gu et al., 2000; Mysore et al., 2002). Over-expression of *Pti4* in *Arabidopsis* caused the activation of several PR genes and increased resistance to the fungal pathogen *Erysiphe orontii* and tolerance to *P. syringae* pv *tomato* (Gu et al., 2002).

Studies by Wu et al., (2002) and Chakravarthy et al., (2003) identified sets of defense-related genes whose expression was affected by Pti4. However, only a small percentage of the genes identified in these studies possessed GCC-box or GCC-box-like *cis*-elements in their promoters. To determine if, as expected, Pti4 bound the promoters of these genes, Chakravarthy et al., (2003) used a Pti4 antibody for chromatin immunoprecipitation (ChIP) assays (Johnson et al., 2001). Surprisingly, although the promoters of many of these genes were bound by Pti4 *in vivo*, most lacked a GCC box. These observations suggest that ERFs such as Pti4 regulate gene expression both directly and indirectly, probably in combination with other transcription factors (Chakravarthy et al., 2003). For example, a rice MYC protein, designated OsBP-5, which contains a putative basic helix-loop-helix-leucine zipper DNA-binding domain acts synergistically, perhaps as a heterodimer with OsEBP-89, a member of

the ERF family of transcription factors, to regulate transcription of the rice waxy (*Wx*) gene (Zhu et al., 2003). In addition the *Arabidopsis* protein AtEBP has been reported to interact with the bZIP transcription factor OBF4 (Buttner and Singh, 1997). Although thus far there are relatively few examples of interactions between transcription factors belonging to different classes, observations suggest that cross-coupling between ERFs and other transcription factors occurs and may therefore be important in regulating gene expression during plant defense response.

Several other members of the ERF subgroup have been shown to be either positive or negative regulators of transcription (Fujimoto et al., 2000; Ohta et al., 2000; Ohta et al., 2001). Five *Arabidopsis* ERFs (AtERF1 to AtERF5) respond to extracellular signals to modulate GCC-box mediated gene expression positively or negatively. AtERF1, AtERF2 and AtERF5 function as activators of GCC-box dependent transcription in *Arabidopsis* leaves. By contrast, AtERF3 and AtERF4 function as active repressors that downregulate not only basal transcription levels of a reporter gene but also the transactivation activity of other transcription factors (Fujimoto et al., 2000).

Based on these collective observations, it is clear that the elucidation of the role of ERFs in the defense response in plants is an emerging important field of study.

1.11 The role of the DRE/CRT subgroup in regulating responses to environmental stress

The ability of plants to tolerate adverse conditions involves many biochemical and physiological changes, among them alterations in gene expression that are mediated in part by the DRE/CRT *cis*-element found in the promoters of dehydration-responsive (*rd*) genes such as *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1994) and cold-regulated (*cor*) genes such as *cor15A* (Baker et al., 1994).

Cor gene expression is induced during cold acclimation, the process by which plants increase their resistance to freezing in response to low but non-freezing temperatures. Over-expression of *cor15A*, which encodes a novel polypeptide that is targeted to the chloroplasts, has been shown to increase the freezing tolerance of chloroplasts *in vivo* and protoplasts *in vitro* (Artus et al., 1996). Whereas over-expression of *cor15A* has a detectable effect on

freezing tolerance of chloroplasts and protoplasts, the effect is small (Artus et al., 1996; Steponkus et al., 1998). Moreover, it has little if any effect on whole-plant freezing tolerance (Jaglo-Ottosen et al., 1998). Expression of the entire battery of COR genes, which includes the *cor6.6*, *cor47/rd17* and *cor78/rd29A* (in addition to the *cor15* gene) however does (Jaglo-Ottosen et al., 1998). The ability to express all of the *cor* genes in concert at warm temperatures was made possible by the discovery of the CBF transcriptional activators (Stockinger et al., 1997; Gilmour et al., 1998; Gilmour et al., 2000) also known as the DREB1 proteins (Liu et al., 1998; Shinwari et al., 1998) that belong to the DRE/CRT subgroup of transcription factors.

Cor6.6, *cor47/rd17* and *cor78/rd29A*, and presumably other yet to be discovered DRE/CRT-regulated *cor* genes, contain in their promoters the DRE/CRT *cis*-regulatory element (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The first DRE/CRT binding factor to be discovered, *CBF1* (CRT/DRE binding factor 1), was initially shown to activate expression of reporter genes in yeast that carried the DRE/CRT element (Stockinger et al., 1997). Over-expression of *CBF1* in *Arabidopsis* was subsequently shown to activate expression of the entire battery of known DRE/CRT-regulated *cor* genes and to enhance whole plant freezing survival without a low-temperature stimulus (Jaglo-Ottosen et al., 1998). Additional studies have shown that *CBF1* is a member of a small gene family encoding nearly identical proteins (Gilmour et al., 1998; Shinwari et al., 1998). The genes, *CBF1*, *CBF2* and *CBF3* (also known as *DREB1B*, *DREB1C*, and *DREB1A*, respectively), are located in tandem on chromosome 4 (Gilmour et al., 1998; Shinwari et al., 1998). Overexpression of *CBF3* in *Arabidopsis*, like over-expression of *CBF1*, activates *cor* gene expression and enhances freezing tolerance at warm nonacclimating temperatures (Liu et al., 1998; Kasuga et al., 1999). All three CBF genes are cold-induced; *CBF* transcript levels increase within 15 min of transferring plants to low-temperature followed at approximately 2 hours by accumulation of transcripts for the target DRE/CRT-regulated *cor* genes (Gilmour et al., 1998; Shinwari et al., 1998).

Because the DRE/CRT element was sufficient for drought-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994) it strongly suggested the existence of drought-inducible transcription factors that bind to the element. Two transcription factors, DREB2A and DREB2B, were identified based on their ability to bind the DRE/CRT element (Liu et al., 1998). Because they are induced by drought stress and are able to induce the expression of

genes that contain the DRE/CRT *cis*-acting element in protoplast transient assays, they were good candidates to be involved in drought signal transduction. Interestingly, over-expression of the DREB2 genes in transgenic plants only caused weak induction of the downstream genes and did not result in obvious phenotypes (Liu et al., 1998). It is proposed that translational modifications are necessary for the activity of those proteins in transgenic plants (Shinozaki and Yamaguchi-Shinozaki, 2000) but this hypothesis remains to be demonstrated experimentally.

Multiple transcription factors and *cis*-acting regulatory elements are involved in controlling low-temperature- and drought-induced gene expression. It has been proposed that the ABA-independent cold- and drought-responsive gene expression is regulated by CBF/DREB1 and DREB2 proteins, respectively (Gilmour et al., 1998; Shinwari et al., 1998; Nakashima et al., 2000). However, a CBF/DREB1 gene, *CBF4/DREB1D*, identified by Haake et al. (2002) is induced by dehydration stress, suggesting the existence of crosstalk between the CBF/DREB1 and DREB2 pathways. The dehydration-inducible expression of *CBF4/DREB1D* is controlled by ABA-dependent pathways suggesting that *CBF4*, may function in the slow response to drought that relies on the accumulation of ABA. A maize DRE-binding protein, DBF1, has been shown to function as a transcriptional activator of the abscisic acid-responsive gene *rab17* by ABA (Kizis and Pages, 2002). This also suggests the existence in some plants of an ABA-dependent pathway for the regulation of stress-inducible genes that involves DRE/CRT (Shinozaki et al., 2003). Analysis of the *cis*-acting elements in *rd29A* gene expression revealed that DRE/CRT functions cooperatively with ABRE as a coupling element in ABA-responsive gene expression in response to drought stress (Narusaka et al., 2003). This indicates that interactions between different transcription machineries function to provide crosstalk between different stress signalling pathways.

Oryza sativa (rice) homologues for *CBF/DREB1* and *DREB2*, four *OsDREB1s* and one *OsDREB2* have been identified in the rice genome sequence and function in stress-inducible gene expression (Dubouzet et al., 2003). Overexpression of *OsDREB1A* in *Arabidopsis* revealed that this gene has a similar function to that of its *Arabidopsis* homolog in stress-responsive gene expression and stress tolerance. This indicates that similar transcription factors function in dicotyledons and monocotyledons (Shinozaki et al., 2003).

Taken together these observations highlight the importance of understanding the relative roles of DRE/CRT-binding proteins in different pathways, their coordinated regulation

and the interactions with other signalling elements. In the longer term, these studies should assist the manipulation of plants in order to improve their stress tolerance and the productivity of crops.

1.12 Objectives of this study

The completion of the genome sequence of *Arabidopsis* made it possible for the first time to characterize the full complement of transcription factor encoding genes in plants. The REGIA (Regulatory Gene Initiative in *Arabidopsis*) project, an EU Framework 5-funded consortium of 29 laboratories was founded with the objective of functionally characterising an estimated 80% of the *Arabidopsis* transcription factor gene complement. The present study, as part of REGIA, set out to functionally characterise two genes coding for putative transcription factors belonging to the AP2/ERF family (*AP2.76* and *AP2.72*) and identified roles for both genes in biotic and abiotic stress response pathways.

Using bioinformatical approaches including database searches, sequence alignments and phylogenetic analysis, chapter 2 aimed to assign putative functions to both *AP2.76* and *AP2.72* and also to determine which transcription factor family both *AP2.76* and *AP2.72* belonged to. In addition by constructing gene fusions comprising *AP2.76* or *AP2.72* and a reporter gene, the subcellular localisation of *AP2.76* and *AP2.72* was investigated.

Chapter 3 investigated the expression of both *AP2.76* and *AP2.72* in response to several potentially inducing stimuli using northern blot analysis. Also by generating transgenic *Arabidopsis* plants engineered to express gene fusions which contained *AP2.76* and *AP2.72* promoter sequences fused with the β -glucuronidase (*GUS*) reporter gene, the promoter mediated control of the expression of these two genes during the *Arabidopsis* life-cycle was investigated.

In chapter 4, in order to gain insight into the function of both *AP2.76* and *AP2.72*, knockout lines for *AP2.76* and *AP2.72* were isolated from publically available libraries and characterised. Transgenic plants in which *AP2.76* and *AP2.72* were ectopically expressed using the CaMV 35S promoter were also generated. A cDNA microarray analysis and/or northern blot analysis were used to identify putative regulatory targets of *AP2.76* and *AP2.72*.

Chapter 2

Sequence analysis and functional characterisation of *AP2.76* and *AP2.72*, *Arabidopsis* genes encoding putative transcription factors of the AP2/ERF family

2.1 Introduction

2.1.1 *Arabidopsis* genes encoding putative transcription factors

With the completion of the *Arabidopsis* genome sequence, the focus has shifted from structural to functional annotation. It has been estimated that the functions of some 2,500 genes have been successfully deduced from experimental data to date (Hilson et al., 2003). The goal set by the scientific community and documented by the Multinational *Arabidopsis* Steering Committee (MASC) is to unambiguously determine the function of the >25, 000 *Arabidopsis* genes by the year 2010 (Osterlund and Paterson, 2002).

Genes encoding transcription factors comprise a substantial fraction of all eukaryotic genomes; *Arabidopsis* contains approximately 1600, representing more than 6% of the identified genes (Riechmann and Ratcliffe, 2000). These regulatory proteins provide the most common and direct mechanism of gene regulation in eukaryotic organisms, i.e. transcriptional control. Furthermore, transcription factors are believed to have played an important role in the evolution of plants, because of their ability to control complex patterns of gene expression (Doebley and Lukens, 1998).

Typical plant transcription factors comprise a DNA binding region, one or more nuclear localization signals (NLSs) and a transcriptional regulation domain (Liu et al., 1999), although some lack either a recognisable regulatory domain (Goff et al., 1992) or a specific DNA-binding region (Hill et al., 1996; Washburn et al., 1997). Having identified a novel candidate

transcription factor sequence, bioinformatics approaches may initially facilitate the identification of DNA binding domains, putative NLS(s) and regulatory domains. However, computer-based analyses are generally not sufficient to define gene function with a high level of confidence, and experimental confirmation is necessary in most cases.

2.1.2 DNA-binding domains

The DNA-binding domains of plant transcription factors, many of which are basic in character, contain amino acid residues that contact DNA bases at *cis*-acting promoter elements, and thus determine the functional specificity of the protein (Aukerman et al., 1991; Huang et al., 1996).

Transcription factors have been broadly classified into a number of families on the basis of the type of DNA-binding domain e.g. bZIP, homeodomain, helix-turn-helix, helix-loop-helix, and zinc-finger domains. Some of these families are large (Singh, 1998) e.g. in the *Arabidopsis* MYB family, members of which contain a specific type of helix-turn-helix motif of about 50 amino acids, more than 80 members have so far been identified (Romero et al., 1998). Some plant transcription factors have DNA-binding domains that appear to be unique to plants. For example, the AP2/ERF family contain a conserved, approximately 60 amino acid region, required for DNA binding (Okamuro et al., 1997).

Although plant transcription factors typically have only a single DNA-binding domain, there are examples, such as the GT-2 and APETALA2 proteins, which contain two related DNA-binding domains (Ni et al., 1996; Okamuro et al., 1997). In the case of GT-2, a rice DNA-binding protein that interacts with GT-box promoter elements in the rice phytochrome A gene, the different DNA-binding domains have been shown to discriminate between closely related GT-box sequences (Ni et al., 1996). For some transcription factors the ability to form either homodimers and/or heterodimers with related family members is a prerequisite for DNA binding. The ability to form specific heterodimers is a form of combinatorial control, and this can expand the number of DNA target sequences that can be recognised, as well as allow different combinations of activation domains to be recruited to a promoter element (Singh, 1998).

The base-recognition residues of DNA-binding domains are often highly conserved (Aukerman et al., 1991; Huang et al., 1996). For example, one arginine residue in bZIP

domains (Aukerman et al., 1991), either one or two cysteine and histidine residues in zinc-finger motifs (Li and Thomas, 1998), and single arginine and lysine residues in MADS domains (Huang et al., 1996) are nearly identical between plants, animals and fungi (Liu et al., 1999).

2.1.3 Nuclear Localisation Signals

Proteins destined for accumulation in the soluble compartment of the nucleus (the nucleoplasm) are synthesised on cytoplasmic ribosomes. From studies in a number of different eukaryotes, with most information coming from yeast and vertebrate systems, it is known that selective targeting to the nucleus occurs through defined channels connecting the inner and outer nuclear membranes, termed nuclear pores, and is regulated by the presence of nuclear localisation sequences (NLS(s)) within the primary structures of the targeted proteins (Hicks and Raikhel, 1995; Gorlich and Mattaj, 1996; Hurt, 1996).

Most NLSs can be assigned to one of three different groups, based on conserved structural features (Silver, 1991; Hicks and Raikhel, 1995). The first group contains members with NLSs similar to that contained within the SV40 large T-antigen. This signal comprises a sequence of basic amino acids (PPKKKRKV) organised as a single cluster (Kalderon et al., 1984). The second, bipartite group comprises proteins that contain a cluster of two basic oligopeptide domains (e.g. nucleoplasmin KRPAATKKAGQAKKKLD) separated by a variable spacer (Robbins et al., 1991). The third group comprises proteins with NLSs similar to that within yeast mating type factor $\alpha 2$ (NKIPIKD) (Robbins et al., 1991) and a variety of other NLSs that cannot be assigned to a specific class (Hicks and Raikhel, 1995).

In higher plants, NLSs have been identified primarily through the use of gene fusion strategies based either on the *E. coli* GUS gene (Jefferson et al., 1987) or the gene encoding the green-fluorescent protein (GFP) of *Aequorea victoria* (Galbraith et al., 1995; Sheen et al., 1995; Grebenok et al., 1997). The GUS system has a number of features ideally suited for use in plant nuclear targeting studies. These include the molecular mass of the GUS enzyme, 68 kDa which ensures its exclusion from the nucleus in the absence of an NLS, the availability of a convenient histochemical assay, the lack of appreciable endogenous GUS activity in most plants and the retention of enzymatic activity when heterologous polypeptides are fused to the GUS N- or C-terminus (Restrepo et al., 1990). Unfortunately however, the GUS assay is not

compatible with cell viability because the various substrates used to visualize GUS activity cannot traverse the membranes of living cells (Grebenok et al., 1997). In contrast, GFP offers the advantage that fusion protein derivatives do not disrupt cellular functions and can be visualized in living cells using conventional fluorescence and confocal microscopy. However, native GFP (25 kDa) and fusion protein derivatives with masses lower than the nuclear size exclusion limit (43 kDa) may accumulate passively in the nucleoplasm thus complicating the interpretation of nuclear-targeting experiments.

2.1.4 Transcription regulation domains

Transcription factors belonging to the same family generally have distinct actions because of differences in their regulatory domains, regions of the proteins that tend to diverge from one another over evolutionary time (Yanagisawa and Sheen, 1998). Regulatory domains and hence transcription factors, function as either repressors or activators, depending on whether they inhibit or stimulate the transcription of target genes (Liu et al., 1999).

In sharp contrast to the DNA-binding domains, the amino acid sequences that comprise activation and repression domains are not conserved, and consensus sequences have yet to be derived from the many eukaryotic activation domains that have been functionally defined to date (Triezenberg, 1995; Hanna-Rose and Hansen, 1996). On the basis of their amino acid composition, activation domains can only be classified as being rich in acidic amino acids, in glutamines, prolines, or serine and threonine residues. However, not all activation domains fit into these general categories, and mutational analyses (also carried out with plant transcription factors) have shown that those amino acids that predominate in activation domains are not necessarily important for their function (Triezenberg, 1995; Sainz et al., 1997; Uesugi et al., 1997). It may be that the precise molecular interactions that lead to transcriptional activation vary between transcription factors in different promoter contexts or that mechanisms other than specific protein-protein interactions govern transcriptional activation (Schwechheimer et al., 1998).

The classification system of transcription factor repression domains is also poorly characterised (Hanna-Rose and Hansen, 1996). This may be because research on repressors is complicated by the fact that it is difficult to distinguish between genuine effects of a repressor protein and the effects caused by a dominant negative form of the protein. It is thought that

repressors act in one of three ways (i) by binding to a cognate promoter site and blocking the binding of general transcription factors or activators, (ii) by blocking transcription by means of inhibitory interactions with general transcription factors or activators, (iii) by altering the higher-order DNA structure in a way that inhibits transcription (Hanna-Rose and Hansen, 1996).

In this chapter, in order to assign putative functions to both the *AP2.76* and *AP2.72* sequences a bioinformatical approach was undertaken. It was determined that both sequences most likely encoded transcription factor genes as the deduced amino acid sequences of both *AP2.76* and *AP2.72* contained an AP2/ERF DNA binding domain and they belong to the DRE/CRT and ERF subgroup within the ERF/AP2 subfamily respectively. A putative NLS along with several putative phosphorylation sites were also identified in the *AP2.76* and *AP2.72* sequences.

The hypothesis that the *AP2.76* and *AP2.72* genes encode transcription factors was investigated by examining the subcellular localisation of fusion proteins comprising *AP2.76* or *AP2.72* and GFP (or GUS).

2.2 Materials and Methods

2.2.1 DNA manipulations

General materials and DNA manipulations have been described in Chapter 5.

2.2.2 Plasmids

AV441496 (Asamizu et al., 2000) contains the AP2.76 cDNA cloned between the *EcoRI* and *XhoI* restriction enzyme sites in the pBluescript II vector., and was obtained from the Nottingham Arabidopsis Stock Centre (NASC).

N97284 (Newman et al., 1994) contains the AP2.72 cDNA cloned between the *Sall* and *Not* restriction enzyme sites in the Lamda Zip-Lox vector and was obtained from NASC.

pRTL22 (Restrepo et al., 1990) is a derivative of pRTL2 (Carrington et al., 1990) which contains the Cauliflower Mosaic Virus (CaMV) 35S promoter, the Tobacco Etch Virus (TEV) 5'-NTR (Non Translated Region), a polylinker sequence for insertion of open reading frames, and the CaMV 35S polyadenylation signal (Figure 2.7). The promoter contains a tandem duplication of the sequence between -90 and -418, which contains enhancer-like control elements (Carrington and Freed, 1990).

pRTL-GFP (a TAK laboratory plasmid) contains the non-fused native GFP gene cloned between the *XbaI* and *KpnI* restriction enzyme sites in the pRTL22 vector.

pK19 (Takeshita et al., 1987) is a derivative of pUC19, containing the neomycin phosphotransferase gene (NPTII) conferring kanamycin resistance as the selectable marker (Figure 2.7).

psmGFP (Davis and Vierstra, 1998) contains a codon-modified GFP gene encoding a soluble-modified GFP (smGFP) and was obtained from the ABRC. The plasmid contains the GFP gene cloned between the CaMV35S promoter and the nopaline synthase (nos) terminator in pUC118.

pGUS1, contains the *E. coli* gene *uidA/GUS* encoding β -glucuronidase in pUC19.

pRTL-GUS (a TAK laboratory plasmid) contains the non-fused *GUS* gene cloned between the *EcoRI* and *BamHI* restriction enzyme sites in the pRTL22 vector.

4XDRE-GUS and **4XGCC-GUS** (Rushton et al., 2002) contain four copies of the DRE/CRT-box (dehydration-responsive/c-repeat element) *cis*-element and GCC-box *cis*-element located upstream of the CaMV 35S TATA box, followed by the *GUS* gene and the *nos* terminator in the plasmid pBT10-GUS (Sprenger-Haussels and Weisshaar, 2000). These two plasmids were donated by Dr. Somssich of the Max Planck Institute for Plant Breeding Research, Cologne, Germany.

pROK219.76 (see Chapter 4 for details) contains the AP2.76 ORF cloned between *BamHI* and *KpnI* sites of the pROK219 vector which contains a CaMV 35S promoter and the nopaline synthase (*nos*) terminator, and the neomycin phosphotransferase gene (NTPII) conferring kanamycin resistance as the selectable marker.

pROK219.72 (see Chapter 4 for details) contains the AP2.72 ORF cloned between *XbaI* and *KpnI* sites of the pROK219 vector.

pROK219-NAN (Kirby and Kavanagh, 2002) contains the synthetic *NAN* reporter gene cloned between the *XbaI* and *SacI* sites of the pROK219 vector. The *NAN* gene is a codon optimized derivative of the *nanH* gene which encodes the ‘small’ cytoplasmic sialidase of *Clostridium perfringens* (Roggentin et al., 1988; Kruse et al., 1996).

2.2.3 Oligonucleotide primers

A) Primers used for sequencing the AP2.76 cDNA.

AP76F: 5’CTCATCCTCCTCTGTTTTCTCAACG3’

AP76F2: 5’GCTAATAGCTCTCAAGAAACGCCGTCG3’

M13 universal forward and reverse primers were used for sequencing both the AP2.76cDNA and AP2.72cDNA.

B) Primers used for sequencing the AP2.72 cDNA.

AP72R: 5’GACCCGCCGGTCAAACAAGAGCTTGAT3’

AP72R2: 5'CCTGGTCCACCTTCAACTACTACTAC3'

C) To amplify the coding region of AP2.76 for construction of AP2.76-GFP gene fusion.

AP76XbaI: 5'TGCTCTAGAATGGAAGAAAGCAATG3'

AP76HindIII: 5'CGAAGCTTATTGGCAAGAACTTCCCAAAT3'

D) To amplify the coding region of AP2.72 for construction of AP2.72-GFP gene fusion.

AP72XbaI: 5'GCTCTAGATGGTCTCCGCTCTCAGCC3'

AP72HindIII: 5'CGAAGCTTGTAACCATAATTCTTCTCTTC3'

E) To amplify smGFP.

smGFPHindF: 5'GGAAGCTTATGAGTAAAGGAGAAGAACTTTTC3'

smGFPkpnR: 5'GGGGTACCTTATTTGTATAGTTCATCCATG3'

F) To sequence the pRTL-AP76-GFP vector.

AP76P1: 5'CGAGTGAGCTACTCGGACTCGGAGAT3'

E) To sequence pRTL-AP72-GFP vector.

AL163491A: 5'CGGGATCCAAGGCCCTACTACCACCAC3'

G) To amplify the coding region of AP2.72 without stop codon for construction of AP2.72-GUS gene fusion.

AP72XbaI: 5'GCTCTAGATGGTCTCCGCTCTCAGCC3'

AP72EcoRI: 5'CGGAATTCGTAACCATAATTCTTCTCT3'

H) To amplify the coding region of AP2.72 with stop codon for construction of GUS-AP2.72 gene fusion.

AP72BamHI: 5'CGGATCCATGGTCTCCGCTCTCAG3'

AP72Kpn: 5'GGGGTACCTTATTCTTGGGTAGTT3'

I) To amplify the coding region of GUS with stop codon for construction of AP2.72-GUS gene fusion.

GUSR1: 5'CGGAATTCATGTTACGTCCTGTAGAAAC3'

GUSB1: 5'CGGGATCCTCATTGTTTGCCTCCCTG3'

J) To amplify the coding region of GUS without stop codon for construction of GUS-AP2.72 gene fusion.

GUSR1: as above

GUSB2: 5'CGGGATCCCTGCTGCGGTTTTTCACCGA3'

where the bold bases were added in order to create restriction enzyme sites.

K) To sequence the pRTL-AP72-GUS vector.

GUS2: 5'CTGCATCGGCGAACTGATCGTTAAACTGCC3'

J) To sequence the pRTL-GUS-AP72 vector.

GUS3: 5'TGAACAGGTATGGAATTTGCCGA3'

2.2.4 DNA and protein sequence analyses

Sequence analyses were performed using BLASTN and BLASTP (<http://ncbi.nlm.nih.gov/BLAST/>). DNA restriction analysis and primer design were carried out using the Vector NTI v.6. (InforMax., 1999). A search for potential splice signals was carried out using the Splice view Web-gene tool (<http://www.itba.mi.cnr.it/webgene/>).

Multiple alignment of protein sequences were done with the ClustalX version 1.81, a Windows version of CLUSTAL W program (Thompson et al., 1997) and edited with the Genedoc program (Nicholas et al., 1997). Phylogenetic relationships between sequences were inferred by the Neighbor-Joining method (Saitou and Nei, 1987). The Molecular Evolutionary Genetics Analysis v.2.1 (MEGA2) program (Kumar et al., 2001) was used to obtain Neighbor-Joining trees using Poisson corrected amino acid distances and the bootstrap support values for 1000 pseudoreplicates using the same distance algorithm as used to generate the Neighbor-Joining tree.

DNA binding domains were identified using the NCBI conserved domain search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Putative serine, threonine and tyrosine phosphorylation sites were identified using the NetPhos 2.0 Prediction Server (<http://www.cbs.dtu.dk/services/NetPhos>). The PSORT prediction program (<http://psort.nibb.ac.jp/form.html>) was used to scan for prediction of protein localisation signals.

2.2.5 Transient transformation of onion epidermal tissue for nuclear localisation experiments

5-10µg of plasmid DNA containing the pRTL-AP76-GFP, pRTL-AP72-GFP and pRTL-GFP constructs described in section 2.3.4 and the pRTL-AP72-GUS, pRTL-GUS-AP2.72 and pRTL-GUS constructs were used to coat washed tungsten particles as described in section 5.4.2. DNA-coated particles were bombarded at 1100p.s.i. as described in Chapter 5 (section 5.4.4) into onion epidermal tissue placed “inner surface down” on support medium (MS+) in Petri dishes. These were placed in a growth room at room temperature for 48 hours.

Bombarded onion epidermal tissue was immersed in GUS staining buffer (50mM NaHPO₄ pH6.8, 1mM 5-bromo-4-chloro-3-indolyl-glucuronide (Research Organics) and histochemical GUS expression patterns were examined as described in section 5.2.4 or examined by fluorescence microscopy to detect GFP expression using a Nikon Eclipse E600 microscope as outlined in section 5.4.5.

2.2.6 Transient transformation of tobacco leaves for the transactivation experiments

A mixture containing 5µg of effector plasmids, 5µg of reporter plasmids and 5µg of internal control plasmids was used to coat washed tungsten particles as described in section 5.4.2. DNA coated particles were bombarded at 1100p.s.i as described in section 5.4.4 and using a stainless steel focusing device which was fitted in the macrocarrier launch assembly which not only concentrated the biolistic particles to a given area but also improved the accuracy and predictability of each shot (Torisky et al., 1996). Tobacco leaves (*Nicotiana tabacum* cv. Petit Havana) were placed abaxial side upwards on support medium (MS+) in Petri dishes in preparation for bombardment. Following bombardment the petiole was pushed into the medium and the plate was sealed and stored in the growth room for two days. At this point the leaves were analysed using histochemical GUS assays as described in section 5.3.2 and analysed with quantitative fluorometric GUS and NAN assays as outlined below.

2.2.7 Quantitative fluorometric GUS and NAN assays

Bombarded tobacco leaf tissue was homogenised in GUS extraction buffer (2:1, buffer:tissue). 10µl aliquots of extracted soluble protein were added to GUS assay buffer (extraction buffer containing 1mM MUG) or to NAN assay buffer (extraction buffer containing 1mM MUN (4-methylumbellifery α -N-neuraminic acid)). The samples were incubated at 37°C for zero hours (control) and one hour or until fluorescence could be observed under a UV transilluminator. Reactions were stopped by the addition of 0.2M Na₂CO₃, which enhances fluorescence. GUS and NAN activity were detected quantitatively using a spectrofluorimeter (Perkin Elmer LS-50B) with excitation at 365nm, emission at 455nm and slit widths set at 5nm.

2.3 Results

2.3.1 Gene Structure

2.3.1.1 The structure of *AP2.76*

The cDNA clone AV441496 was sequenced using the primers outlined in section 2.2.3 and found to contain a single ORF of 1005 bp flanked by a 40 bp 5'UTR and a 286 bp 3'UTR (Figure 2.2). The *AP2.76* cDNA encodes a putative protein of 335 amino acids with a predicted molecular weight of 36.56kDa, an isoelectric point of 7.59, and a charge at pH7.0 of 1.3. Database searches using the NCBI conserved domain search revealed that the deduced amino acid sequence of *AP2.76* contained a conserved DNA binding domain of 58 amino acids (Figure 2.1 (A) and Figure 2.2), termed the AP2/ERF domain that is present in a large subfamily of plant DNA binding proteins, known as the ERF/AP2 subfamily (Okamuro et al., 1997; Riechmann and Meyerowitz, 1998). This domain possesses a charge at pH7.0 of 6.85. In contrast, a region in the C-terminus of *AP2.76*, comprising 68 amino acids with a charge at pH7.0, of -11.22, was classified as an acidic region (Figure 2.1 (A) and Figure 2.2) and may act as a transcriptional activator domain (Hahn, 1993). Several putative serine (ser), threonine (thr), and tyrosine (tyr) phosphorylation sites were identified in the *AP2.76* protein (Figure 2.1 (A)). The N-terminal region of the *AP2.76* protein contains a stretch of basic amino acids, KKKK (Figure 2.1 (A) and Figure 2.2), that resembles the SV40-type nuclear localisation signal (Kalderon et al., 1984).

A comparison of the nucleotide sequences of the *AP2.76* cDNA and the chromosomal *AP2.76* gene (ID: At1g64380) showed the ORF to be uninterrupted by introns.

2.3.1.2 The structure of *AP2.72*

The *AP2.72* cDNA clone N97284 was sequenced using the primers outlined in section 2.2.3 and shown to contain an ORF of 637 bp flanked by a 90 bp 5'UTR and a 3'UTR of 198 bp (Figure 2.3). The ORF encodes a putative protein of 214 amino acids with a predicted molecular weight of 24.28 kDa, an isoelectric point of 8.72, and a charge at pH7.0 of 2.05.

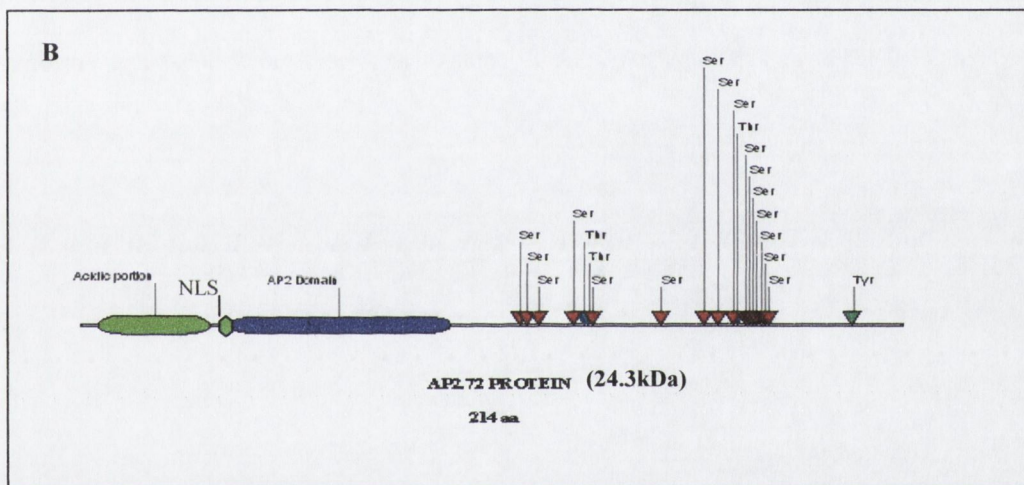
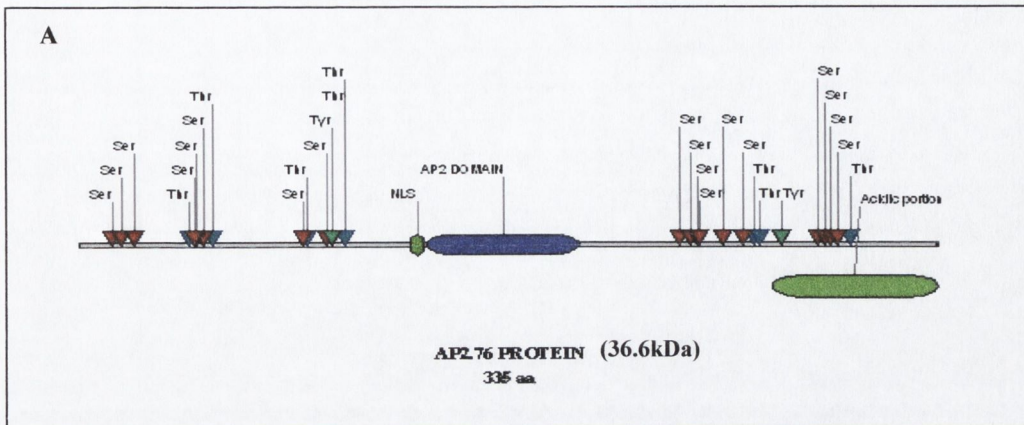


Figure 2.1. AP2.76 and AP2.72 protein structure.

A) The AP2.76 protein comprises 335 amino acids, B) The AP2.72 protein comprises 214 amino acids.

The AP2/ERF domains are shown in blue and acidic rich regions are shown in green. Putative phosphorylation of serine (ser), threonine (thr), and tyrosine (tyr) are shown in red, blue and green triangles respectively. Putative NLS(s) are shown with green hexagons.

									M E E S
1	NTTTTATCTT	ATACTCCACG	AAACGTCTGG	TTGAGAAAAA	ATGGAAGAAA				
	N D I	F Q N	N F S P	K I S	E I R				
51	GCAATGATAT	TTTTCAGAAC	AATTTTCAGTC	CTAAAATCTC	AGAAATCAGA				
	A S L S	Q I I	L A G	G P N T	L D S				
101	GCATCTCTGT	CTCAGATCAT	ATTAGCAGGA	GGACCAAACA	CACTCGACTC				
	I F S	L L T P	S S V	E S A	T T S F				
151	AATCTTCTCA	CTTCTCACTC	CCTCCTCCGT	AGAATCCGCC	ACAACATCAT				
	N T H	N P P	P P P Q	L G S	S V Y				
201	TCAACACTCA	CAATCCTCCG	CCACCGCCGC	AGCTCGGGTC	CTCCGTCTAT				
	L R Q R	D I I	E K F	H L Q N	R A I				
251	CTCCGCCAAC	GAGATATCAT	CGAGAAGTTC	CACCTTCAGA	ACAGAGCAAT				
	S T P	H P P L	F S S	T Y D	H H Q T				
301	CTCCACTCCT	CATCCTCCTC	TGTTTTCTC	AACGTACGAT	CATCATCAAA				
	S E L	M L Q	A A A G	S P A	A A F				
351	CTTCCGAGCT	AATGCTCCAA	GCGGCGGCCG	GGTCTCCAGC	CGCCGCTTTC				
	A A A L	A A G	R V T	K K K K	L Y R				
401	GCCGCTGCGT	TAGCGGCTGG	GAGGGTGACG	AAAAAGAAGA	AACTTTACAG				
	G V R	Q R H W	G K W	V A E	I R L P				
451	AGGAGTGAGG	CAGAGACATT	GGGGAAAATG	GGTTGCGGAG	ATAAGATTGC				
	Q N R	M R V	W L G T	Y D T	A E A				
501	CGCAAAACAG	AATGAGGGTT	TGGTTAGGTA	CTTACGACAC	GGCGGAAGCC				
	A A Y A	Y D R	A A Y	K L R G	E Y A				
551	GCCGCTTACG	CTTACGATCG	CGCCGCCTAT	AAGCTCCGTG	GAGATACGC				
	R L N	F P N L	K D P	S E L	L G L G				
601	TCGTCTCAAT	TTTCTAATC	TCAAAGACCC	GAGTGAGCTA	CTCGGACTCG				
	D S S	K L I	A L K N	A V D	G K I				
651	GAGATTCTCT	TAAGCTAATA	GCTCTCAAGA	ACGCCGTCGA	CGGGAAGATC				
	Q S I C	Q R V	R K E	R A K K	S V K				
701	CAATCCATTT	GCCAGAGAGT	CAGAAAAGAG	AGAGCAAAAA	AGAGCGTAAA				
	V S K	N S S A	T A D	S S C	L S S P				
751	AGTGAGCAAA	AACTCGTCGG	CCACGGCGGA	TTCTTCGTGT	TTGTCATCGC				
	E I L	S S S	P V T T	T T T	A V T				
801	CGGAGATTCT	GTCGTCGTCT	CCGGTGACGA	CAACTACTAC	TGCGGTTACT				
	S E D S	Y W V	S P M	G L C N	S E N				
851	TCAGAGGATA	GTTATTGGGT	TTCACCTATG	GGTTTGTGTA	ACAGTGAAAA				
	S S P	V S V S	V P S	E V P	A T A E				
901	TAGTTCTCCG	GTATCAGTTT	CGGTCCCTAG	TGAAGTACCG	GCGACGGCGG				
	E E A	M M G	V D T D	G F L	L A R				
951	AGGAAGAGGC	AATGATGGGA	GTGGACACTG	ATGGGTTTTT	ATTAGCGAGG				
	M P S F	D P E	L I W	E V L A	N *				
1001	ATGCCATCGT	TCGATCCAGA	GCTGATTTGG	GAAGTTCTTG	CCAATTAATA				
1051	CTACACAAAG	AATGTGAATT	TTGATCAAAA	TTGCAACAAG	AAGAAGAAAA				
1101	AAAAAAGTTT	GGAATTAGGA	CAAAAATGAT	GAGAAAAATT	AGGGTTTTAG				
1151	GTGTTAATTT	AGGGTTTAAA	GTAAAACCGG	ATCTTTTGTT	AAAATCATTC				
1201	GAAAGTTGTT	AAATAATATA	ATTAGGGTTT	CTCAAATACA	ATGTAAAGCC				
1251	TCGTGGTTTT	TGAAAGAGGT	TGTGAAAGCT	CCTTTTTTTT	AATTTCTGTT				
1301	TTAGATCATC	GAAGGACTTT	CTTTCTGATT	GTTN					

Figure 2.2. Analysis of an *Arabidopsis* cDNA encoding the AP2.76 protein.

The nucleotide and deduced amino acid sequences of the *AP2.76* gene are shown. The AP2/ERF DNA binding domain is shown in bold. The basic amino acids that potentially act as a nuclear localisation signal are shown in red. An acidic rich region is shown in italics. The termination codon is denoted by an asterisk.

1	GATCGGAGGT	ATGGAAAGGG	ACCGGTTCCA	CCGGAACAT	CGGCGGCGGC	
						M V S A
51	GGATGATAAT	TCGTCTTGA	ACGAGACTGA	TGTCACCGCC	ATGGTCTCCG	
	<i>L S R</i>	<i>V I E</i>	<i>N P T D</i>	<i>P P V</i>	<i>K Q E</i>	
101	CTCTCAGCCG	TGTCATAGAG	AATCCGACAG	ACCCGCCGGT	CAAACAAGAG	
	<i>L D K S</i>	<i>D Q H</i>	<i>Q P D</i>	<i>Q D Q P</i>	<i>R R R</i>	
151	CTTGATAAAT	CGGATCAACA	TCAACCAGAC	CAAGATCAAC	CAAGAAGAAG	
	H Y R	G V R Q	R P W	G K W	A A E I	
201	AACTATAGA	GGCGTAAGGC	AGAGACCATG	GGGTAAATGG	GCGGCAGAAA	
	R D P	K K A	A R V W	L G T	F E T	
251	TCCGCGATCC	AAAGAAAGCA	GCCCGTGTCT	GGCTCGGGAC	TTTCGAGACG	
	A E E A	A L A	Y D R	A A L K	F K G	
301	GCAGAGGAAG	CTGCTTTAGC	CTATGACCGA	GCTGCCCTCA	AATTCAAAGG	
	T K A	K L N F	P E R	V Q G	P T T T	
351	CACCAAGGCT	AAACTGAAC	TCCCTGAACG	GGTCCAAGGC	CCTACTACCA	
	<i>T T I</i>	<i>S H A</i>	<i>P R G V</i>	<i>S E S</i>	<i>M N S</i>	
401	CCACAACCAT	TTCTCATGCA	CCAAGAGGAG	TTAGTGAATC	CATGAACTCA	
	<i>P P P R</i>	<i>P G P</i>	<i>P S T</i>	<i>T T T S</i>	<i>W P M</i>	
451	CCTCCTCCTC	GACCTGGTCC	ACCTTCAACT	ACTACTACTT	CGTGGCCAAT	
	<i>T Y N</i>	<i>Q D I L</i>	<i>Q Y A</i>	<i>Q L L</i>	<i>T S N N</i>	
501	GACTTATAAC	CAGGACATAC	TTCAATACGC	TCAGTTGCTT	ACGAGTAACA	
	<i>E V D</i>	<i>L S Y</i>	<i>Y T S T</i>	<i>L F S</i>	<i>Q P F</i>	
551	ATGAGGTTGA	TTTATCATAC	TACACGTCGA	CTCTCTTCAG	TCAACCTTTT	
	<i>S T P S</i>	<i>S S S</i>	<i>S S S</i>	<i>Q Q T Q</i>	<i>Q Q Q</i>	
601	TCAACGCCTT	CTTCATCTTC	TTCTTCCTCC	CAACAGACGC	AGCAACAGCA	
	<i>L Q Q</i>	<i>Q Q Q Q</i>	<i>R E E</i>	<i>E E K</i>	<i>N Y G Y</i>	
651	GCTACAACAA	CAACAACAGC	AGCGTGAAGA	AGAAGAGAAG	AATTATGGTT	
	<i>N Y Y</i>	<i>N Y P</i>	<i>R E *</i>			
701	ACAATTATTA	TAACTACCCA	AGAGAATAAT	CTAATTATTA	TTGTTGGTCG	
751	AATCAGTTTT	ATAAATAGCT	ATCATAGTTT	CATTTTTGGT	TTCCGTAACC	
801	TTTGTTGCAT	GGAAAAATATG	AATGAACGAG	GGACATGTGT	AACAATTTGT	
851	TTGTGTTTTT	CCGTAACCTT	TGTTGCATGG	AAAAATATGAA	TGAACGAGGG	
901	ACATGTGTAA	CAATTTGTTT	GTGTTT			

Figure 2.3. Analysis of an *Arabidopsis* cDNA encoding the AP2.72 protein.

The nucleotide and deduced amino acid sequences of the *AP2.72* gene are shown. The ERF/AP2 DNA binding domain is shown in bold. The basic amino acids that potentially act as a nuclear localisation signal are shown in red. The acidic rich region is shown in italics. The termination codon is denoted by an asterisk.

Database searches using the NCBI conserved domain search revealed that the deduced amino acid sequence of AP2.72 like the AP2.76 protein contained a conserved DNA binding domain of 58 amino acids (Figure 2.1 (B) and Figure 2.3), known as the AP2/ERF domain, that is found in the ERF/AP2 transcription factor subfamily (Riechmann and Meyerowitz, 1998). This domain possesses a charge at pH7.0 of 6.76. In contrast, the N-terminus of AP2.72, comprising 38 amino acids with a charge at pH7.0, of -4.14, was classified as an acidic region (Figure 2.1 (B) and Figure 2.3) and may act as a transcription activation domain (Hahn, 1993). Several putative serine (ser), threonine (thr), and tyrosine (tyr) phosphorylation sites were identified in the AP2.72 protein (Figure 2.1 (B)). The N-terminal region of the AP2.72 protein contained a stretch of basic amino acids PRRRH resembling an SV40-type nuclear localisation signal (Kalderon et al., 1984).

A comparison of the AP2.72 cDNA and chromosomal (ID: At5g13330) sequences identified the presence of a single intron of 1440 bp interrupting the ORF, with the 5' splice junction at position + 91 bp relative to the ATG.

2.3.2 Sequence comparison of the ERF/AP2 domains of the AP2.76 and AP2.72 proteins with those of other members of the ERF/AP2 transcription factor family

The AP2 domains of the AP2.76 and AP2.72 proteins were aligned with those of other previously characterized members of the ERF/AP2 subfamily (Figure 2.4). The ERF/AP2 domain typically comprises 58 or 59 amino acids (Ohme-Takagi and Shinshi, 1995) and this domain in AP2.76 and AP2.72 shows the same conserved amino acids found in other ERF/AP2 domain-containing proteins (Figure 2.4).

As described by Sakuma et al., (2002), on the basis of similarities of the amino acid sequence of their DNA binding domains, the ERF/AP2 subfamily can be further divided into two subgroups, the “DRE/CRT box” binding proteins and the “GCC box” binding proteins. The valine at position 14 (V14) and the glutamic acid at position 19 (E19) are conserved in the DRE/CRT-box binding proteins (DRE/CRT subgroup), whereas alanine (A14) and aspartic acid (D19) occupy the corresponding positions in the GCC-box binding proteins (ERF subgroup). The sequence alignment shown in Figure 2.4 shows that the AP2.72 protein

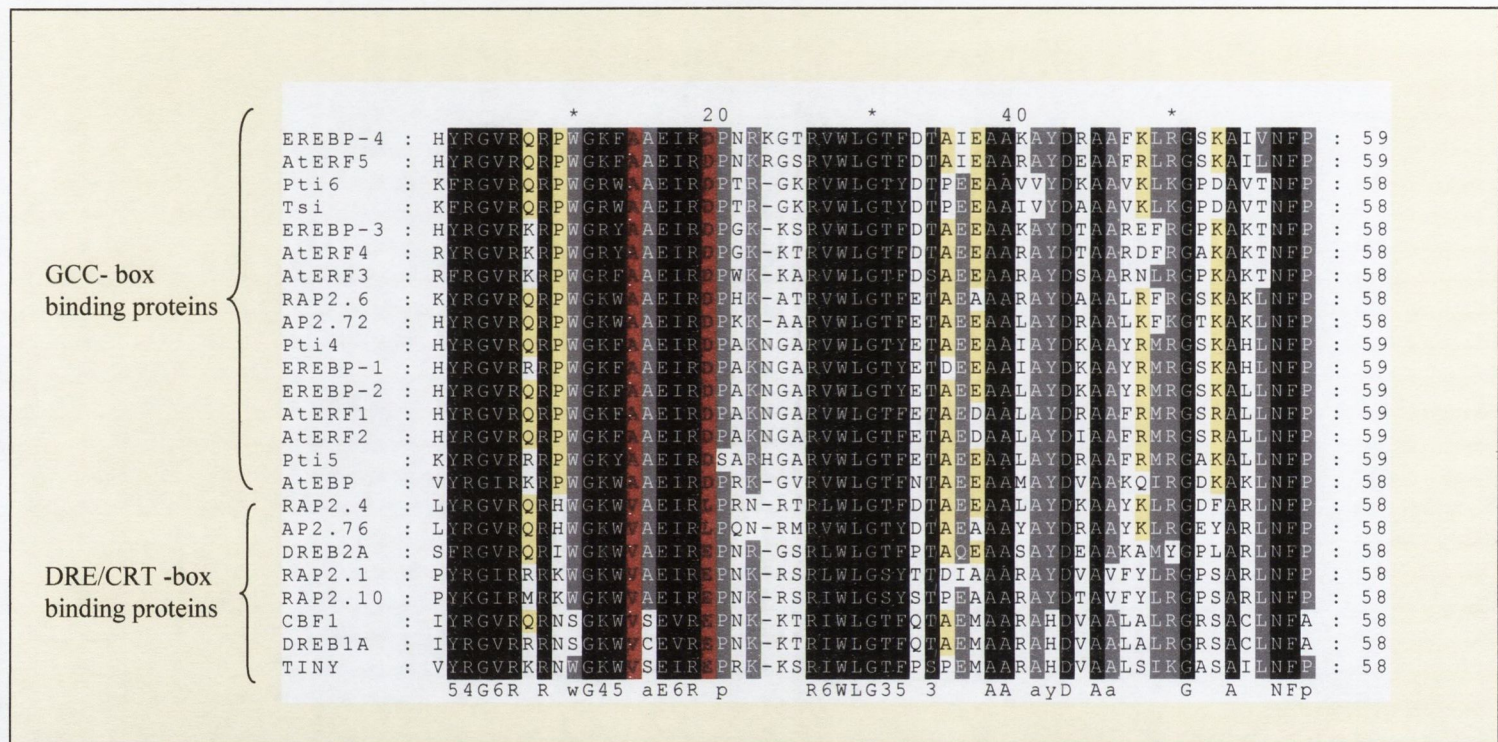


Figure 2.4. Comparison of the deduced amino acid sequences of the DNA binding domains of AP2.72 and AP2.76 with those of other AP2/ERF related proteins. The deduced amino acid sequence of the DNA binding domains of AP2.76 and AP2.72 was compared with those of RAP2.1, RAP2.4, RAP2.6, RAP2.10, CBF1, DREB1A, DREB2A, TINY, AtERF1, AtERF2, AtERF3, AtERF4, AtERF5 and AtEBP from *Arabidopsis* (AF003094, AF003097, AF003099, AF003103, U77378, AB007787, AB007790, X94689, AB008103, AB008104, AB008105, AB008106, AB008107 and Y00942 respectively); EREBP-1, EREBP-2, EREBP-3, EREBP-4, and Tsi of tobacco (D38123, D38126, D38124, D38125, and AF058827 respectively); and Pti4, Pti5 and Pti6 of tomato (U89255, U89256 and U89257 respectively). These genes were classified into two groups on the basis of similarities of the amino acid sequence of their binding domains. The degree of conservation is distinguished at four levels (100%-80%), (80%-60%), (60%-40%) and not conserved, which are shown in black, grey, yellow and white colours respectively. The red background represents amino acids that have are thought to have important roles in DNA-binding specificity (Sakuma et al., 2002). Dashes indicate gaps in the amino acid sequences introduced to optimise their alignment. The Alignment were performed using ClustalX and edited using the GeneDoc program.

contains the conserved A14 and D19 residues, and therefore belongs to the ERF subgroup several members of which have been reported to bind the GCC-box *cis*-element (Fujimoto et al., 2000; Park et al., 2001; Gu et al., 2002). Figure 2.4 also shows that the AP2.76 protein contains the conserved V14 (although not the E19) residue and therefore belongs to the DRE/CRT subgroup, several members of which have been shown to bind to the DRE/CRT-box *cis*-element (Liu et al., 1998; Sakuma et al., 2002).

2.3.3 Phylogenetic analysis of AP2.72 and AP2.76

The alignment generated in Figure 2.4, was used to construct a phylogenetic tree that includes the AP2.76 and AP2.72 proteins (Figure 2.5). The unrooted tree reveals two major groupings, corresponding to the ERF subgroup which contains AP2.72 and the DRE subgroup which contains AP2.76. These two subgroups had previously been reported (Riechmann and Meyerowitz, 1998; Sakuma et al., 2002).

Within the DRE subgroup, the AP2/ERF domain encoded by the *Arabidopsis* gene *RAP2.4* (Related to *A*petala2 *P*rotein4) showed the highest level of similarity with the AP2.76 DNA binding domain (Figure 2.5). *RAP2.4* (ID: At1g78080) encodes a predicted protein of 335 amino acids (Okamuro et al., 1997). The ERF/AP2 domains of AP2.76 and *RAP2.4* share 63% amino acid identity and 71% similarity. Moreover, the ERF/AP2 domain was the only part of these proteins that showed significant similarity.

Furthermore within the ERF subgroup, the ERF/AP2 domain of AP2.72 was found to share 70% amino acid identity and 75% similarity to *RAP2.6* (Related to *A*petala2 *P*rotein6). The *RAP2.6* gene (ID: At1g43160) encodes a predicted protein of 193 amino acids (Town et al., 2003; GenBank-unpublished).

2.3.4 Subcellular localization of the AP2.72 and AP2.76 proteins: *in vivo* transient expression of gene fusion constructs in onion cells

To examine the subcellular localisation of AP2.76 and AP2.72, an *AP2.76-GFP* gene fusion, an *AP2.72-GFP* and *AP2.72-GUS* gene fusions were constructed and were transiently expressed in onion epidermal cells. The monolayer of transparent cells in onion epidermal

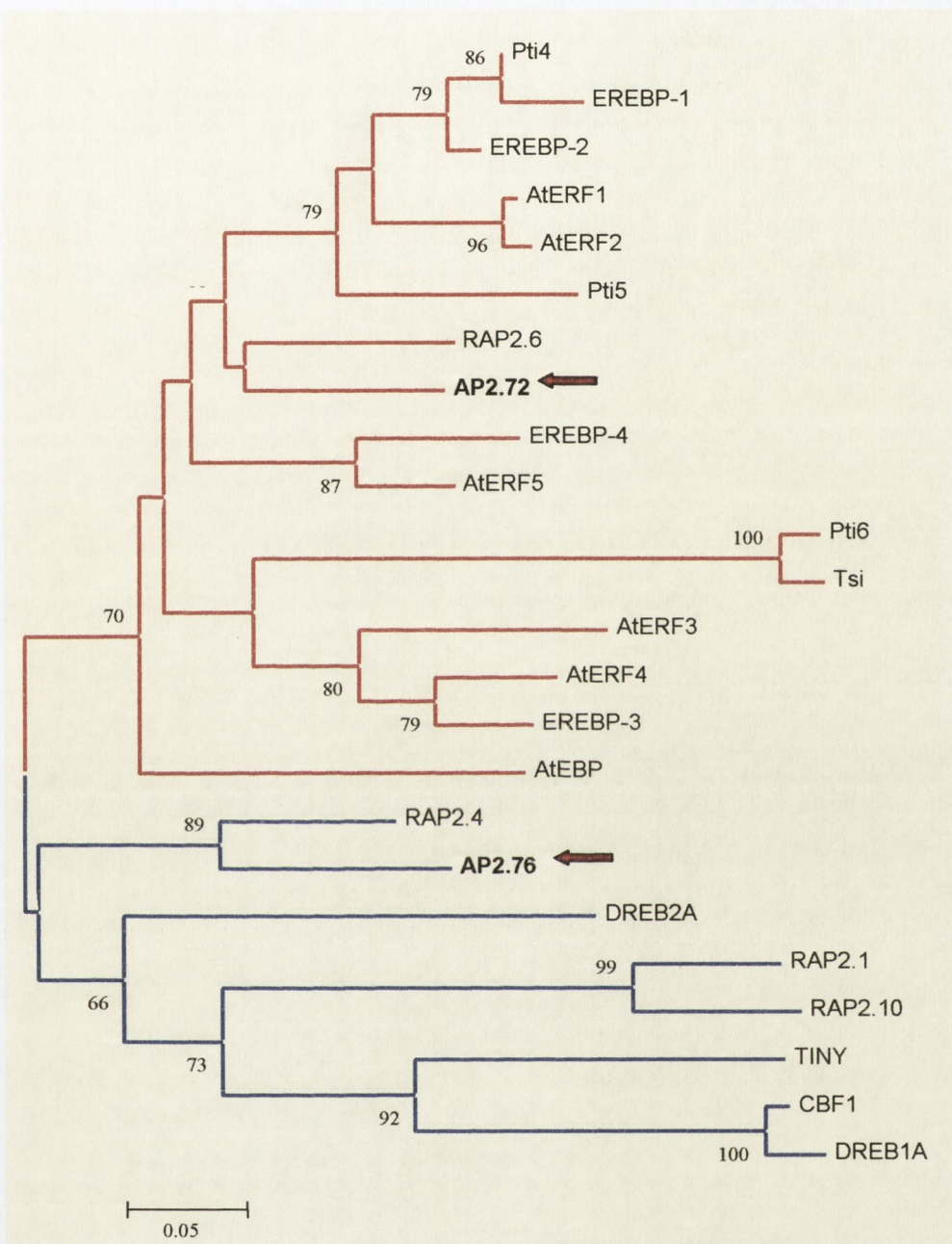


Figure 2.5. Phylogenetic analysis of AP2/ERF family members.

Members of the ERF/AP2 subfamily members from two distinguishable subgroups, the ERF subgroup (shown in red) and the DRE/CRT subgroup (shown in blue). The neighbor-joining method was used to generate the phylogenetic tree drawn using the MEGA2 program. Numbers on the nodes correspond to bootstrap values based on 1000 pseudoreplicates. Values greater than 70% are shown. The gene accession numbers are cited in Figure 2.4. AP2.76 and AP2.72 are shown in bold and indicated with a red arrow.

cells enables easy visualization of the GFP and GUS reporter gene expression in the nucleus and cytoplasm and onion cells have previously been used successfully in analysis of nucleocytoplasmic partitioning of *Arabidopsis* proteins (von Arnim and Deng, 1994).

2.3.4.1 GFP fusion constructs

To generate the various GFP fusions, the smGFP gene fragment was amplified from the plasmid pSmGFP (encoding soluble modified (sm) GFP) by PCR using *Pfu* polymerase and primers engineered to contain *HindIII* (5') and *KpnI* (3') sites. The entire coding regions of AP2.72 and AP2.76 without the stop codons were also amplified by PCR using *Pfu* polymerase and primers engineered to contain *XbaI* (5') and *HindIII* (3') sites. The smGFP fragment obtained by PCR was cloned into the pK19 plasmid and verified by restriction digestion at the *HindIII* and *KpnI* sites (Figure 2.6). The new vector was called pK19GFP. The coding regions of AP2.76 and AP2.72 that were obtained by PCR were cloned into the pK19 plasmid and verified by restriction digestion at the *XbaI* and *HindIII* sites (Figure 2.7). The new vectors were called pK19AP2.76 and pK19AP2.72.

To assemble the various fusion constructs, triple ligations were performed which contained (i) the pK19GFP vector digested with *HindIII* and *KpnI*, (ii) the pK19AP2.76 or pK19AP2.72 vectors digested with the *XbaI* and *HindIII* restriction enzymes (iii) the pRTL22 vector (3.9kb) which contains the cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer region digested with *XbaI* and *KpnI* restriction enzymes. The two resulting constructs were called pRTL-AP76-GFP (AP2.76-GFP: 5.6kb) and pRTL-AP72-GFP (AP2.72-GFP: 5.2kb). All constructs were verified by restriction enzyme analysis (Figure 2.7). Sequencing using the AP76P1 and AL163491A primers showed that the coding region of AP2.76 and AP2.72 was fused in-frame with *GFP* in the AP2.76-GFP gene fusion and the AP2.72-GFP gene fusion respectively.

The pRTL-AP76-GFP and pRTL-AP72-GFP constructs along with a control pRTL-GFP that contained the non-fused smGFP gene fragment were introduced individually by particle bombardment into onion epidermal cells.

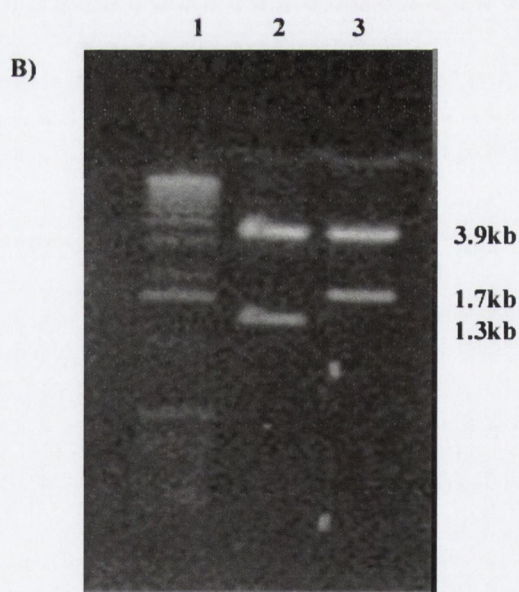
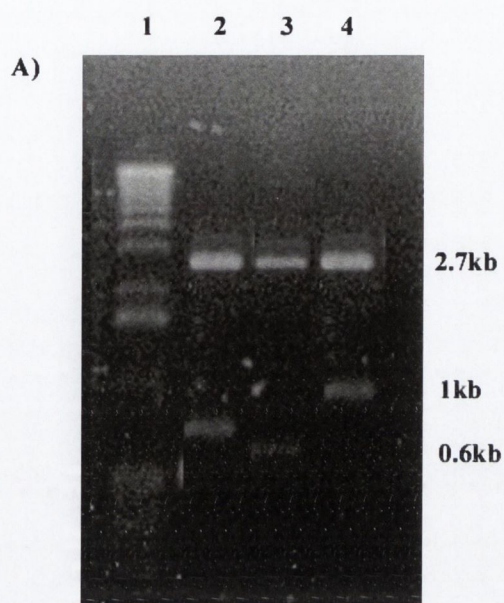


Figure 2.7. Confirmation of the structure of the AP2.76-GFP and AP2.72-GFP gene fusion plasmids.

A) Restriction digest of transformant clones containing pK19AP2.76 (lane 4) and pK19AP2.72 (lane 3) digested with *Xba*I/*Hind*III and pK19GFP (lane 2) digested with *Hind*III/*Kpn*I.

B) Restriction digest of transformant clones containing pRTL-AP76-GFP (lane 3) and pRTL-AP72-GFP (lane 2) digested with *Xba*I/*kpn*I.

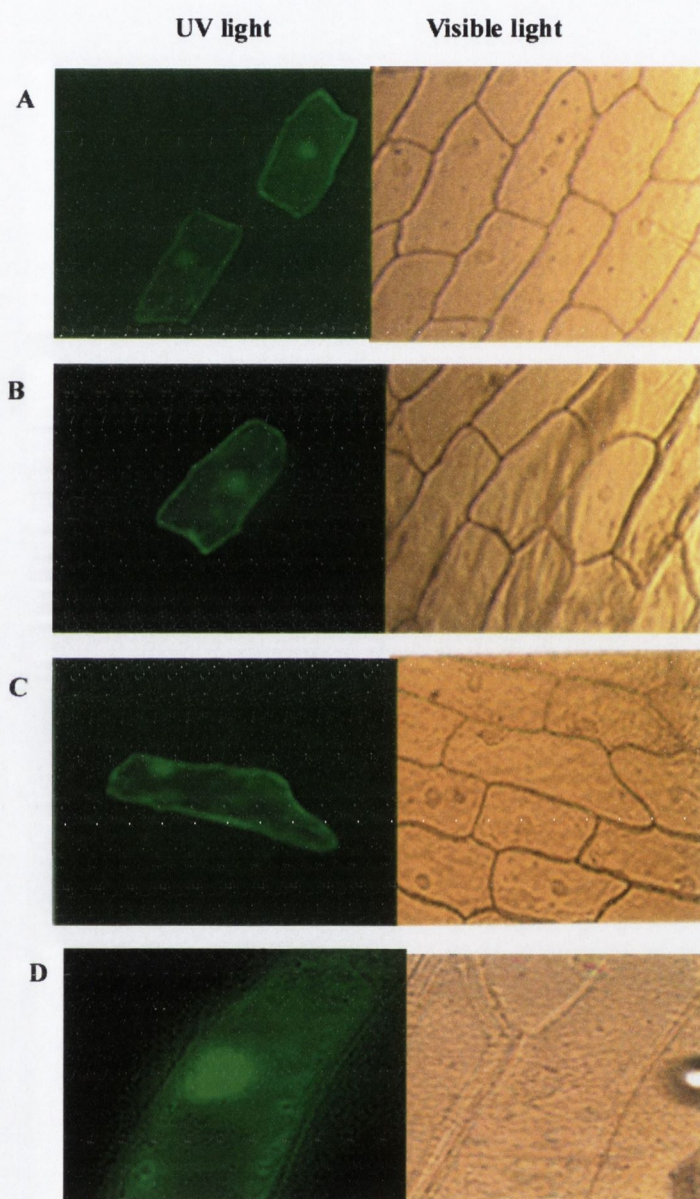


Figure 2.8. Subcellular localisation of native smGFP.

Subcellular distribution of green fluorescence within onion cells transiently expressing a non-fused smGFP protein. All images were analysed by a fluorescence microscopy at 100X (A, B & C) and 400x (D).

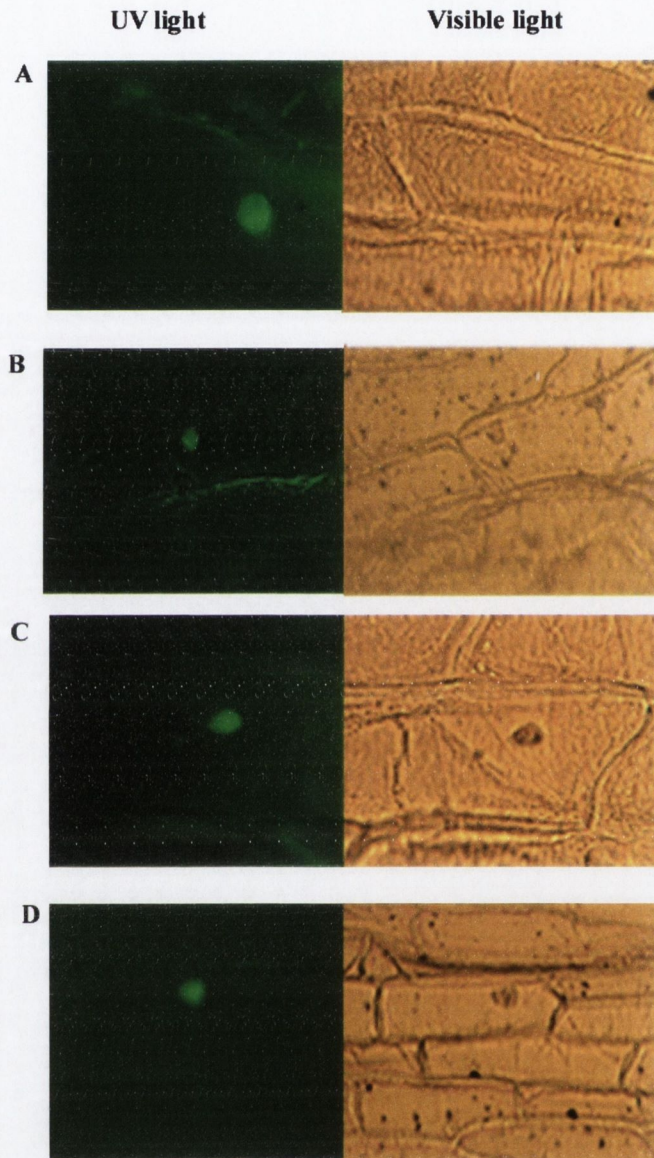


Figure 2.9. Subcellular localisation of AP2.76-GFP fusion protein.

Subcellular distribution of green fluorescence within onion cells transiently transformed with the AP2.76-GFP gene fusion. All images were analysed by fluorescence microscopy at 400X magnification (A, B, C & D).

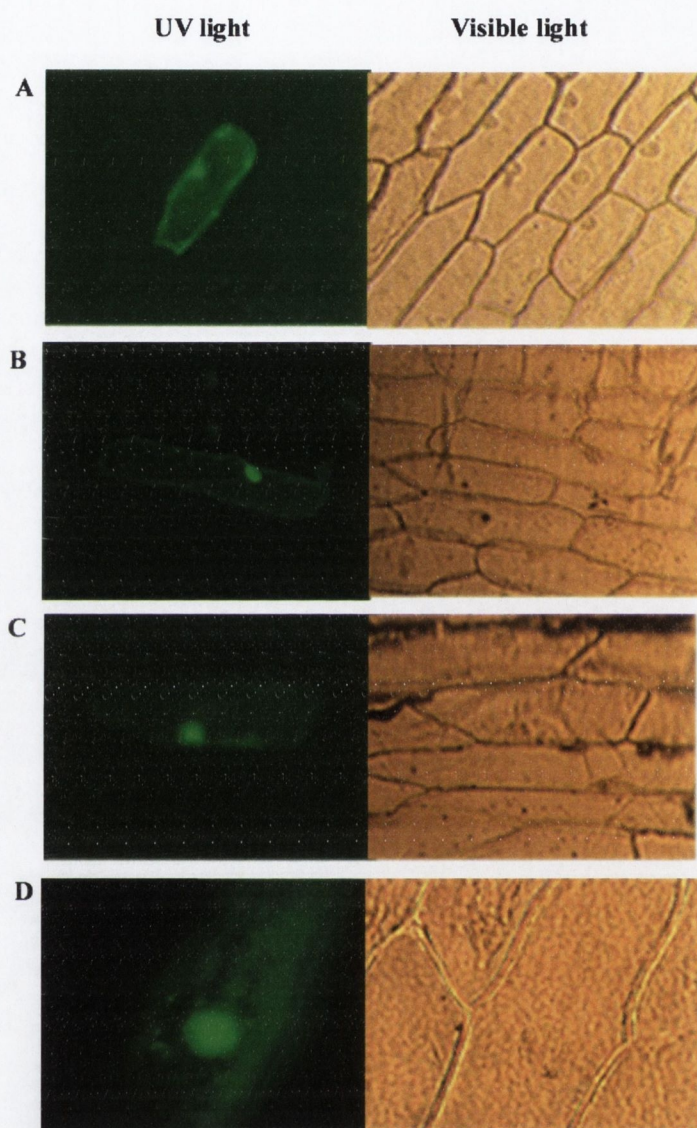


Figure 2.10. Subcellular localisation of the AP2.72-GFP fusion protein.

Subcellular distribution of green fluorescence within onion cells transiently transformed with a AP2.72-GFP fusion. All images were analysed by fluorescence microscopy at 100X (A, B & C) and 400X (D) magnification.

2.3.4.2 GFP expression in bombarded onion cells

Distinct patterns of GFP expression were observed 48 hours following bombardment of onion epidermal tissue with pRTL-AP76-GFP, pRTL-AP72-GFP and pRTL-GFP constructs. Fusion protein localization was determined by fluorescence microscopy at 100X and 400X magnifications.

pRTL-GFP, which encodes a nonfused native GFP protein, served as a control. The native GFP protein (26kDa) was sufficiently small to allow passive GFP diffusion into the nucleus (Grebenok et al., 1997). Figure 2.8 shows that when pRTL-GFP was introduced into cells of onion epidermis by particle bombardment, GFP was targeted to both the nucleus and the cytoplasm. As the native GFP protein contained no NLS and was below the diffusion exclusion limit of 46kDa, the diffusion of GFP into the nucleus of the transiently transformed onion epidermal cells shown in Figure 2.8, was assumed to be passive.

GFP was fused to the AP2.76 and AP2.72 proteins which contain putative NLSs (Figure 2.6). The molecular mass of the fusion proteins (62kDa and 50kDa respectively) is sufficient to prevent passive GFP diffusion into the nucleus. Targeting of the fusion proteins to the nucleus would be expected only if the AP2.76 and AP2.72 portion contains a NLS. Figure 2.9 shows that the AP2.76-GFP fusion protein was localised exclusively to the nucleus of the cell, suggesting that AP2.76 contains an NLS.

On the other hand, the AP2.72-GFP fusion protein showed both cytoplasmic and nuclear localisation of GFP (Figure 2.10). Since the mass of the AP2.72-GFP fusion protein (50kDa) exceeded the size exclusion limit required to prevent passive diffusion into the nucleus (Gorlich and Mattaj, 1996; Grebenok et al., 1997), the observed nucleocytoplasmic partitioning of GFP was most likely controlled by sequences in AP2.72, suggesting that as well as functioning in the nucleus, the AP2.72 protein may also have a cytoplasmic function.

2.3.5 In vivo transient expression of GUS fusion constructs in onion cells

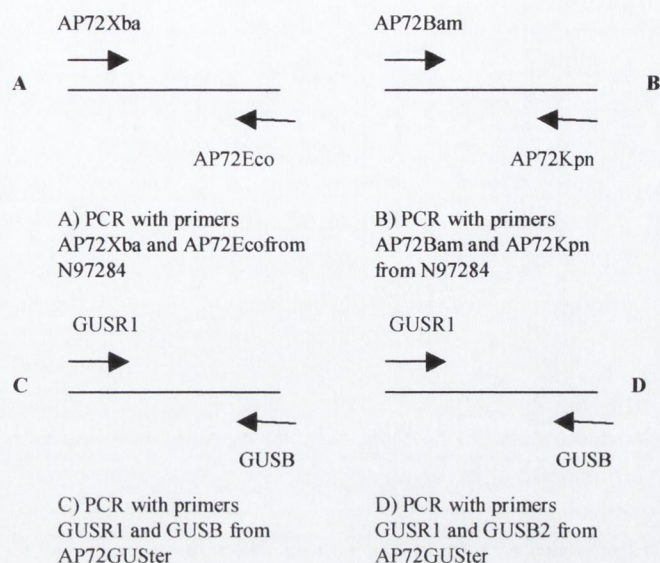
To examine the subcellular localisation of AP2.72, an AP2.72-GUS gene fusion and a GUS-AP2.72 fusion were constructed. Both gene fusions were used to transiently transform onion cells.

2.3.5.1 GUS fusion constructs

To generate the various GUS fusions, two versions of the GUS coding region were amplified from the vector pGUS1-3'NOS by PCR using *Pfu* polymerase: the first, containing a stop codon was amplified using primers engineered to contain the restriction sites *EcoRI*(5') and *BamHI*(3'); this PCR fragment was referred to as GUSRB1 and was used for construction of the AP2.72-GUS gene fusion. The second version, lacking a stop codon was amplified using primers engineered to contain the restriction sites *EcoRI*(5') and *BamHI*(3'); this PCR fragment was referred to as GUSRB2 and was used in the assembly of the GUS-AP2.72 gene fusion. Similarly, two versions of the entire coding region of AP2.72 were amplified by PCR using *Pfu* polymerase: the first, lacking a stop codon was amplified using primers engineered to contain the restriction sites *XbaI*(5') and *EcoRI*(3'); this PCR fragment was referred to as AP72XE and was used to assemble the AP2.72-GUS gene fusion. The second version containing a stop codon was amplified using primers engineered to contain the restriction sites *BamHI*(5') and *KpnI*(3'); this PCR fragment was referred to as AP72BK and was used to assemble the GUS-AP2.72 gene fusion.

The various GUS fusions were assembled by triple ligations which contained (i) one of the AP2.72 coding regions described above: AP72XE or AP72BK, digested with *XbaI*, *EcoRI* and *BamHI*, *KpnI* respectively, (ii) the appropriate GUS fragment: GUSRB or GUSRB2 digested with *EcoRI* and *BamHI*, and (iii) the pRTL22 vector digested with *XbaI* and *BamHI* (for assembly of the AP2.72-GUS gene fusion) or the pRTL22 vector digested with *EcoRI* and *KpnI* (for assembly of the GUS-AP2.72 gene fusion). The two resulting constructs were called pRTL-AP72-GUS (AP2.72-GUS: 6.3kb) and pRTL-GUS-AP72 (GUS-AP2.72: 6.3kb) (Figure 2.11). All constructs were verified by restriction enzyme analysis (Figure 2.12). Sequencing using the GUS2 and GUS3 primers (Figure 2.11) showed that AP2.72 was fused in-frame with GUS in the AP2.72-GUS gene fusion and that GUS was fused in-frame with AP2.72 in the GUS-AP2.72 gene fusion, respectively.

The pRTL-GUS-AP72, pRTL-AP72-GUS and pRTL-GUS constructs were introduced individually into onion epidermal cells by particle bombardment.



1) pRTL2AP72GUS (6.3kb) Triple ligation of PCR fragments A and C and pRTL22 at XbaI, EcoRI and BamHI restriction enzyme sites

2) pRTL2GUSAP72 (6.3kb) Triple ligation of PCR fragments B and D and pRTL22 at EcoRI, BamHI and KpnI restriction enzyme sites

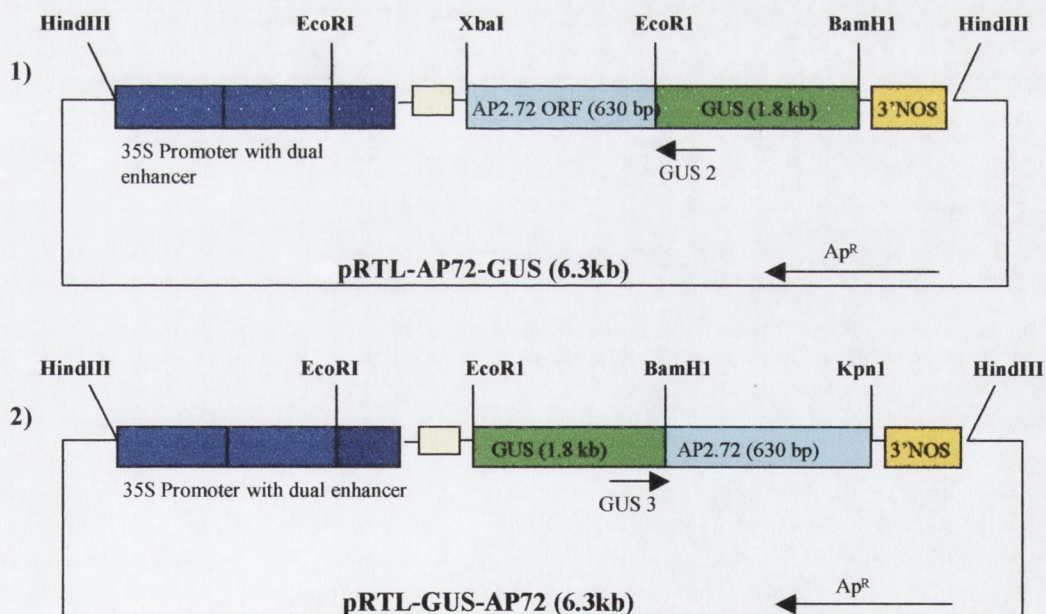


Figure 2.11. Construction of the (1) AP2.72-GUS and the (2) GUS-AP2.72 gene fusions.

The restriction sites shown in bold were used for cloning fragments into the corresponding vectors. Primers used for PCR amplifications and sequencing are indicated with black arrows.

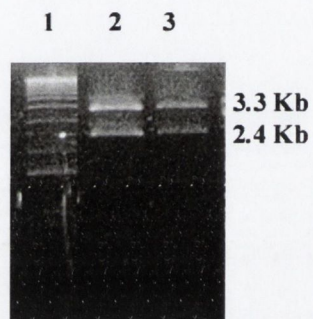


Figure 2.12. Verification of the structure of the AP2.72-GUS and GUS-AP2.72 gene fusions.

Restriction digests of transformant clones containing the pRTL-AP72-GUS vector (lane 2) and the pRTL-GUS-AP72 vector (lane 3) digested with *Xba*I/*Bam*HI and *Eco*RI/*Kpn*I respectively.

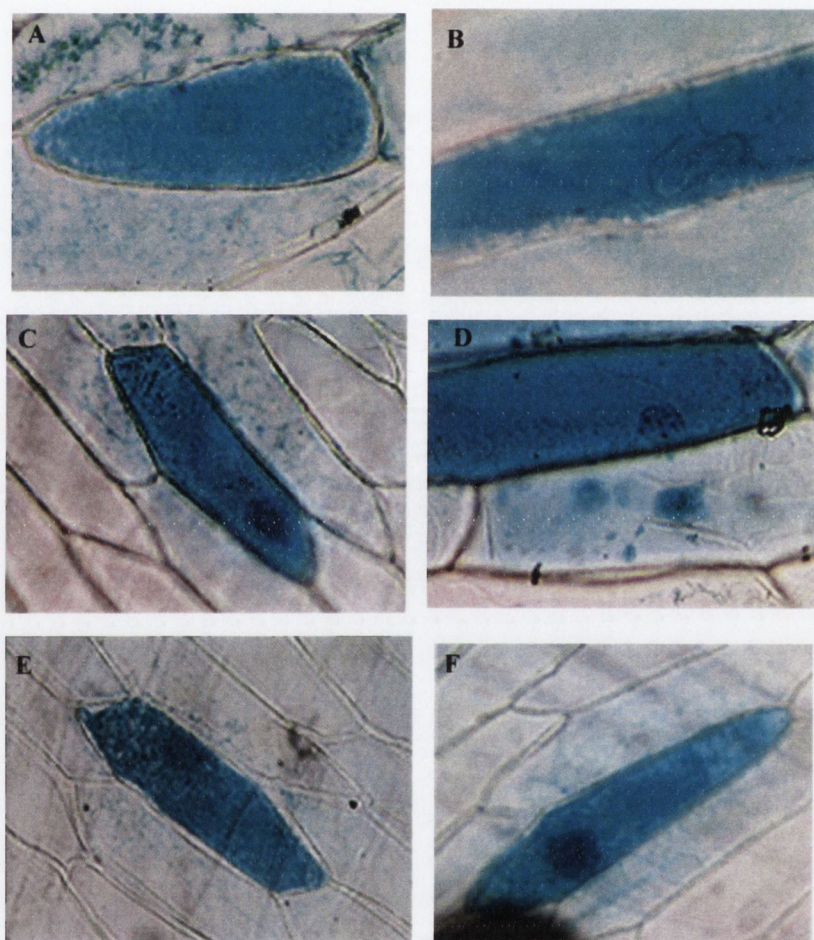


Figure 2.13. Subcellular localisation of AP2.72-GUS fusion protein activity.

Subcellular distribution of GUS activity within onion cells transiently transformed with a control non-fused *GUS* gene (A & B), an AP72-GUS gene fusion (C & D), and an GUS-AP72 gene fusion (E & F). All images were analysed using an Olympus BX40 (Optical Co.,LTD) light microscope and Olympus SZX9 (Optical Co., LTD). 100X (A,C,E&F)

2.3.5.2 GUS expression in bombarded onion cells

To examine the GUS activity of onion epidermal tissue transiently transformed with pRTL-AP72-GUS, pRTL-GUS-AP72 and pRTL-GUS constructs, a histochemical assay was employed because of its high sensitivity and precision. The assay was carried out as described in section 5.3.2.

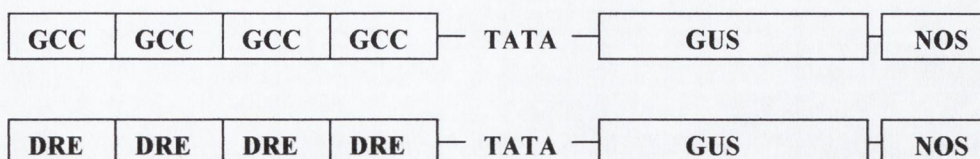
Since the 68kDa GUS protein, when expressed transiently in its native (nonfused) form, is located in the cytoplasm, and cannot enter the nucleus (Restrepo et al., 1990; von Arnim and Deng, 1994), histochemical or biochemical detection of intranuclear GUS implies the presence of an NLS.

Onion cells transiently transformed with pRTL-GUS (Figure 2.13), which encodes the nonfused GUS protein, served as a cytosolic control and showed as expected GUS activity localised predominantly in the cytoplasm. Figure 2.13 also shows that the GUS activity of the AP2.72-GUS and GUS-AP2.72 fusion proteins was localised to both the nucleus and the cytoplasm. The GUS staining observed in the nucleus of onion cells expressing the non-fused GUS protein was very faint compared with onion cells expressing the AP2.72-GUS and GUS-AP2.72 fusion proteins strongly suggesting that AP2.72 contains a NLS that is sufficient to target the fusion proteins into the nucleus at a significant level. However GUS activity was also observed in the cytoplasm of onion cells expressing the fusion proteins, indicating that some retention of AP2.72 in the cytosol. These results served to further confirm the observed nucleocytoplasmic partitioning of the AP2.72-GFP fusion protein observed in Figure 2.10.

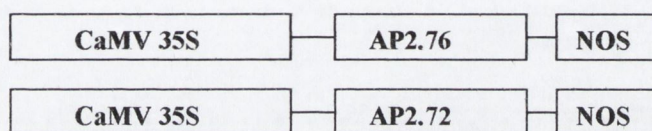
2.3.6 Investigating the DNA binding specificity of the AP2.76 and AP2.72 proteins using a transactivation strategy

It has been shown that the ERF and DRE/CRT proteins contain an AP2/ERF DNA binding domain that binds to the GCC- and DRE/CRT-box *cis*-elements respectively (Buttner and Singh, 1997; Stockinger et al., 1997; Sakuma et al., 2002). To determine if AP2.76 and AP2.72 can bind the DRE/CRT-box and GCC-box *cis*-elements and function as activators of DRE/CRT- and GCC-box-mediated transcription respectively, tobacco leaves (*Nicotiana tabacum* cv Petit Havana) were cotransformed with (i) a reporter plasmid containing the GUS

A) Reporter constructs



B) Effector constructs



C) Control constructs

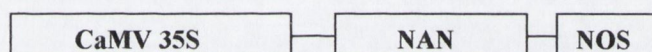


Figure 2.14. Schemes of the effector and reporter constructs used in the cotransformation experiments.

- A) The reporter constructs contain four copies of the DRE/CRT-box (4XDRE-GUS) or GCC-box (4XGCC-GUS) in tandem that were fused upstream to the CaMV 35S minimal TATA promoter, the coding region from the *GUS* gene, and the nopaline synthase (NOS) terminator in the pBT10 vector.
- B) The effector constructs contain a CaMV 35S promoter fused to the coding regions of AP2.76 (35S-AP2.76) and AP2.72 (35S-AP2.72) in the pROK219 vector.
- C) The internal control constructs (35S-NAN) contain a CaMV 35S promoter fused to the synthetic *NAN* gene in the pROK219 vector.

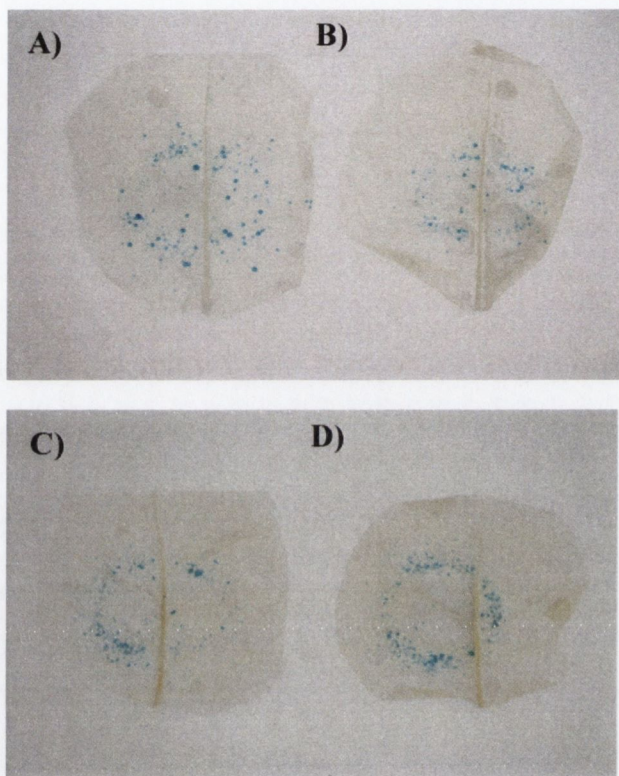


Figure 2.15. GUS expression patterns directed by synthetic promoters in biolistically bombarded tobacco leaves.

Expression patterns (GUS activity) in leaves co bombarded with
A) Reporter 4XDRE-GUS, effector 35S-AP76, and internal control 35S-NAN constructs.

B) Reporter 4XDRE-GUS, and internal control 35S-NAN constructs.

C) Reporter 4XGCC-GUS, effector 35S-AP72, and control 35S-NAN constructs.

D) Reporter 4XGCC-GUS, and internal control 35S-NAN constructs.

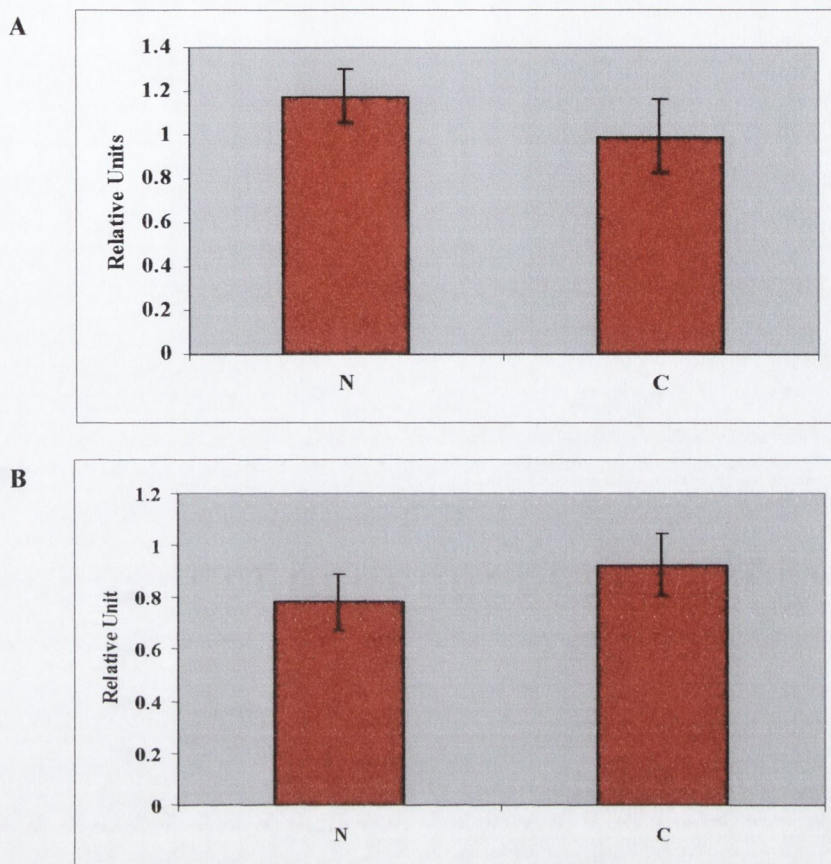


Figure 2.16. Transactivation of DRE box and GCC box mediated transcription by AP2.76 and AP2.72 respectively.

- A)** Induction of GUS activity relative to NAN activity in tobacco leaves cotransformed with a mixture of plasmids containing reporter constructs (4XDRE-GUS), effector construct (35S-AP2.76), and an internal control (35S-NAN) construct (N). For controls (C) tobacco leaves were cotransformed with plasmids containing the reporter and internal control constructs only. Each bar represents data derived from six individual bombardments. Error bars indicating the standard deviations for the relative GUS activity of the six leaves bombarded are shown
- B)** Induction of GUS activity relative to NAN activity in tobacco leaves cotransformed with a mixture of plasmids containing reporter constructs (4XGCC-GUS), effector construct (35S-AP2.72), and an internal control (35S-NAN) construct (N). For controls (C) tobacco leaves were cotransformed with plasmids containing reporter and internal control constructs only. Each bar represents data derived from six individual bombardments. Error bars indicating the standard deviations for the relative GUS activity of the six leaves bombarded are shown

gene and an upstream synthetic promoter containing four copies of either the DRE-box *cis*-element (4XDRE-GUS) or the GCC-box *cis*-element (4XGCC-GUS) and (ii) an effector plasmid expressing AP2.76 (pROK219-76; 35S-AP2.76) or AP2.72 (pROK219-72; 35S-AP2.72) driven by the CaMV35S promoter, along with (iii) an internal control plasmid expressing NAN driven by the CaMV 35S promoter (pROK219-NAN; 35S-NAN) (Figure 2.14). NAN was used as an internal control to standardise the transformation efficiency. Co-transforming the tobacco leaves with (i) the reporter plasmids (4XDRE-GUS or 4XGCC-GUS) and with the (iii) internal control plasmids (pROK219-NAN) but without an effector plasmid served as a negative control. To ensure reproducibility and to account for variations between individual transformations, NAN was employed as a second reporter gene whose expression would not be affected by the transcription factors under investigation (Kirby and Kavanagh, 2002).

The GCC-box *cis*-element and the DRE/CRT-box *cis*-elements alone can direct pathogen-inducible expression and abiotic inducible expression respectively *in planta* when removed from their native promoter context (Rushton et al., 2002). The reporter constructs 4XDRE-GUS and 4XGCC-GUS were designed to contain four copies of the DRE-box and GCC-box respectively to ensure levels of transcription high enough to permit detection of GUS activity by histochemical staining and to respond to a number of abiotic and biotic stresses (Rushton et al., 2002).

In the experiments reported here, both vectors even in the absence of the effector plasmid, were found to confer high background levels of GUS activity during transient transformation experiments (Figure 2.15 & 2.16). This high background level of GUS activity in the controls made it impossible to determine if the AP2.76 and AP2.72 effector plasmids could transactivate the reporter constructs 4XDRE-GUS and 4XGCC-GUS respectively.

2.4 Discussion

2.4.1 Sequence analysis of *AP2.76* and *AP2.72*

Sequence analysis of *AP2.76* and *AP2.72* revealed that each encoded a conserved DNA binding domain of 58 amino acids previously identified in a large family of DNA binding proteins known as AP2/ERF (Riechmann and Meyerowitz, 1998; Sakuma et al., 2002). The analysis further revealed that both *AP2.76* and *AP2.72* belong to the ERF/AP2 subfamily. The deduced amino acid sequences of *AP2.76* and *AP2.72* shared no significant sequence identity with other members of this subfamily, except within the conserved DNA binding domain. However both *AP2.76* and *AP2.72* contained an acidic region that might act as a transcriptional activation domain (Hahn, 1993), and a putative nuclear localisation signal. These data suggest that both *AP2.76* and *AP2.72* cDNAs each encode transcription factors. Comparisons of the amino acid sequences of the DNA binding domains of *AP2.76* and *AP2.72* with those of other members of the ERF/AP2 subfamily revealed on the basis of conserved amino acid residues at positions 14 (valine vs. alanine) and 19 (glutamic acid vs. aspartic acid or leucine) that *AP2.76* belongs to the DRE/CRT subgroup while *AP2.72* belongs to the ERF subgroup originally identified by Sakuma et al., (2002).

Several members of the DRE/CRT subgroup have been shown to specifically bind to the DRE/CRT-box *cis*-element found in the promoter regions of many dehydration and low-temperature-stress-inducible target genes such as *rd29A*, *kin1*, *cor6.6* and *rd17* (Yamaguchi-Shinozaki and Shinozaki, 1994; Wang et al., 1995; Seki et al., 2001). All transcription factors analysed in this group contained the conserved V14 and E19 residues with the exception of *AP2.76* and *RAP2.4* in which E19 is replaced by leucine (L). V14 and E19 have been shown to be important for recognition of the DRE/CRT-box *cis*-element as single amino acid substitutions at V14 or E19 reduced the DNA-binding activity and changed the DNA-binding specificity of several members of the DRE/CRT subgroup with a V14 substitution having a more drastic effect on the DNA-binding than an E19 substitution (Sakuma et al., 2002). Furthermore, a DREB1A (DRE Binding 1A) homologue in *Oryza sativa*, OsDREB1A, which contains the conserved V at position 14 but lacks E at position 19, nevertheless binds specifically to the DRE/CRT *cis*-element in the *rd29A* promoter of *Arabidopsis*. Therefore because V14 is more important than E19 for determining the DNA-binding specificity of the

AP2/ERF domain in members of the DRE/CRT subgroup, and because AP2.76 showed conservation of V14 (but not E19), it was hypothesized that AP2.76 might bind to the DRE/CRT-box *cis*-element and regulate the expression of target genes in response to abiotic stresses such as dehydration and low temperatures.

Several members of the ERF subgroup have been shown to interact specifically with the GCC sequence and to activate transcription of genes containing the GCC-box *cis*-element (Park et al., 2001; Gu et al., 2002), found in the promoter regions of many pathogenesis-related (PR) genes such as those encoding the acidic proteins PR1, PR2 and PR5, the basic PR proteins (PR3 and PR5) and plant defensins (PDF1.2) (Ohme-Takagi and Shinshi, 1995; Penninckx et al., 1996). The AP2/ERF DNA binding domains of proteins belonging to the ERF subgroup contain alanine at position 14 (A14) and aspartic acid at position 19 (D19) and these residues are thought to be important for recognition of the GCC-box *cis*-element (Sakuma et al., 2002). AP2.72 showed strong similarity with other members of the ERF subfamily and contained the conserved A14 and D19 residues suggesting that AP2.72 might bind to the GCC-box *cis*-element and play a role in mediating the plant's response to biotic stresses by regulating PR gene expression. The DNA binding domains of AP2.76 and AP2.72 showed high levels of similarity with those of RAP2.4 and RAP2.6, respectively. Both these genes have been shown to be induced by mechanical wounding (Cheong et al., 2002) and also by infection with pathogens (Chen et al., 2002) suggesting that AP2.76 and AP2.72 might be similarly induced.

Some members of the ERF/AP2 subfamily such as *Tsi1* (for *Tobacco stress-induced gene1*), a member of the ERF subgroup could bind specifically to the GCC and the DRE sequences, although the binding activity to the former was stronger than to the latter, and over-expression of the *Tsi1* gene in *Tobacco* resulted in improved tolerance to salt and pathogens (Park et al., 2001). Although AP2.76 was shown to belong to the DRE subfamily of proteins and predicted to bind the DRE/CRT sequence and AP2.72 was shown to belong to the ERF subfamily and predicted to bind the GCC sequence, it is possible that AP2.76 and AP2.72 also bind the GCC and DRE/CRT sequences respectively and like the *Tsi1* gene be involved in responses to both abiotic and biotic stress. Determining the binding specificity of both AP2.76 and AP2.72 proteins will give valuable insights into the signaling transduction pathways that AP2.76 and AP2.72 are involved in.

2.4.2 Nuclear Localisation of AP2.76 and AP2.72

Proteins destined for the nucleus are translocated from the cytoplasm where they are synthesized. Translocation into the nucleus, unlike other organelles, does not require traversal of lipid bilayers. Rather, translocation involves active passage through nuclear pore structures spanning the nuclear envelope. The pores are composed of two stacked coaxial rings, each composed of eight subassemblies arranged symmetrically around a central plug (Hicks and Raikhel, 1995; Gorlich and Mattaj, 1996; Hurt, 1996). Although the diffusion exclusion limit of the pore is relatively high, permitting passage of macromolecules of up to 43kDa, transport of physiologically relevant proteins is dependent on cytosolic and pore-associated receptors that interact with one or more NLS(s), short sequences that are typically rich in basic amino acids, within the translocated protein (Silver, 1991; Gorlich and Mattaj, 1996).

AP2.72 and AP2.76 both contained NLS-like stretches of basic amino acids (PRRRH and KKKK respectively). The AP2.76-GFP fusion protein showed almost complete accumulation within the nucleus of onion epidermal cells. This result is consistent with the observation that the AP2.76 sequence encodes a putative transcriptional regulator. AP2.72 accumulated in both the nucleus and the cytoplasm suggesting possible nucleo-cytoplasmic partitioning of the protein. Both AP2.72 and AP2.76 contain several putative phosphorylation sites. Post-translational modification by phosphorylation has been shown to activate the nuclear import of transcription factors and to stimulate or repress their DNA-binding affinity or their activation potential (Karin and Hunter, 1995). For example, phosphorylation of the maize abscisic acid-responsive Rab17 by protein kinase CK2 is a key step in its nuclear targeting (Jensen et al., 1998). In addition, the Pto kinase is known to interact with the resistance elicitor of *Pseudomonas syringae*, AvrPto, and subsequently phosphorylate several Pti transcription factors belonging to the ERF/AP2 subfamily. This results in an enhanced ability of the Pti proteins to activate the expression of GCC-box-containing PR genes in tomato (Gu et al., 2000; Gu et al., 2002; Wu et al., 2002). Furthermore, several reports have suggested that the regulation of nuclear import in higher plants is complex and NLS efficiency can be tissue-specific (Citovsky et al., 1994), or environmentally-regulated (von Arnim and Deng, 1994), and is likely mediated by protein-protein interactions in the nuclear and cytoplasmic compartments (Sanderfoot and Lazarowitz, 1995). It will be important to investigate whether the nucleo-cytoplasmic partitioning of AP2.72 observed here in onion

(monocotyledonous) epidermal cells also occurs in *Arabidopsis* cells and if so, whether it is mediated by environmental factors. These more detailed studies should ideally be based on immunodetection of the AP2.72 protein.

2.4.3 Investigating the DNA binding specificity of AP2.76 and AP2.72

The synthetic promoters 4XGCC and 4XDRE direct transcription from a minimal CaMV35S promoter in response to a number of biotic and abiotic stresses (Rushton et al., 2002). The preparation of tobacco leaves for bombardment and the bombardment procedure itself exposes the plant tissue to significant levels of abiotic stress (e.g. wounding) that may have activated GCC-box and DRE-box mediated transcription in the absence of AP2.76 and AP2.72. Therefore the strong background levels of DRE- and GCC-box mediated transcription that caused high level expression of *GUS* relative to *NAN* in the absence of the AP2.76 or AP2.72 effector plasmids was probably due to the abiotic stresses imposed by the biolistic bombardment procedure or/and preparation of the tissue. This high background level of *GUS* expression made it impossible to determine if AP2.76 and AP2.72 could specifically activate DRE/CRT or GCC-box mediated transcription, respectively.

Several studies have successfully determined the DNA binding specificity of various ERF/AP2 proteins using a gel band-shift assay (Fujimoto et al., 2000; Park et al., 2001; Sakuma et al., 2002), a method that has been used widely in the study of sequence-specific DNA-binding proteins. The assay is based on the observation that protein-DNA complexes migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. Employing a gel band-shift assay to determine the DNA binding preferences of recombinant AP2.76 and AP2.72 proteins would eliminate competition from other members of the ERF/AP2 family of proteins that may also induce DRE/CRT and GCC-box mediated transcription in *in vivo* experiments.

Chapter 3

Expression analysis of *AP2.72* and *AP2.76*

3.1 Introduction

Plants respond to adverse environmental conditions and pathogen attack by synthesising a large number of stress- and pathogenesis-related proteins that increase their ability to tolerate stress and resist pathogens, respectively (Skriver and Mundy, 1990; Reymond and Farmer, 1998). There is evidence for commonalities between plant responses to pathogens (referred to as defense responses) and environmental stresses (referred to as stress responses) (Mahalingam et al., 2003). Despite similarities, however, a plant's response to each environmental challenge is unique and tailored to increasing the plant's ability to survive the inciting stress (Reymond et al., 2000; Xiong and Zhu, 2001; Mahalingam et al., 2003).

3.1.1 Defense responses

The development of SAR results in the transcriptional activation of many pathogenesis-related (PR) genes (eg., *PR1*, *PR2* and *PR5*) which enhance resistance to a broad spectrum of virulent pathogens (Ward et al., 1991; Uknes et al., 1992; Ryals et al., 1996). SAR induction requires the signalling molecule SA (SA), which accumulates in plants prior to the onset of SAR and several studies have demonstrated that SA signalling is mediated by an ankyrin repeat protein, NPR1 (Cao et al., 1994; Cao et al., 1997; Li et al., 1999).

Some defense responses are activated by signal transduction networks that require JA and ET as signalling molecules and are distinct from the classic SA-mediated SAR pathway (Pieterse and van Loon, 1999; Glazebrook, 2001; Kunkel and Brooks, 2002). JA and ET co-

regulate a subset of PR genes in *Arabidopsis* that encode the antimicrobial proteins PR3 (a basic chitinase) and PR4 (a hevein-like protein) (Thomma et al., 1998) along with plant defensin and thionin (*PDF1.2* and *Thi2.1*) genes (Penninckx et al., 1996; Epple et al., 1997). This regulation is clearly distinct from that of the SA-dependent PR genes such as *PR-1*, *PR-2* and *PR-5*, because it does not depend on NPR1 (Penninckx et al., 1998; Thomma et al., 2001). There also appears to be considerable cross-talk between these signal transduction networks, with at least some SA-dependent responses limited by JA/ET-dependent responses and *vice versa* (Doares et al., 1995b; Doares et al., 1995a; Spoel et al., 2003).

Members of the ERF/AP2 subfamily are known to play important roles in plant defense responses (Riechmann and Meyerowitz, 1998; Park et al., 2001; Gu et al., 2002). The expression of many ERF/AP2 genes is induced by pathogen infection, ET, JA and SA. Several members of the ERF/AP2 subfamily bind the GCC box (TAAGAGCCGCC) that is present in the promoters of many PR genes (Ohme-Takagi and Shinshi, 1995; Fujimoto et al., 2000; Ohme-Takagi et al., 2000; Gu et al., 2002) and when over-expressed constitutively in *Arabidopsis* plants they induce the expression of downstream genes under non-stress conditions and confer pathogen resistance to the transgenic plants (Park et al., 2001; Gu et al., 2002).

3.1.2 Environmental stress responses

Plant growth is greatly affected by environmental abiotic stresses, such as drought, high salinity and low temperature. Plants respond to these stresses at molecular and cellular levels as well as the physiological level. Expression of a variety of genes is induced by these stresses (Shinozaki and Yamaguchi-Shinozaki, 1996), the products of which are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction during the stress response (Shinozaki and Yamaguchi-Shinozaki, 1997). Promoter analyses of drought- and/or cold-inducible genes have identified at least four independent regulatory systems for gene expression (Shinozaki and Yamaguchi-Shinozaki, 2000) two of which are ABA-dependent and two ABA-independent.

Crosstalk between these regulatory systems has been suggested by genetic and molecular analyses e.g. genomic analyses of stress-inducible genes using microarrays have recently revealed crosstalk in stress-responsive gene expression (Seki et al., 2001; Kreps et al., 2002;

Seki et al., 2002). Most dehydration-inducible genes are also induced by high-salinity stress, and many dehydration-inducible genes are also induced by ABA. However, only 10% of drought-inducible genes are also cold-inducible. In fact, although ABA is involved in osmotic-stress-responsive gene expression it does not play an important role in the low-temperature stress response (Shinozaki et al., 2003). Interactions between different transcriptional machineries facilitate crosstalk between the different stress-signalling pathways.

3.1.3 Investigating the role of *AP2.76* and *AP2.72* in defense and environmental stress responses

This chapter investigates *AP2.76* and *AP2.72* expression in response to various potentially inducing stimuli (chemical and environmental) using a northern blotting approach. The response to the exogenous signal molecules SA, ACC and JA was examined to elucidate the potential involvement of *AP2.76* and *AP2.72* in plant defense responses. The putative involvement of both genes in environmental stress responses, specifically dehydration, high salinity, low-temperature, wounding and ABA was also investigated.

In addition, having access to the genomic and cDNA sequence of *AP2.76* and *AP2.72* enabled us to undertake a preliminary investigation of promoter-mediated control of expression of these two genes during the *Arabidopsis* life-cycle. This was achieved by generating transgenic *Arabidopsis* plants engineered to express gene fusions comprising *AP2.76* and *AP2.72* promoter sequences fused with the *GUS* reporter gene (Jefferson et al., 1987).

Finally, putative *cis*-acting regulatory elements within the promoter regions of *AP2.76* and *AP2.72* were identified through interrogation of the PLACE (The Plants *Cis*-acting Regulatory DNA Elements) database (<http://www.dna.affrc.go.jp/htdocs/PLACE>).

3.2 Materials and Methods

3.2.1 DNA manipulations

General materials and DNA manipulations have been described in Chapter 5.

3.2.2 Plasmids

AV441496 (Asamizu et al., 2000), and see section 2.2.2

N97284 (Newman et al., 1994), and see section 2.2.2

pGUS1, containing the *E.coli* beta-glucuronide gene (*uidA/GUS*) in pUC19 vector (Figure 3.12).

pGUS1ter, containing the nopaline synthase terminator (3'NOS) in pGUS1 (Figure 3.13), which was available from the laboratory.

pTAK1, also known as pBI101; (Jefferson et al., 1987), and contains a polylinker cloning site upstream of *GUS*, followed by the nopaline synthase terminator (3'NOS) in pBIN19.

3.2.3 Oligonucleotide primers

A) To generate the DIG-AP76 probe for northern blot analysis

AP76P1: 5'CGAGTGAGCTACTCGGACTCGGAGAT3'

AP76P2: 5'CTTCCCAAATCAGCTCTGGATCGAAC3'

B) To generate the DIG-AP72 probe for northern blot analysis

AL163491A: 5'CGGGATCCAAGGCCCTACTACCACCAC3'

AL163491B: 5'GGGGTACCTTCTCTTCTTCTTACGCTG3'

C) To amplify the AP2.76 promoter region

AP76X: 5'GCTCTAGATGAGAGACGAATGGAGAGAG3'

AC66689D: 5'CGGGATCCCAGAGATGCTCTGATTTCTG3'

D) To amplify the AP2.72 promoter region

AL163491A: 5'GCGTCGACGTCGTCTTAAGCATCGATTC3'

AL163491B: 5'CGGGAT**TC**CTGTCGGATTCTCTATGAC3'

C) For sequencing constructs

GUS3: 5' TGAACAGGTATGGAATTT**CG**CCGA 3'

GUS2: 5' CTGCATCGGCGAACTGATCGTTAA**AA**ACTGC3'

D) For PCR analysis of plants transformed with pTAK1-AP76 vector

AP76GUS:5'CCTTTCTGGTTTGT**TTT**TCCTTGTCTATCTG3'

GUSR:5'TTGCCCGGCTTTCTTGTAA**CG**C3'

E) For PCR analysis of plants transformed with pTAK1-AP72 vector

AP72GUS:5'CGGTCCTACCCCGCACCA**TT**CCAAAGATT3'

GUSR:5'TTGCCCGGCTTTCTTGTAA**CG**C3'

Bold bases were added in order to create restriction enzyme sites.

3.2.4 Plant material

3.2.4.1 ABA-deficient mutants

aba3-1: this mutation reduces the level of endogenous ABA (ABA) biosynthesis during various stages of plant and seed development by impairing the conversion of ABA-aldehyde to ABA (Leon-Kloosterziel et al., 1996).

aba2-4: this mutation reduces the levels of endogenous ABA by blocking the conversion of xanthoxin to ABA-aldehyde. Mutants are slightly darker green than wild-type plants (Leon-Kloosterziel et al., 1996).

aba1-5: this mutation impairs ABA biosynthesis by blocking the conversion of the precursor zeaxanthin to the epoxy-carotenoids violaxanthin and neoxanthin. Mutants are slightly darker green than wild-type plants (Xiong et al., 2002b).

All three mutants show an increased leaf transpiration rate, which leads to a wilted phenotype with reduced growth and reduced plant size and vigour especially under low relative humidity and water stress; the wilted phenotype can be restored to normal by spraying plants with an

ABA solution. All three mutants show reduced seed dormancy. Fresh seeds germinate at high frequency and show a decreased sensitivity to the presence of GA biosynthesis inhibitors such as paclobutrazol. *Arabidopsis* seeds (ecotype Columbia) homozygous for the *aba1-5*, *aba2-4* and *aba3-1* mutations were obtained from NASC and were germinated on MS medium and maintained as described in section 5.1.4.

3.2.4.2 NPR1 mutants

npr1-1 This mutation converts a highly conserved histidine residue in the third ankyrin-repeat consensus to a tyrosine, and causes complete loss-of-function of the NPR protein. Consequently plants show little expression of PR genes, are unable to respond to various SAR-inducing treatments and show increased susceptibility to infection by various pathogens (Cao et al., 1997).

npr1-2 This mutation converts a cysteine residue to a tyrosine in the NPR amino terminal region. This mutation results in partial loss-of-function and some inducible PR1 expression is observed in the mutant. However, the mutation reduces the level of expression of pathogenesis-related (PR) genes in response to SA and enhances susceptibility to pathogen infections (Cao et al., 1997).

Arabidopsis seeds (ecotype Columbia) homozygous for the *npr1-1* and *npr1-2* mutations were obtained from NASC and were germinated on MS medium and maintained as described in section 5.1.4.

3.2.5 Northern blot analysis of AP2.76 and AP2.72 expression

Organ-specific expression patterns of AP2.76 and AP2.72 were examined in *Arabidopsis thaliana* ecotype Columbia (wild-type). Total RNA was extracted from whole seedlings and the roots, leaves, stems, flowers and siliques of mature plants grown on soil as described in section 5.1.4.2. An AP2.76-specific double stranded probe called DIG-AP76 (synthesised using the primers AP76P1 (1µM) and AP76P2 (1µM) and using the plasmid AV441496 as a template) and an AP2.72-specific single stranded probe called DIG-AP72

(synthesised using the primers AL163491A (0.1 μ M) and AL163491B (1 μ M) and using the plasmid N97284 as a template) were generated by PCR using the digoxigen-labelled (DIG) Probe synthesis kit (Roche Molecular Biochemicals) as outlined in section 5.2.10.2. Northern blot analysis was carried out as described in section 5.2.15. The DIG-AP76 probe encoded the C-terminal region (404bp), downstream of the AP2/ERF domain of AP2.76. The DIG-AP2.72 probe was a single stranded DIG labelled AP2.72 cDNA fragment encoding the C-terminal region (307 bp) downstream of the AP2/ERF domain of AP2.72. The single strand probe was used to avoid unspecific hybridisation.

Expression patterns of both AP2.76 and AP2.72 in response to various chemical treatments ABA, SA, ACC, JA and NaCl and various abiotic stress treatments (dehydration, cold and wounding) for varying lengths of time were investigated by northern blot analysis (section 5.2.15) of total RNA isolated from three week old *Arabidopsis* seedlings germinated on MS medium.

The expression pattern of AP2.76 in response to wounding, dehydration and NaCl treatment was examined in three ABA-deficient mutants' *aba1-5*, *aba2-4* and *aba3-1* that were germinated on MS medium. The expression pattern of AP2.72 in response to SA treatment was examined in two *NPRI* mutants' *npr1-1* and *npr1-2* that were germinated on MS medium.

3.2.5.1 Chemical treatments

For treatment with SA, ACC and NaCl, three to four week old *Arabidopsis* seedlings growing on MS medium were sprayed with 2mM SA, 5 μ M ACC (a natural precursor of ET, used to increase ET levels in plant tissues (Wada et al., 1997)), and 200mM NaCl. Water was used as a control and RNA was isolated at various times. JA treatment was performed by spraying the plants with 100 μ M JA dissolved in 0.01% ethanol (control was 0.01% ethanol). ABA treatment was performed by spraying the plants with 100 μ M ABA and RNA was isolated at various times after treatment.

3.2.5.2 Dehydration, cold and wounding treatments

Three to four week old *Arabidopsis* seedlings growing on MS medium were dehydrated in Petri dishes on Whatman 3MM paper at 60% relative humidity and 22°C under dim light, or for cold treatment were grown at 4°C under dim light. For wounding experiments, rosette leaves of seedlings were damaged by crushing with a pair of fine tweezers and RNA was isolated at various times after treatment.

3.2.5.3 Densitometry of northern blots

Relative amounts of mRNA transcripts in northern blots were determined by scanning the image of blots as a TIFF file and relative signal intensities were determined using UVP GelWorks for Windows software.

3.2.6 Transformation of *Arabidopsis* plants

The promoter-GUS fusion vectors pTAK1-AP76 and pTAK1-AP72 were electroporated into *Agrobacterium tumefaciens* strain AGL1. Cells were plated on YM agar plates containing amp (100µg/ml) and km (50µg/ml). The presence of each vector in *Agrobacterium* was confirmed by plasmid isolation and restriction digest analysis. The bacterial strains of interest were called AGL1/ pTAK1.AP76 and AGL1/ pTAK1.AP72, and were used to transform *Arabidopsis thaliana* ecotype Columbia (germinated on soil and maintained in greenhouse) by the “Floral Dip” method (section 5.2.11).

3.2.7 PCR analysis and histochemical analysis

Putative transgenic plants were selected for PCR and histochemical analysis. For plants putatively transformed with (i) the AP76-GUS fusion, PCR amplification was performed using the primers AP76GUS and GUSR and (ii) the AP72-GUS fusion, PCR amplification was performed using the primers AP72GUS and GUSR.

Whole siliques, flowers, leaves, roots of plants grown in the greenhouse and seedlings germinated on MS+ containing km (50µg/ml) were used for histochemical analysis of *GUS* expression. The tissues were immersed in GUS staining buffer (50mM NaHPO₄ pH6.8, 1mM 5-bromo-4-chloro-3-indolyl-glucuronide (Kirby and Kavanagh, 2002) (Research Organics) followed by vacuum-infiltration for 20 minutes and incubation overnight at 37°C. The chlorophyll was removed with absolute ethanol or cleared in 20% glycerol/20% lactic acid (for roots) and the samples were stored in 20% ethanol at 4°C. The GUS expression patterns were observed using an Olympus BX40 (Optical CO, LTD) light microscope and an Olympus SZX9 (Optical Co, LTD) stereomicroscope. Pictures were taken with an Olympus DP 10 digital camera (Optical Co, LTD).

3.2.8 Sequence analysis

The PLACE (plant cis-acting elements) database (Higo et al., 1999) was used to identify candidate cis-acting sequence elements in the promoter regions of *AP2.72* and *AP2.76*.

3.3 Results

3.3.1 Organ-specific expression of *AP2.76* and *AP2.72*

Organ-specific expression of *AP2.76* and *AP2.72* was examined in *Arabidopsis* ecotype Columbia wild-type plants by northern blot analysis of total RNA extracted from seedlings, flowers, siliques, leaves, stems, and roots and using the DIG-*AP2.76* and DIG-*AP2.72* probes respectively.

Northern blot analysis using the DIG-*AP2.76* probe, detected a single band of approximately 1.3kb in wild-type plants, consistent with the predicted length of the cDNA (Figure 3.1). *AP2.76* transcripts were detected at a low level in flowers and siliques (Figure 3.1; lane 2 & 3), and not at all in roots, stems, or leaves (Figure 3.1).

Northern blot analysis using the DIG-*AP2.72* probe, detected a single band of approximately 900bp, consistent with the predicted length of the cDNA (Figure 3.1). *AP2.72* was expressed in roots and flowers (Figure 3.1; lanes 1 & 2, respectively), and not at all in stems, siliques or leaves (Figure 3.1; lanes 3, 4 & 5 respectively).

3.3.2 *AP2.72* expression in response to various mediators of abiotic stress

AP2.72 expression was analysed following treatment with various chemical mediators of abiotic stress. As shown in Figure 3.2, expression was strongly induced eight hours after treatment with SA, JA, ABA, NaCl, and ACC. When control plants were sprayed with water, very low level of accumulation of *AP2.72* mRNA was detected. A densitometry analysis of relative transcript accumulation for the various stress treatments as compared to the control showed that *AP2.72* was most strongly induced (at least 10-fold) after treatment with SA, and was significantly induced by JA, ABA and ACC approximately 8 hours after treatment. However, only low-level accumulation of *AP2.72* transcripts was detected after treatment with NaCl.

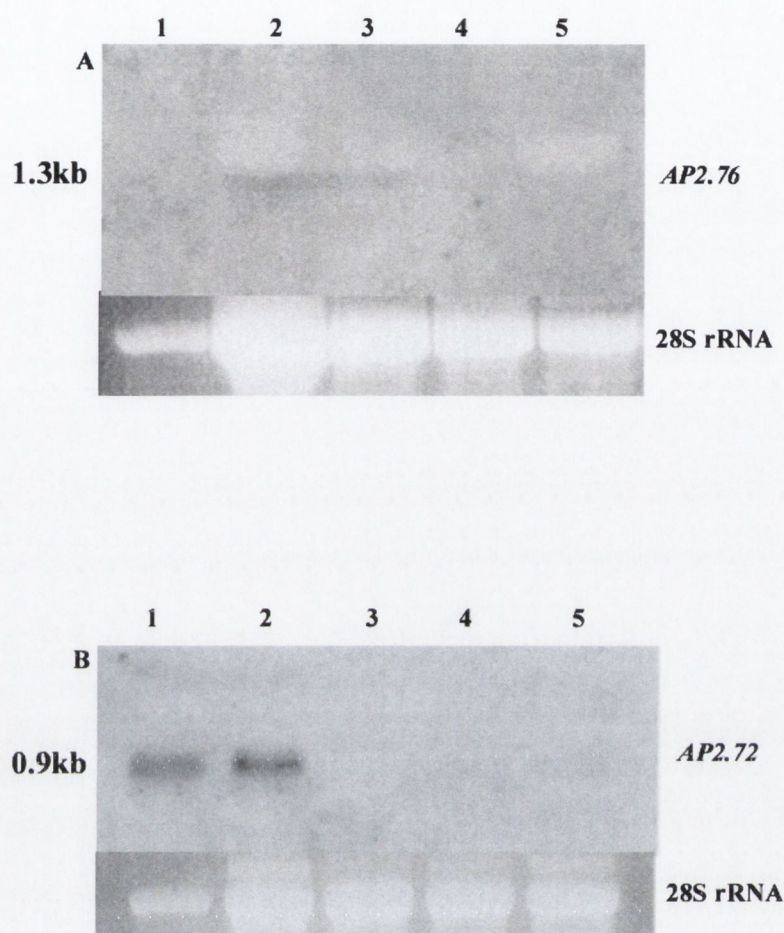


Figure 3.1. Organ-specific expression of *AP2.76* and *AP2.72* in *Arabidopsis*.

Total RNA was isolated from roots (lane 1), flowers (lane 2), siliques (lane 3), stems (lane 4) and leaves (lane 5) of mature plants. Hybridisation was performed using a A) *AP2.76* gene specific probe (DIG-AP76) and B) *AP2.72* gene specific probe, as indicated on right. rRNA is shown as a loading control.

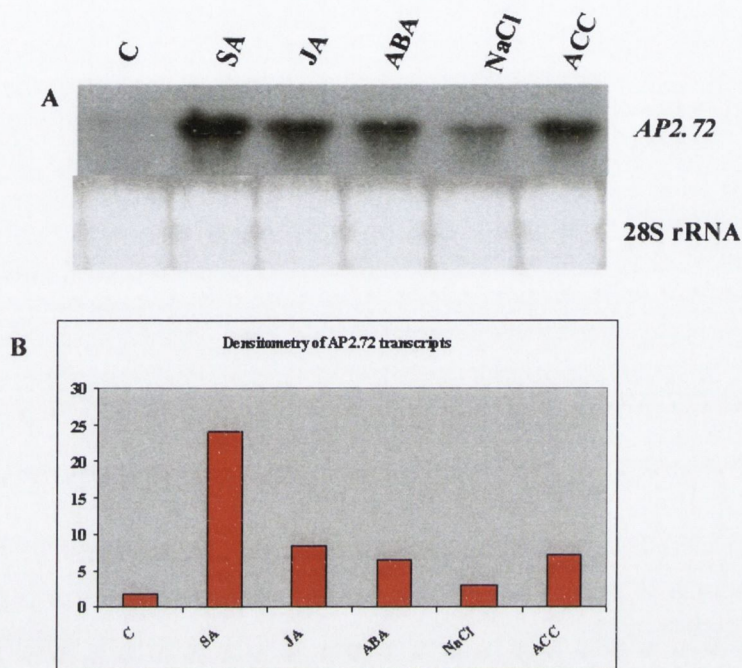


Figure 3.2. Induction of the AP2.72 gene in response to various abiotic stresses.

A) Total RNA was isolated from three to four week old *Arabidopsis* plants that had been treated for 8 hours with 2mM SA; 100μM JA; 100μM ABA; 200mM NaCl and using water as the control (C). RNA gel blots were hybridised with an AP2.72 gene specific probe (DIG-AP72) as indicated at right. rRNA is shown as a loading control.

B) AP2.72 relative transcript accumulation as determined by densitometry.

3.3.3 Time-course of *AP2.76* and *AP2.72* induction by SA and JA

The time-dependence of *AP2.76* and *AP2.72* expression in response to SA and JA treatments was investigated by northern blot analysis. As shown in Figure 3.3, while *AP2.76* mRNA showed significant accumulation (at least 4-fold) following a 1 hour treatment with JA, little or no mRNA was detected following 7, 10 and 24 hours treatment. Furthermore, *AP2.76* mRNA did not accumulate in response to treatment with SA for any of the time-points investigated.

AP2.72 mRNA showed high-level accumulation (approximately 10-fold) following 5 hours treatment with SA which declined after 10 and 24 hours treatment (Figure 3.4). A similar but weaker induction pattern was observed following JA treatment (approximately 3-fold induction), except that *AP2.72* mRNA accumulation peaked between 7 and 10 hours and declined slightly following 24 hours treatment.

When seedlings were treated with 0.01% ethanol or water as controls for JA and SA treatment respectively, no accumulation of *AP2.76* or *AP2.72* mRNA was apparent.

These results indicate that *AP2.76* expression was rapidly but transiently induced by JA treatment but was not induced by SA. In contrast, although expression of *AP2.72* was activated more slowly after both treatments, expression persisted for at least 24 hours.

3.3.4 Time-course of *AP2.76* and *AP2.72* induction by ABA and ACC

As shown in Figure 3.5, *AP2.76* mRNA accumulated to similar high levels (approximately 10-fold induction) following a 1 hour and 7 hour treatment with ABA but declined to almost undetectable levels following treatment for 10 and 24 hours. Figure 3.5 also shows that significant levels of *AP2.76* mRNA accumulated after a 1 hour treatment with ACC but was undetectable following treatment for 7, 10 and 24 hours.

AP2.72 mRNA accumulated at a low level following ABA treatment reaching a maximum at approximately 7 hours (Figure 3.6). ACC caused a similar low-level induction of *AP2.72* expression that peaked after 10 hours of treatment.

Control seedlings for the ABA and ACC treatments, showed no significant accumulation of either *AP2.76* or *AP2.72* mRNA.

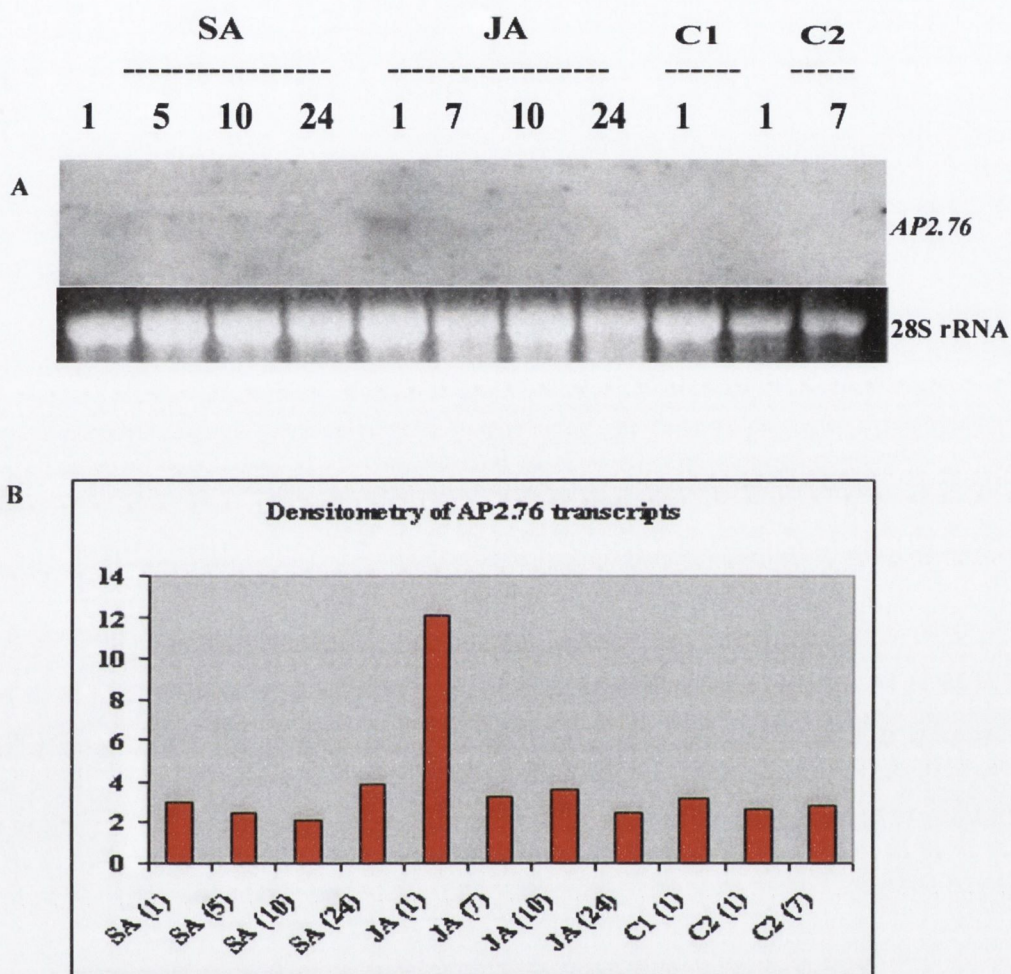


Figure 3.3. Time course of induction of *AP2.76* in response to SA and JA.

A) Total RNA was isolated from three to four week old *Arabidopsis* plants that had been treated with 2mM SA for 1, 5, 10 & 24 hours; 100μM JA for 1, 7, 10 and 24 hours; C1 and C2 are treatment controls for SA and JA respectively. RNA gel blots were hybridised with a *AP2.76* gene specific probe (DIG-AP76) as indicated at right. Numbers indicate duration of treatments in hours. rRNA is shown as a loading control.

B) *AP2.76* relative transcript accumulation as determined by densitometry.

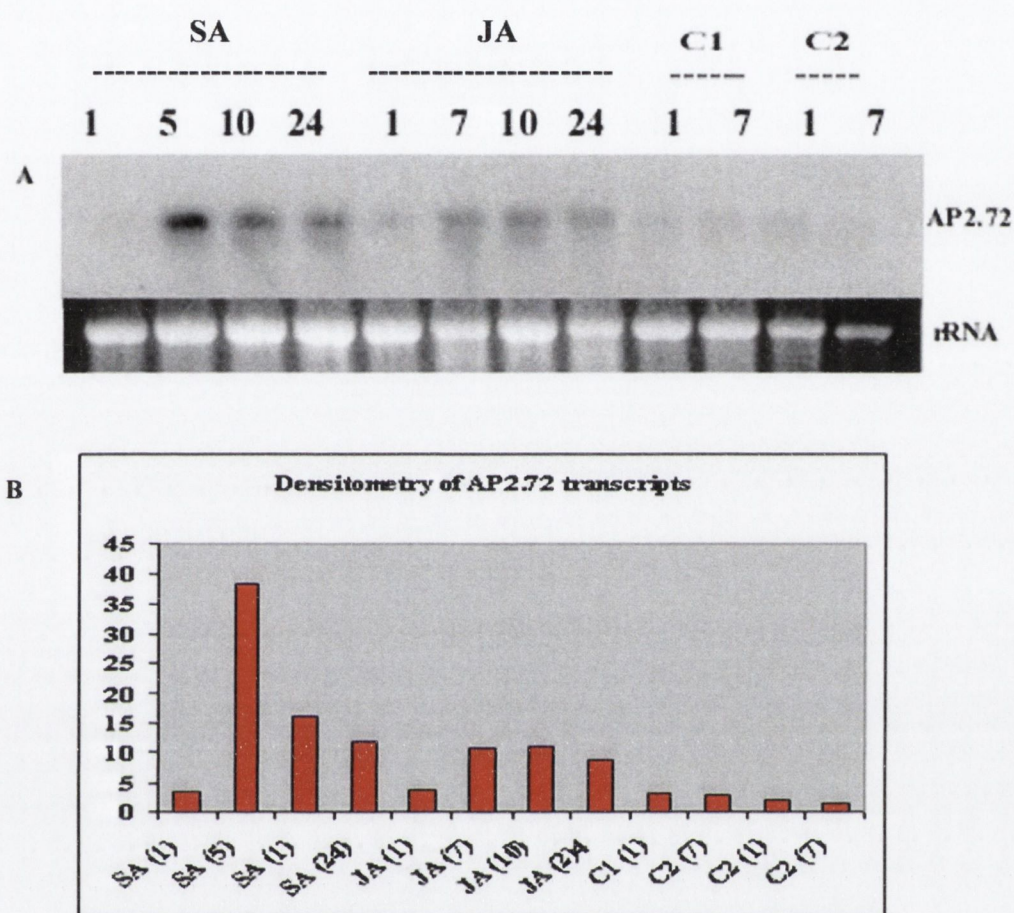


Figure 3.4. Time course of induction of *AP2.72* in response to SA and JA.

- A) Total RNA was isolated from three to four week old *Arabidopsis* plants that had been treated with 2mM SA for 1, 5, 10 & 24 hours; 100μM JA for 1, 7, 10 and 24 hours, C1 and C2 are treatment controls for SA and JA respectively. RNA gel blots were hybridised with a *AP2.72* gene specific probe as indicated at right. Numbers indicate duration of treatments in hours. rRNA is shown as a loading control.
- B) *AP2.72* relative transcript accumulation as determined by densitometry.

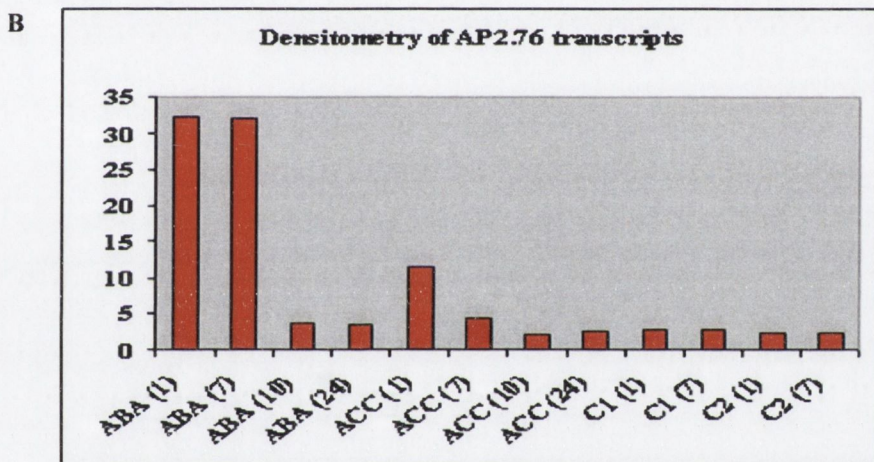
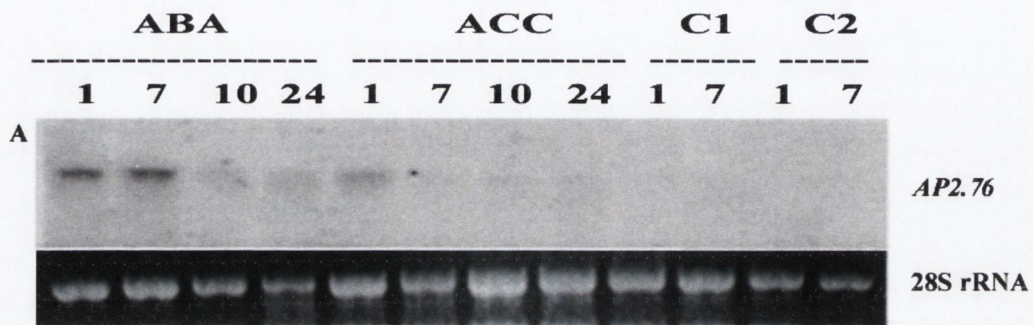


Figure 3.5. Time course induction of *AP2.76* in response to ABA and ACC.

A) Total RNA was isolated from three to four week old *Arabidopsis* plants that had been treated with 100 μ M ABA for 1, 7, 10 & 24 hours; 5 μ M ACC for 1, 7, 10 and 24 hours; C1 and C2 are treatment controls for ABA and ACC respectively. RNA gel blots were hybridised with a *AP2.76* gene specific probe (DIG-*AP76*) as indicated at right. Numbers indicate duration of treatments in hours. rRNA is shown as a loading control.

B) *AP2.76* relative transcript abundance as determined by densitometry.

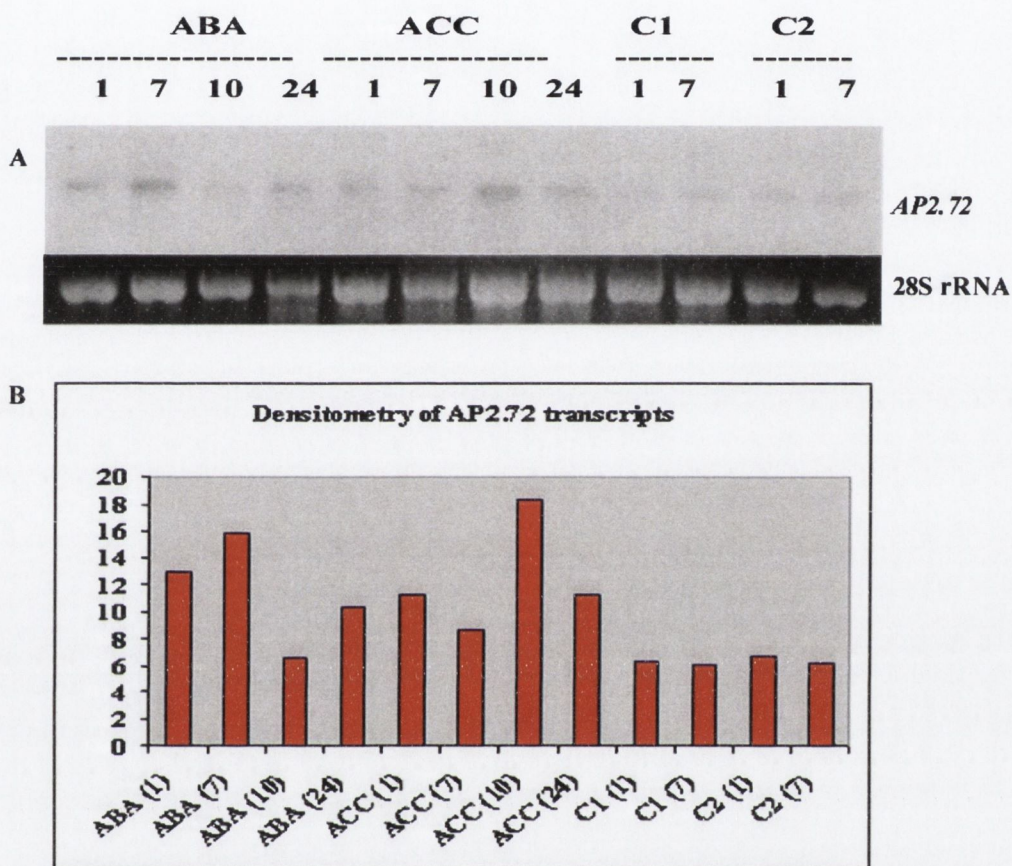


Figure 3.6. Time course induction of *AP2.72* in response to ABA and ACC.

A) Total RNA was isolated from three to four week old *Arabidopsis* plants that had been treated with 100 μ M ABA for 1, 7, 10 & 24 hours; 5 μ M ACC for 1, 7, 10 and 24 hours; C1 and C2 are treatment controls for ABA and ACC respectively. RNA gel blots were hybridised with a *AP2.72* gene specific probe (DIG-*AP2.72*) as indicated at left. Numbers indicate duration of treatments in hours. rRNA is shown as a loading control.

B) *AP2.72* relative transcript abundance as determined by densitometry.

3.3.5 Induction of *AP2.76* and *AP2.72* expression by wounding

AP2.76 mRNA showed high-level accumulation 1 hour after wounding but was undetectable after 10 and 24 hours (Figure 3.7A). A further time-point analysis (Figure 3.7B) confirmed the accumulation of *AP2.76* mRNA at 1 hour and a slight accumulation 30 minutes following treatment and also showed that the transcript was not detectable at 10 minutes or at 5 and 10 hours after wounding.

In contrast, Figure 3.7 (C) shows that *AP2.72* expression was significantly induced 10 hours after wounding but not at 10 and 30 minutes or 1 and 5 hours

These results indicate a rapid, transient induction of *AP2.76* expression, but a considerably slower induction of *AP2.72* in response to mechanical wounding.

3.3.6 Induction of *AP2.76* and *AP2.72* expression by NaCl

The accumulation of *AP2.76* and *AP2.72* mRNA was investigated following treatment of plants with NaCl over a 1 – 24 hour period. *AP2.76* mRNA showed a rapid, strong accumulation after one hour (an approximately 10-fold induction) that declined gradually until the transcript became undetectable after 6 hours.

In contrast, similar high levels of *AP2.72* mRNA were detected 1 and 2 hours after treatment; these decreased to low levels at 3 and 6 hours but increased to high levels again after 12 hours treatment. No significant *AP2.76* mRNA accumulation was apparent after 24 hours (Figure 3.8).

When seedlings were treated with water as a control, no accumulation of *AP2.76* or *AP2.72* mRNA was apparent.

3.3.7 Induction of *AP2.76* and *AP2.72* by cold and dehydration

The accumulation of *AP2.72* and *AP2.76* mRNAs in response to cold and dehydration treatments is shown in Figure 3.9. Neither gene showed any detectable expression in response to low temperature (4°C) treatment over a 5-hour period and *AP2.72* showed no detectable expression in response to dehydration. However, *AP2.76* mRNA was strongly induced

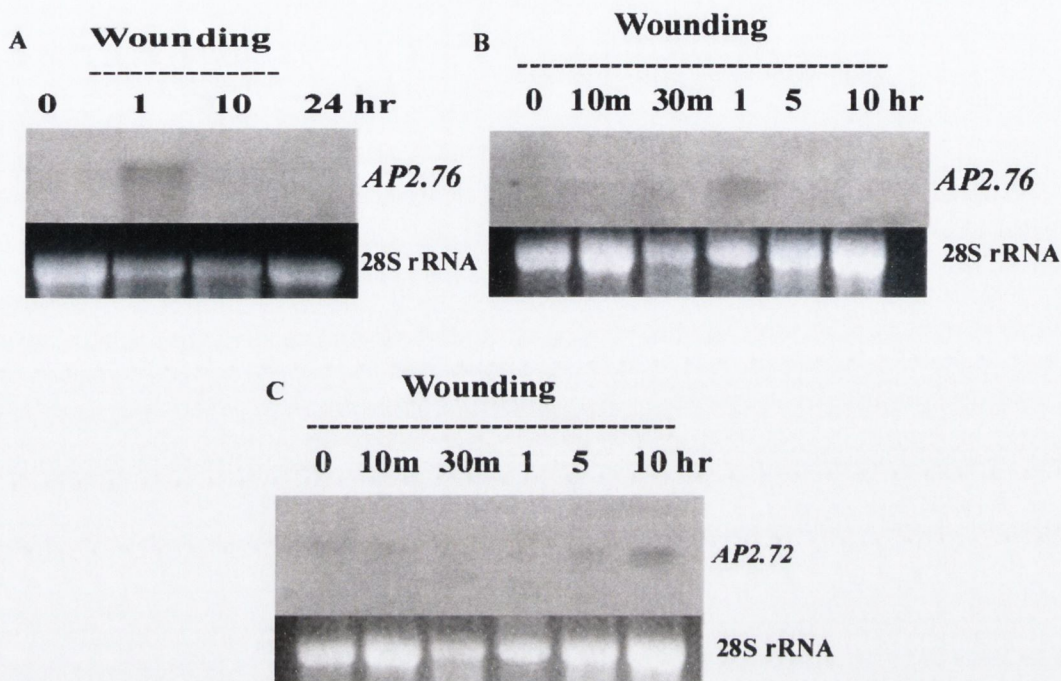


Figure 3.7. Time course induction of *AP2.76* and *AP2.72* by wounding.

Total RNA was isolated from mechanically wounded *Arabidopsis* plants at the indicated times following treatment. RNA gel blots were hybridised with **A)** and **B)** an *AP2.76* specific probe (DIG-AP76) and **C)** an *AP2.72* specific (DIG-AP72) probe. Approximate equal loading was verified by visualising rRNA on a gel stained with ethidium bromide.

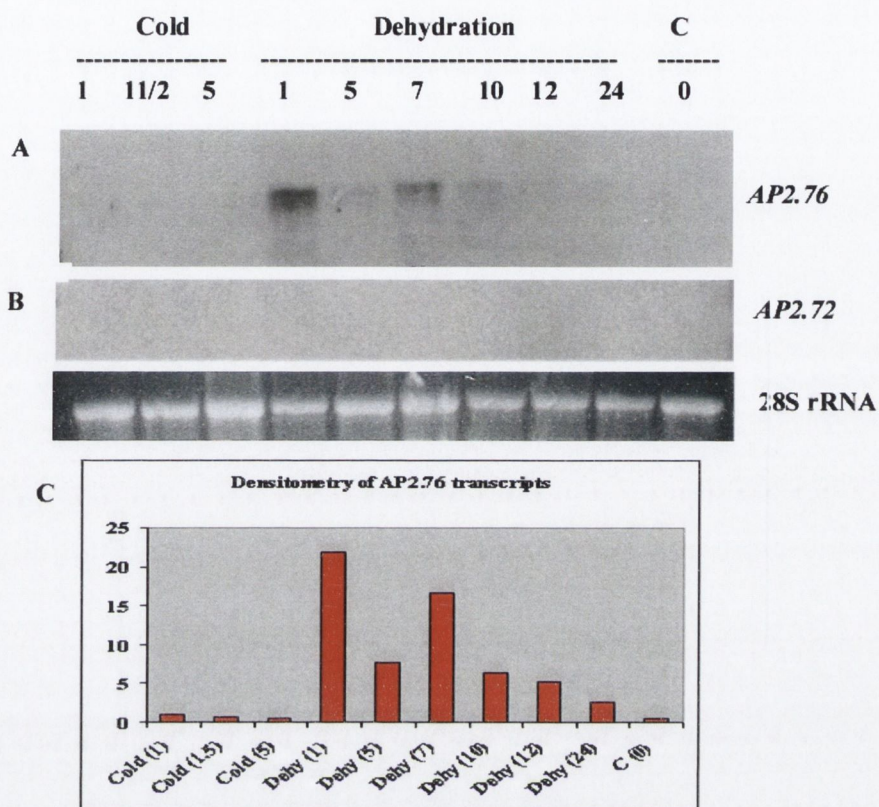


Figure 3.9. Time course induction of *AP2.76* and *AP2.72* in response to cold and dehydration treatments.

Total RNA was isolated from three to four week old *Arabidopsis* plants that had been cold treated for 1, 11/2 & 5 hours and dehydrated for 1, 5, 7, 10, 12 & 24 hours. Total RNA was isolated at zero hours for the control (C). The RNA gel blot was hybridised with a (A) *AP2.76* gene specific probe and a (B) *AP2.72* gene specific probe as indicated at right. Numbers indicate duration of treatments in hours.

C) *AP2.76* relative transcript accumulation as determined by densitometry.

(approximately 20-fold) following a 1 hour dehydration treatment. Transcript levels declined rapidly within 5 hours of treatment, but showed a second increase which reached a maximum at 7 hours. At 12 hours *AP2.76* transcripts were virtually undetectable.

These results clearly indicate that *AP2.72* and *AP2.76* are differentially regulated by dehydration.

3.3.8 Expression of *AP2.76* in three ABA-deficient mutants in response to various abiotic stresses

To investigate whether ABA plays a role in the dehydration-, wounding- and NaCl-mediated induction of *AP2.76*, three ABA-deficient mutants (*aba1-5*, *aba3-1* and *aba2-4*) were investigated by northern blot analysis for their ability to accumulate *AP2.76* transcripts in response to these stresses.

The level of accumulation of *AP2.76* mRNA at 0 and 1 hours after wounding (Figure 3.10(A)) or at 2 and 6 hours following treatment with NaCl (Figure 3.10(B)) was not altered in the three ABA-deficient mutants as compared with wild-type plants treated in the same way.

Figure 3.10 (C) shows that the level of accumulation of *AP2.76* mRNA at 0, 1 and 11/2 hours after commencing a dehydration treatment was also not altered in the three ABA-deficient mutants as compared to wild-type plants.

These results suggest that the changes in *AP2.76* expression in response to wounding, NaCl and dehydration stresses are not mediated via ABA signalling.

3.3.9 Expression of *AP2.72* in two *npr1* mutants in response to SA

NPR1 has been shown to be a key component of the SA-mediated pathway that activates PR gene expression and results in SAR. Although *npr1* mutants still accumulate SA upon pathogen challenge they fail to induce SAR and hence expression of the marker genes PR1, PR2 and PR5.

To investigate if *NPR1* plays a role in the SA-mediated expression of the *AP2.72* gene, two *NPR1*-mutants (*npr1-2* and *npr1-1*) were analysed by RNA gel blot analysis for their ability to accumulate *AP2.72* transcripts in response to SA. Since *npr1-2* is a partial loss-of-

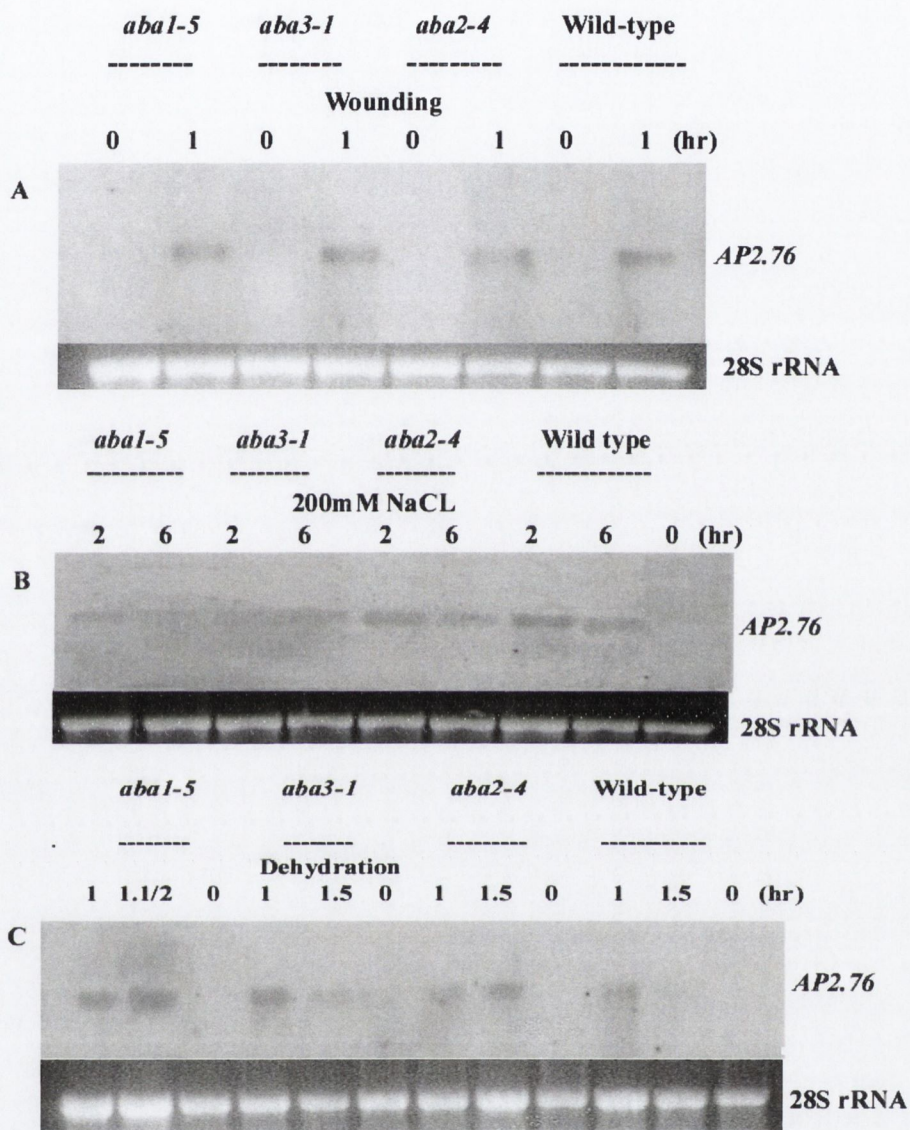


Figure 3.10. Expression patterns of the *AP2.76* gene in response to wounding, dehydration and NaCl treatment in three ABA-deficient mutants.

Total RNA was isolated from three to four week old ABA-deficient mutants (*aba1-5*, *aba3-1* and *aba2-4*) along with wild-type *Arabidopsis* plants that had been treated with **A)** wounding for 0 & 1 hours **B)** 200mM NaCl for 0, 2 & 6 hours and **C)** dehydration treatment for 0, 1 and 1.5 hours. RNA gel blots were hybridised with an *AP2.76* gene specific probe as indicated on right. Numbers indicate duration of treatment in hours. Ethidium bromide staining of the rRNA gel was used to show equal loading.

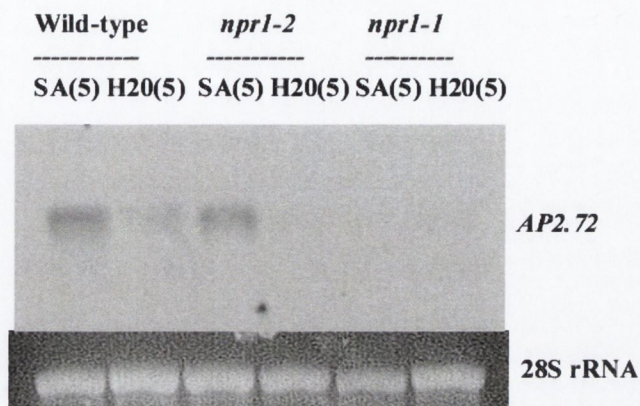


Figure 3.11. Expression patterns of the AP2.72 gene in response to SA treatment in two *NPR1* mutants.

Total RNA was isolated from three to four week old *NPR1* mutants (*npr1-2* and *npr1-1*) along with wild-type *Arabidopsis* plants that had been treated with SA for 5 hours. RNA gel blots were hybridised with an AP2.72 gene specific probe as indicated on right. Numbers indicate duration of treatment in hours. Ethidium bromide staining of the rRNA gel was used to show equal loading.

function mutation, some SA-mediated PR gene expression is observed in the mutant (Cao et al., 1997). However the *npr1-1* mutant causes destabilization of the protein and no SA-mediated PR gene expression is observed.

Figure 3.11 shows that while the level of accumulation of *AP2.72* mRNA 5 hours after treatment with SA was slightly reduced in the *npr1-2* mutant as compared to the wild-type plants, no significant accumulation of *AP2.72* mRNA was evident in the *npr1-1* mutant treated with SA. This clearly indicates that SA-mediated induction of *AP2.72* depends on NPR1.

3.3.10 Construction and analysis of *AP2.76* and *AP2.72* promoter-GUS translational fusions

In order to characterise the spatial and temporal expression patterns directed by the promoter regions of *AP2.76* and *AP2.72* in *planta*, promoter-GUS translational fusions were constructed.

An 1859bp region upstream of and including the 23rd codon of *AP2.76* obtained by PCR amplification was cloned in-frame with *GUS* in the vector pGUS1 (Figure 3.12) and verified by restriction digestion analysis and sequencing. The resulting intermediate vector was called pAP76GUS1. The fragment of pAP76GUS1 carrying the *AP2.76-GUS* fusion was subcloned into the pTAK1 binary vector and further verified by restriction digestion (Figure 3.14; lanes 2, 3 & 4). The resulting construct was called pTAK1-AP76.

A 1998bp region of and including the 13th codon of *AP2.72* obtained by PCR was cloned in-frame with *GUS* in the vector pGUS1-3'NOS (Figure 3.13) and verified by restriction digestion and sequencing. The resulting intermediate vector was called pAP72GUS1-3'NOS. The fragment of pAP72GUS1-3'NOS carrying the *AP2.72-GUS* fusion plus the 3'NOS terminator was subcloned into the pTAK1 binary vector, verified by restriction digest (Figure 3.14; lanes 5,6,7 & 8) and called pTAK1-AP72.

The pTAK1-AP76 and pTAK1-AP72 constructs were introduced into *Agrobacterium tumefaciens* strain AGL1 by electroporation and the resulting strains used to transform individual *Arabidopsis* ecotype Columbia plants by the "Floral Dip" method. After two months, the seeds of primary transgenic plants were harvested and subjected to further analysis.

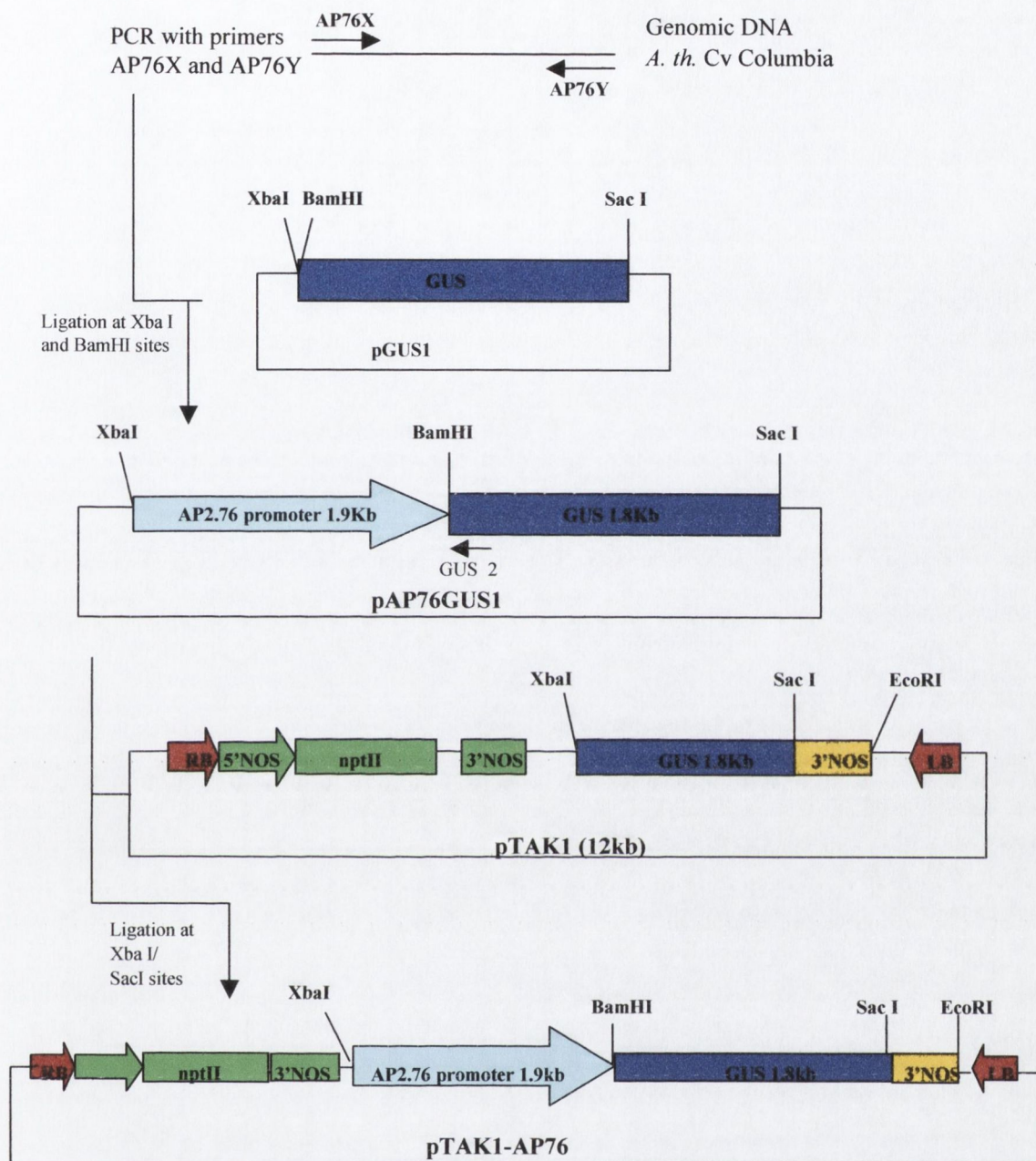


Figure 3.12. Construction of the pTAK1-AP76 binary vector that contains an AP2.76-GUS fusion.

The restriction sites shown were used for cloning fragments into the appropriate vectors. Primers used for PCR amplifications are indicated with black arrows.

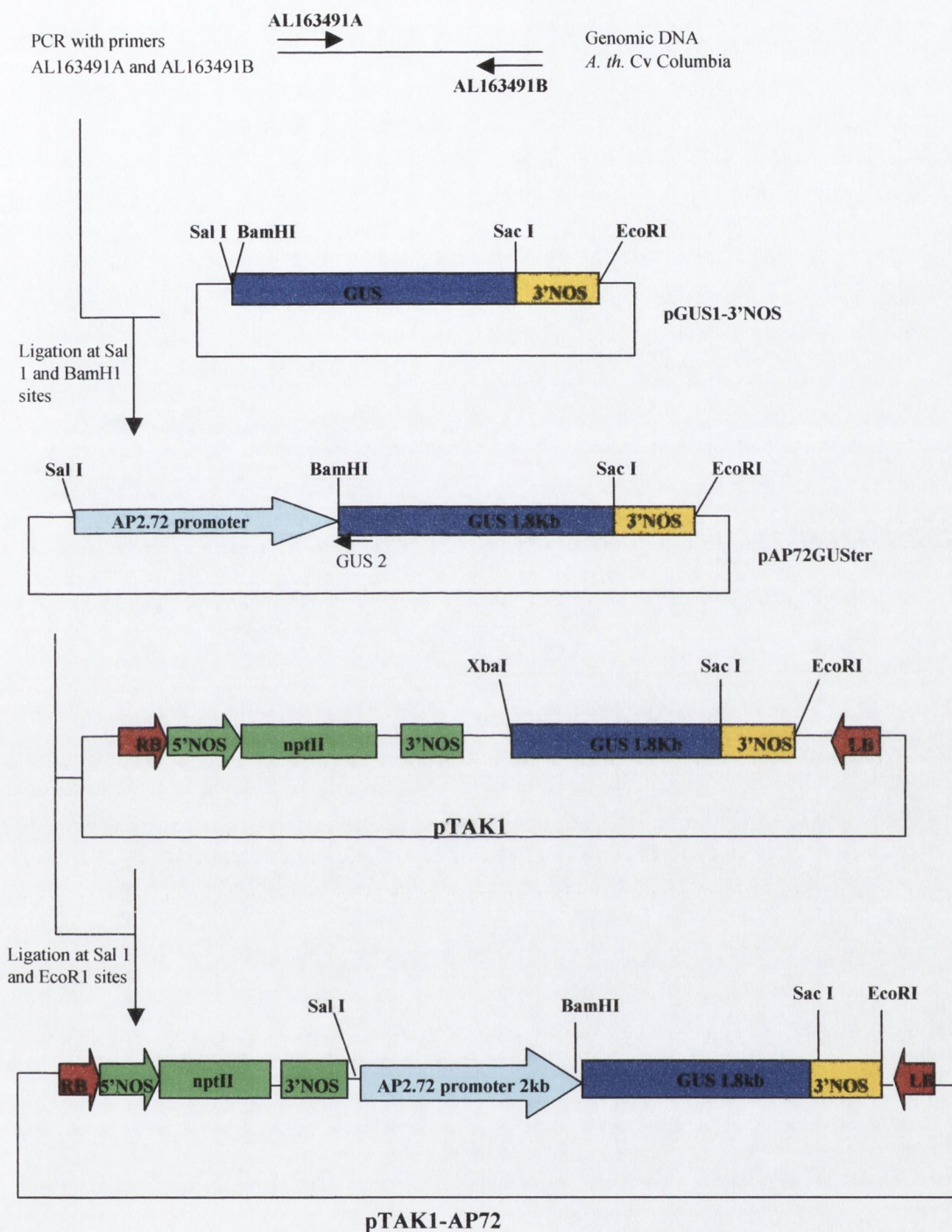


Figure 3.13. Construction of the pTAK1-AP72 that contains the *AP2.76-GUS* fusion.

The restriction sites shown were used for cloning fragments into the appropriate vector. Primers used for PCR amplifications are indicated with black arrows.

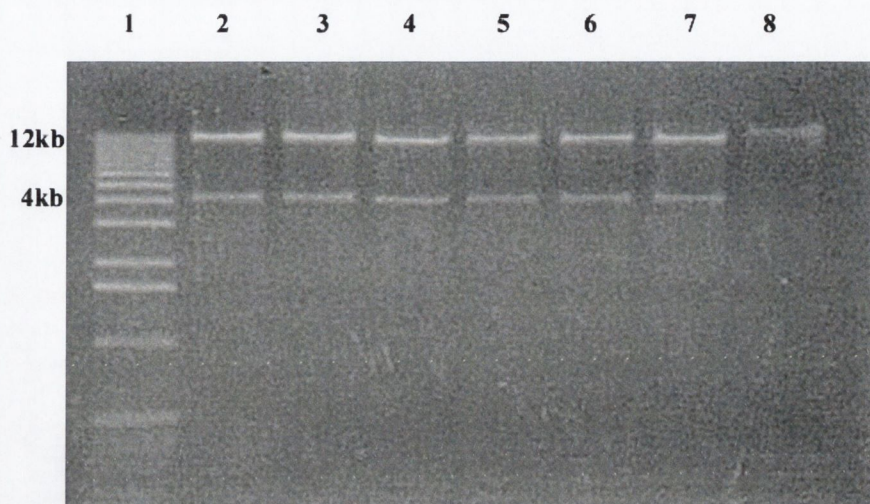


Figure 3.14. Verification of the structure of the pTAK1-AP76 and pTAK1-AP72 binary vectors that contain the *AP2.76-GUS* and *AP2.72-GUS* fusions respectively.

Restriction digest of plasmid DNA from transformant clones containing pTAK1-AP76 binary vector (lanes 2, 3 & 4) with *XbaI* and *EcoRI*. Restriction digest of transformant clones containing pTAK1-AP72 binary vector (lanes 5, 6, 7 & 8) with *SalI* and *BamHI*; 1 kb ladder (lane 1).

3.3.11 Screening for primary transgenic plants

Approximately 3,000 putative transgenic seeds of each transformed plant were sterilised by the Vapour-phase method (section 5.2.12) and selected in MS+ agar plates containing km (50µg/ml). Several putative transformed plants were obtained by kanamycin selection consistent with the reported transformation frequency of 0.1 - 1% (Clough and Bent, 1998). Two to three resistant seedlings for each of the 10 primary transformant parental lines were transferred to soil and grown in greenhouse conditions.

A histochemical analysis was performed on flowers of these plants, and after incubation overnight at 37°C in GUS staining buffer, *GUS* expression was observed. One plant from each of the primary transformant lines showing strong GUS activity was selected to facilitate further analysis.

Primary transgenic plants (T0) transformed with the pTAK1-AP76 vector showing strong GUS activity were also analysed for the presence of the *AP2.76-GUS* cassette by PCR analysis using specific primers for the *AP2.76* promoter (AP76GUS) and for *GUS* (GUSR). The results presented in Figure 3.15 (A) show that six *GUS* positive plants contained the expected 450bp fragment following amplification with the *AP2.76*- and *GUS*-specific primers. Similarly, primary transgenic plants transformed with the pTAK1-AP72 vector were also analysed using primers specific for the *AP2.72* promoter (AP72GUS) and for *GUS* (GUSR). The results presented in Figure 3.15 (B) show that six *GUS* positive plants contained the expected 620bp fragment amplified with the *AP2.72* and *GUS* specific primers.

3.3.12 Expression of the *AP2.76-GUS* and *AP2.72-GUS* transgenes in progeny plants

GUS expression patterns in T1 generation progeny of the *AP2.76-GUS* and *AP2.72-GUS* primary transformant lines described above were characterised in detail at the tissue level and at different stages of development.

Transgenic plants carrying the *AP2.76-GUS* fusion showed *GUS* activity in floral organs and siliques (Figure 3.15(C) and 3.16). No *GUS* expression was observed in leaf, stems, roots and seedlings (Figure 3.15 (C)). In floral organs, *GUS* activity was detected only

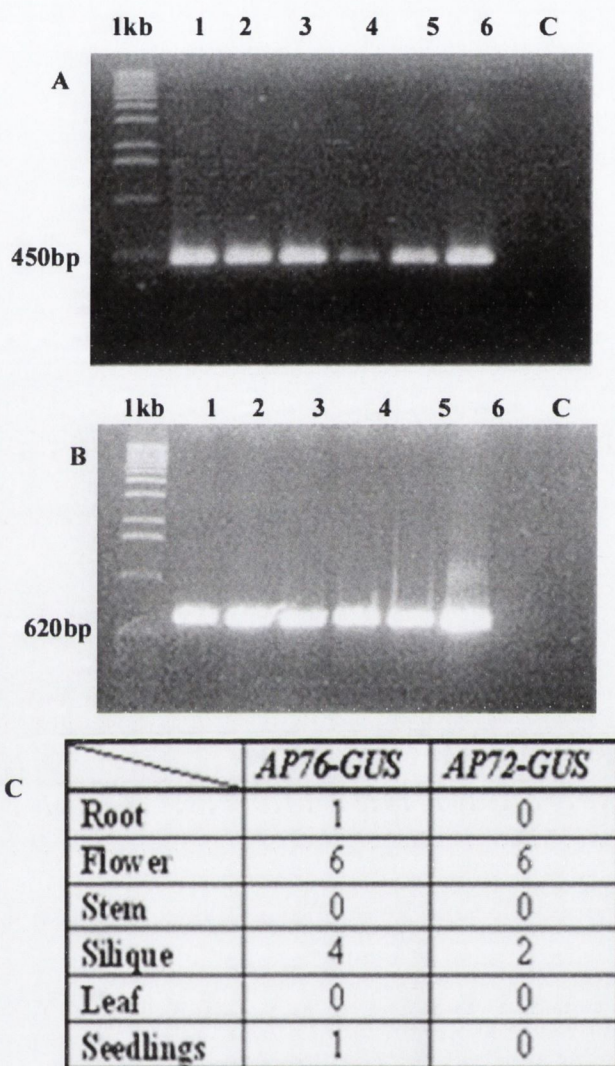


Figure 3.15. Analysis of transgenic plants expressing *AP76-GUS* and *AP72-GUS* fusions.

- A) PCR analysis of putative transgenic plants that were transformed with pTAK1-AP76 binary vector and showed strong *GUS* activity. Primers specific for the *AP2.76-GUS* fusion (*AP76GUS* and *GUSR*) amplified a 450bp fragment.
- B) PCR analysis of putative transgenic plants that were transformed with pTAK1-AP72 binary vector and showed strong *GUS* activity. Primers specific for the *AP2.72-GUS* fusion (*AP72* and *GUSR*) amplified a 620bp fragment.
- C) Table showing tissue specific *GUS* expression in 6 individual transgenic plants expressing the *AP2.76-GUS* and *AP2.72-GUS* fusions.

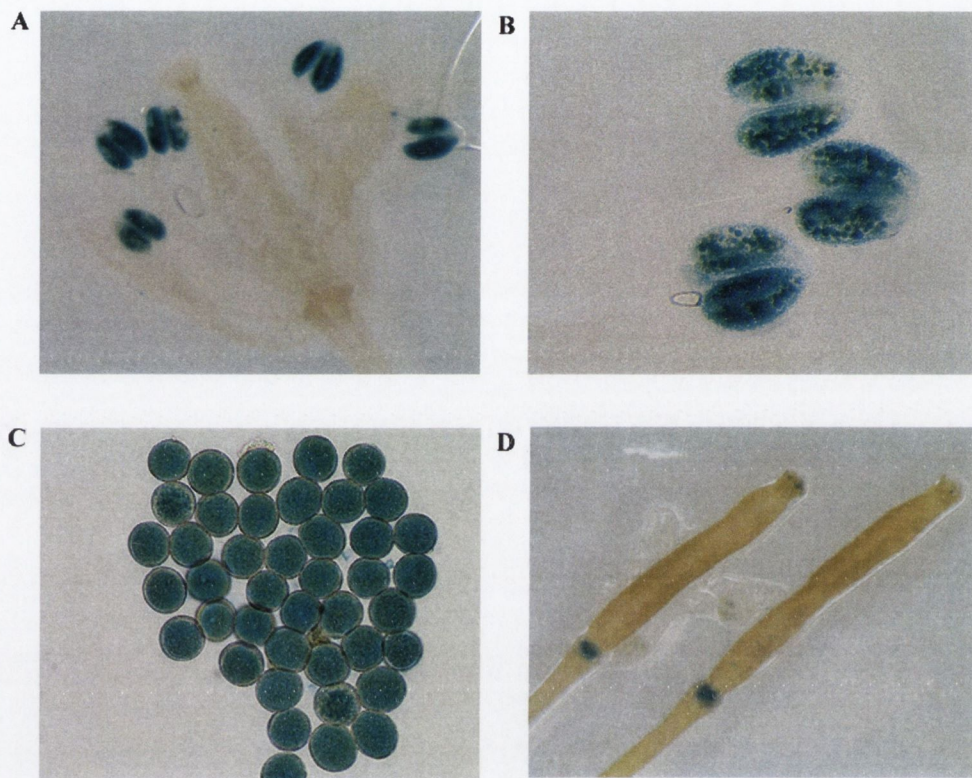


Figure 3.16. Histochemical detection of GUS activity in transgenic plants transformed with pTAK1-AP76 binary vector and expressing the *AP76-GUS* fusion.

Transgenic plants expressing the *AP76-GUS* fusion, grown in the green house showed GUS activity in flowers (A) pollen grains (B) & (C) mature siliques (D).

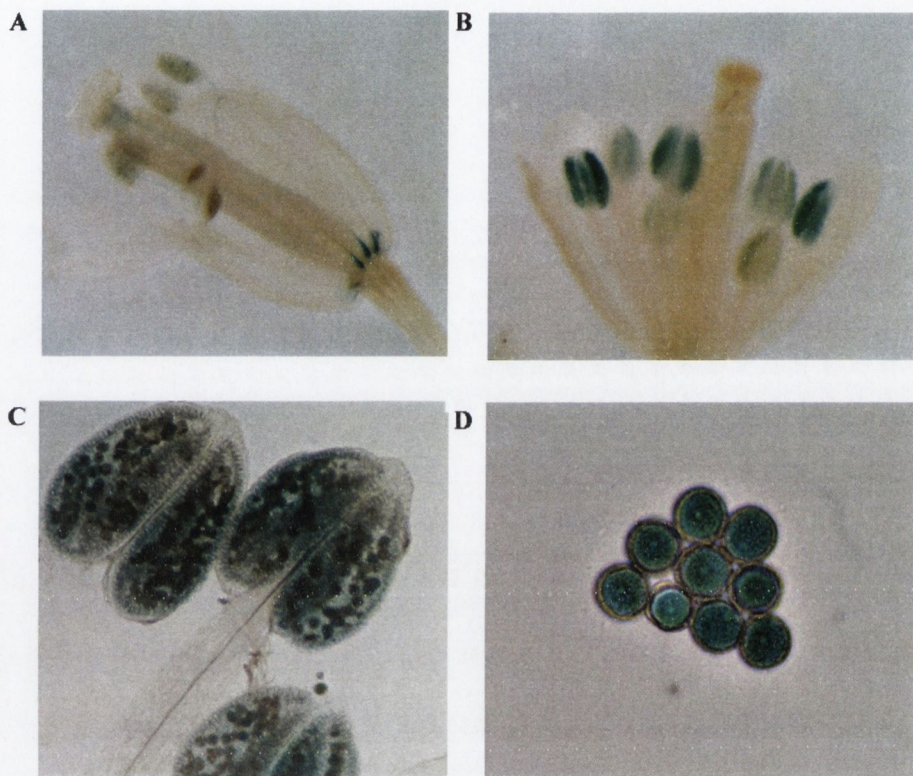


Figure 3.17. Histochemical detection of GUS activity in transgenic plants transformed with the pTAK1-AP72 binary vector and expressing the *AP72-GUS* fusion.

Transgenic plants expressing the *AP2.72-GUS* fusion grown in the green house showed GUS activity in petals (A), anthers (B) & (C) and pollen grains (D).

in pollen grains (Figure 3.16 (A, B & C) and between the base of mature siliques and the pedicel (Figure 3.16 (D)).

Transgenic plants carrying the *AP2.72-GUS* fusion showed GUS activity in floral organs (Figure 3.15 (C)). No *GUS* expression was observed in roots, stems, siliques, leaf or seedlings (Figure 3.15(C)). In floral organs, GUS activity was detected only in pollen grains and at the base of the petals (Figure 3.17).

3.3.13 Promoter *cis*-acting regulatory elements in *AP2.76* and *AP2.72*

Approximately 2kb upstream of the predicted translation start site (ATG) of both *AP2.76* and *AP2.72* was investigated, because the relevant *cis*-elements in the promoter upstream regions of most *Arabidopsis* genes are located within the first 1kb and most of the 5' untranslated sequences are less than 150bp (Maleck et al., 2000).

The 1895bp region upstream of the 23rd codon of *AP2.76* and the 1998bp region upstream of the 13th codon of *AP2.72* were analysed in order to identify candidate sequences corresponding to alternative translational start sites, CAAT and TATA box elements, transcription start sites and promoter *cis*-acting enhancer elements. The results of what are considered to be significant identifications are presented in Figures 3.18 and 3.19.

Figure 3.18 shows that the promoter region of *AP2.76* contains putative CAAT elements upstream of the 5' end of the *AP2.76* cDNA (which may represent the transcription start site). Two stringent W-boxes (TTGACC/T) were found at positions -250bp and 1538bp, which are essential for function and binding of WRKY transcription factors (Eulgem et al., 2000). The statistical expectation for WRKY binding sites (a TTGACC/T hexamer) in the pathogenesis related (PR-1) regulon is 2.6 copies per promoter and the W-box can be arranged in a palindromic or directly repeated manner on many promoters from the PR-1 regulon (Maleck et al., 2000). These results suggest that the W-boxes identified in the *AP2.76* promoter are likely to be of biological significance. In addition, three putative motifs (CTGTTA at -557bp; TAACAG at -1387bp and CGGTTG at -1532bp) that resemble a MYB recognition site C/TAACNG/A, (Nakashima et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997) and two putative motifs (CATGTG at -238bp and CAAGTG at -1065bp) that resembled a MYC recognition site CANNTG, (Murre et al., 1989; Shinozaki and Yamaguchi-Shinozaki, 1997) were also identified in the *AP2.76* promoter region.



Figure 3.18. The nucleotide sequence of the promoter region of the AP2.76 gene. Putative CAAT (green colour) and ATG initiation codon (red colour); W-box (TTGACC/T) in direct or palindromic repeated manner (blue colour). MYC-like binding motifs (CANNTG) and MYB-like binding motifs ((C/TAACNG/A) and core (CNGTTG/A)) in direct or palindromic repeated manner) are shown in brown and purple, respectively. Restriction sites for *XbaI* and *BamHI* (red colour). The 5' end of the AP2.76 cDNA that may represent the transcription start site is shown with an arrow. Numbers refer to nucleotides relative to the 5' end of the cDNA.

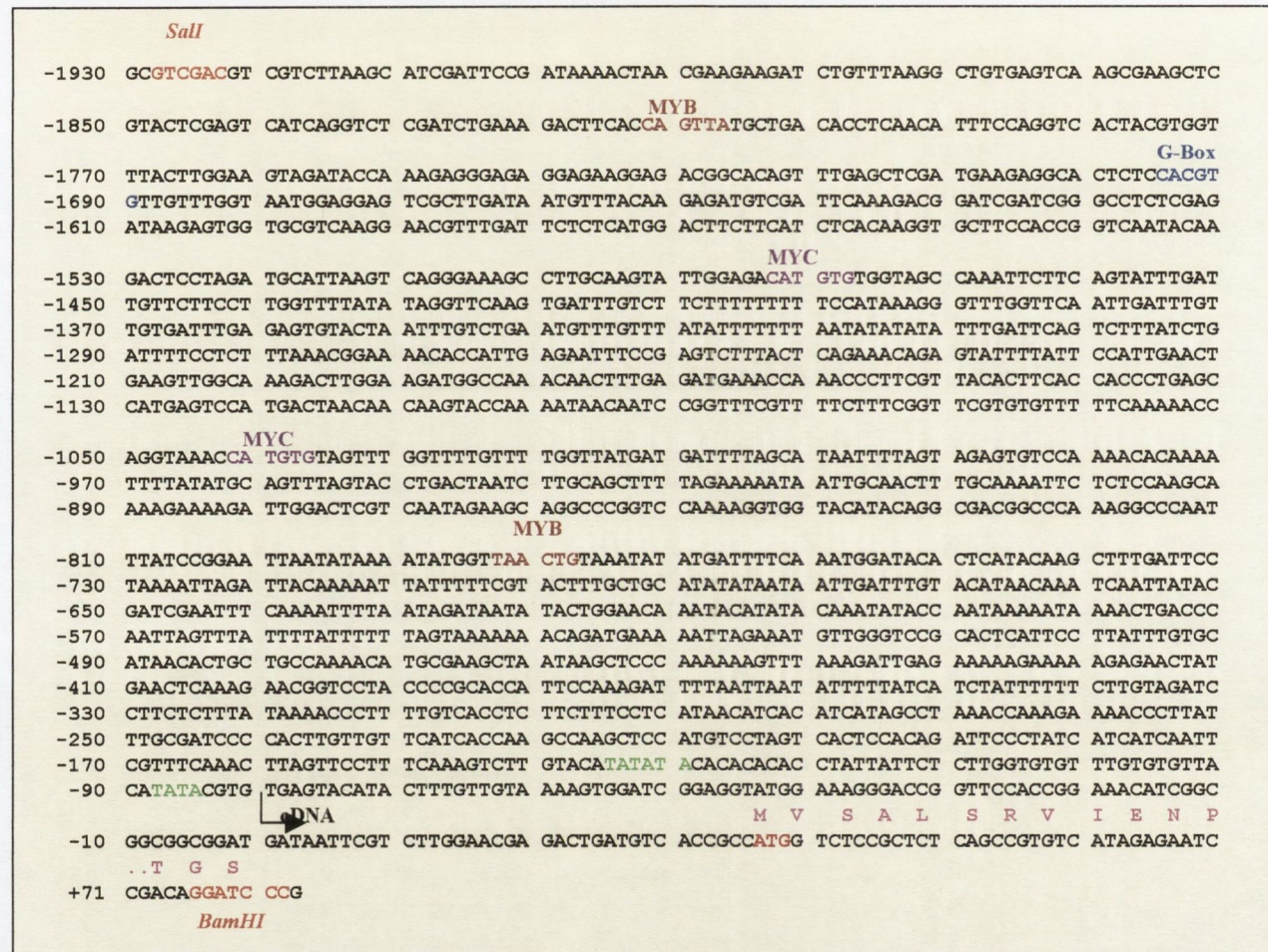


Figure 3.19. The nucleotide sequence of the promoter region of the AP2.72 gene. Putative TATA elements (green colour) and ATG initiation codon (red colour); G-box (CACGTG) is shown by blue colour. MYC-like binding motifs (CANNTG) and MYB-like binding motifs ((C/TAACNG/A) and core (CNGTTG/A)) are shown in purple and brown, respectively. Restriction sites for *SalI* and *BamHI* (red colour). The 5' end of the AP2.72 cDNA that may represent a transcription start site is shown with an arrow. Numbers refer to nucleotides relative to the 5' end of the cDNA.

Figure 3.19 shows that the promoter region of *AP2.72* contains putative TATA elements upstream of the 5' end of the *AP2.72* cDNA (which may represent the transcription start site). The G-box sequence CACGTG (Shinozaki and Yamaguchi-Shinozaki, 1997) was found at position -1690bp. In addition, two putative motifs (TAACTG at -767bp and CAGTTA at -1806bp) that resemble a MYB recognition site C/TAACNG/A, (Nakashima et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997) and two putative motifs (CATGTG at -1036bp; CATGTG at -1477bp) that resembled a MYC recognition site CANNTG, (Murre et al., 1989; Shinozaki and Yamaguchi-Shinozaki, 1997), were identified in the *AP2.72* promoter region.

3.4 Discussion

Several published studies have shown that members of the ERF/AP2 subfamily of transcription factors are induced by biotic and abiotic stresses (Liu et al., 1999; Gu et al., 2002) and regulate GCC-box mediated gene expression (Fujimoto et al., 2000) and DRE/CRT box-mediated gene expression (Liu et al., 1998). Some members such as *Tsi1* are rapidly induced after salt, wounding, SA, ethephon and methyl jasmonate treatment and in turn induce the expression of several pathogenesis-related (PR) genes such as PR1, PR2, PR5, PR3 and PR4 through a specific interaction with the GCC-box *cis*-element found in the promoters of these genes (Park et al., 2001). Moreover, other members such as *DREB1A* and *DREB2A* are induced by cold and dehydration stresses respectively and induce the expression of the cold- and drought-inducible gene *rd29A* through interaction with the DRE/CRT-box *cis*-element found in the promoter of this gene (Liu et al., 1998).

The AP2.76 gene responded at the transcriptional level to JA, ET, ABA, NaCl, wounding and dehydration, suggesting that AP2.76, like other members of the ERF/AP2 subfamily of plant transcription factors may also play a role in regulating gene expression in response to abiotic stresses and pathogen defense. In addition to the SA-mediated induction of systemic acquired resistance (Zhang et al., 1999) evidence has emerged demonstrating that JA and ET are important signals in the induction of systemic defense responses independently of SA (Thomma et al., 1998). Since the AP2.76 gene was rapidly induced by both JA and ET it is likely that it may play a role in the JA/ET-mediated signalling pathway that leads to the transcriptional activation of plant defensin and thionin (PDF1.2 and Thi2.1) genes and the expression of some basic PR genes in *Arabidopsis* (PR3 and PR4) during necrotising infections (Penninckx et al., 1996; Thomma et al., 1998; Pieterse and van Loon, 1999; Wu et al., 2002).

The ISR response triggered in roots by rhizobacteria is also regulated by JA and ET (Pieterse et al., 1996) but is not associated with the activation of genes encoding plant defensins, thionins or PRs. The responsiveness of AP2.76 to both JA and ET suggests it may also play a role in the signal transduction pathway leading to ISR.

Both JA and ET are also known to mediate the wound response pathway and act synergistically to induce the wound response genes (Xu et al., 1994) and also stimulate each other's biosynthesis (O'Donnell et al., 1996). Wounding rapidly and transiently induced the

transcription of the *AP2.76* gene in an ABA-independent manner and this induction correlated with its induction by JA and ET, suggesting a possible role for *AP2.76* in the JA- and ET-regulated wound- and insect-induced pathway that leads to accumulation of proteinase inhibitors and the expression of other wound responsive (WR) genes such as *JR1*, *JR2* and *VSP* (Rojo et al., 1999; Leon et al., 2001).

Wounding and pathogen responses share a number of components in their signalling pathways (Maleck and Dietrich, 1999), including several plant hormones such as JA and ET (O'Donnell et al., 1996; Creelman and Mullet, 1997). Transcriptional profiling has also revealed that a large fraction of wound-responsive genes are known pathogen responsive genes and many osmotic stress genes are highly responsive to wounding (Cheong et al., 2002). However, despite similarities a plants response to each environmental challenge is unique and tailored to increasing the plants ability to survive the inciting stress (Reymond et al., 2000; Xiong and Zhu, 2002). *AP2.76* expression was induced by wounding and by hormones associated with the pathogen response pathway (JA and ET), and may therefore be involved in the activation of distinct sets of defense-related genes depending on the type of attacker encountered.

ABA, dehydration and NaCl also induced expression of *AP2.76*. Expression analysis in ABA-deficient mutants revealed that early induction of *AP2.76* in response to dehydration was ABA-independent and that the induction in response to NaCl was at least in part ABA independent. These findings suggest that *AP2.76* is involved in both ABA-independent as well as ABA-dependent signal transduction cascades between the initial signal of drought or high salt stress and the expression of specific genes. The DRE/CRT-box binding *cis*-element is found in the promoter regions of many dehydration- and low-temperature stress-inducible target genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Shinozaki and Yamaguchi-Shinozaki, 2000) and is responsible for ABA-independent rapid induction by dehydration, high salt, or low temperature (Yamaguchi-Shinozaki and Shinozaki, 1994; Shinozaki and Yamaguchi-Shinozaki, 2000) *AP2.76* was rapidly induced by dehydration (peaked at 1 hour) in an ABA-independent manner, and based on sequence comparisons with other members of the ERF/AP2 transcription factor family was proposed to bind to the DRE/CRT-box *cis*-element in target gene promoters. These results suggest that *AP2.76* may function in the ABA-independent signal transduction cascade through an interaction with the DRE/CRT-box *cis*-element and therefore may mediate transcription of several dehydration- inducible genes that

do not require ABA for their expression under drought and high salt conditions but are known to be regulated by the DRE/CRT-box *cis*-acting element such as *rd29A*, *cor15a*, *rd17/cor47*, and *erd10* (Yamaguchi-Shinozaki and Shinozaki, 1994; Seki et al., 2001).

Many ABA-inducible genes contain a conserved ABA-responsive *cis*-acting element named ABA-responsive element (ABRE; ACGTGG/TC) in their promoter regions (Grill and Himmelbach, 1998) which is involved in the ABA-associated slow response to dehydration (Yamaguchi-Shinozaki and Shinozaki, 1994). Several bZIP-type transcription factors have been isolated as ABRE-binding proteins and are upregulated by drought, high-salinity, and ABA treatments (Guiltinan et al., 1990). Cross coupling of transcription factors is thought to play an important role in mediating responses to various signalling events. Interestingly, ABRE-binding proteins (AREBs) and DRE/CRT-box binding proteins have been shown to function as interactive transcription activators in the ABA-responsive expression of the *rd29A* gene by dehydration, high-salinity, and ABA (Narusaka et al., 2003). Since *AP2.76* showed a second slow induction response to dehydration (that peaked after 7 hours) and high-salinity as well as responding to ABA, it is conceivable that it may also be involved in ABA-dependent gene expression, perhaps by cooperating with the AREBs. Further examination of the dehydration induced expression of *AP2.76* in ABA-deficient mutants may help to determine if *AP2.76* functions in ABA-dependent gene expression. In addition determining if *AP2.76* can regulate gene expression in combination with other transcription factors may prove vital in unmasking its functions in stress responses.

AP2.72 responded at the transcriptional level to SA, JA, ET, ABA, NaCl, and wounding, suggesting that like other members of the ERF/AP2 subfamily, it may also play a role in regulating gene expression in response to biotic (pathogen) and abiotic stresses. Induction of *AP2.72* by SA suggests it may be involved in transducing the SA signal by, for example, regulating PR gene expression. Moreover, the expression of *AP2.72* in response to SA was reduced in the *npr1-2* mutant and abolished in the *npr1-1* mutant, indicating that *AP2.72* may act downstream of *NPR1* in the induction of SAR. The *npr1-1* mutation is in the ankyrin-repeat domain while the *npr1-2* mutation is in the amino-terminal region, two regions that are known to be required for the interaction of NPR1 with bZIP transcription factors that bind *cis*-acting promoter sequences required for SA induction of PR genes (Zhang et al., 1999). Several ERF/AP2-type transcription factors have been implicated in SAR and are known to regulate PR gene expression through GCC-box mediated transcription (Gu et al.,

2000; Park et al., 2001; Gu et al., 2002); however a direct or indirect interaction between NPR1 and ERF/AP2 transcription factors has not so far been reported.

NPR1 has also been shown to be essential for induction of ISR by root-colonising bacteria, a JA- and ET-dependent process. Since *AP2.72* is induced by JA and ET and is also regulated at the transcriptional level by NPR1, a role for the *AP2.72* transcription factor in regulating gene expression during ISR development is also possible. Additionally, the induction of *AP2.72* expression by JA and ET suggests it might be involved in transcriptional regulation of JA- and ET-dependent genes encoding plant defensins, thionins and basic PR genes (Thomma et al., 1998).

The antagonistic relationship between the JA and SA pathways has been well documented in studies of wound and pathogen responses in plants (Doares et al., 1995b; Doares et al., 1995a; Spoel et al., 2003). For example, cytosolic NPR1 plays a crucial role in the SA-mediated negative regulation of JA signalling in *Arabidopsis* (Spoel et al., 2003). Moreover, studies with *Nicotiana tabacum* (tobacco) and *Arabidopsis* reveal that JA- and SA-mediated defense responses can be mutually antagonistic (Norman-Setterblad et al., 2000). Although induction of *AP2.72* in response to SA was dependent on NPR1, a general role for *AP2.72* in the negative regulation of JA signalling is unlikely since *AP2.72* was also induced by JA. Additionally, *Arabidopsis* cDNA array analyses have shown that a relatively large group of genes are inducible by both JA and SA (Schenk et al., 2000). Moreover, because both JA and SA contribute to resistance against the fungus *Plectosphaerella cucumerina* (Thomma et al., 2000) a general antagonism appears to be unlikely and instead the major endogenous signal molecules SA and JA appear to form a network of synergistic and antagonistic interactions (Glazebrook, 2001; Spoel et al., 2003). Furthermore, it is likely the two regulatory networks controlling plant defense signalling contain several nodes of interaction (Li et al., 2004) but the identity of those nodes remains to be elucidated. One of the open questions is whether transcription factors are involved in the cross talk between SA and JA signalling. It would be interesting to determine whether *AP2.72* contributes to the cross-talk between the two pathways.

In addition to their pathogen-related induction in leaves, several pathogenesis-related proteins such as PR1, PR2 and PR3 were found to be present in healthy tobacco flowers, irrespective of microbial attack or other environmental stresses (Lotan et al., 1989; Neale et al., 1990). Although a specific biological function for these proteins in flowers is unknown, it

is thought that during certain stages of flower development, there may be an increased susceptibility to foreign organisms as well as environmental stresses and the presence of PR proteins may then perform an ancillary protective function as part of a general defense mechanism (Neale et al., 1990). The endogenous *AP2.76* and *AP2.72* genes were both expressed in the flowers of unstressed *Arabidopsis* plants (although at quite different levels) and GUS activity was detected in pollen grains of transgenic plants carrying promoter-*GUS* fusions for each gene. Since *AP2.76* and *AP2.72* have both been implicated in abiotic and biotic stress responses and may potentially act as regulators of PR gene expression, *AP2.76* and *AP2.72* may perform an additional defense or stress-protective role in pollen grains. GUS expression driven by the *AP2.76* promoter was also detected at the base of siliques, a region that functions as an abscission zone for petals and sepals. Abscission is known to be promoted by ET; GUS expression in the abscission zone may simply reflect the responsiveness of *AP2.76* to ET (Ecker and Theologis, 1994).

The endogenous *AP2.72* gene was expressed in the roots of wild-type unstressed plants. However, no GUS activity was detected in roots of transgenic plants carrying an *AP2.72* promoter-*GUS* fusion. There are several possible explanations for this contradictory result. One is that the 2kb promoter region used in the promoter-*GUS* fusion did not include all of the relevant *cis*-acting regulatory elements located upstream from the transcription start site (TSS), necessary to confer the full promoter activity of the endogenous *AP2.72* gene. Secondly, regulatory elements can also be located downstream of the TSS, for example in introns (Gidekel et al., 1996; de Boer et al., 1999; Dorsett, 1999) or in the 3'-untranslated region (3'UTR); the *AP2.72* promoter-*GUS* fusion would not have contained such elements. Finally, *AP2.72* transcripts might be subject to translational control. If the *AP2.72* promoter-*GUS* fusion described herein (a translational fusion) contained such signals, GUS activity might be absent despite the presence of GUS transcripts.

Sequence analysis of the promoter regions of both *AP2.76* and *AP2.72* revealed the presence of several putative MYC and MYB recognition sites which act as *cis*-acting elements in the dehydration- and ABA-inducible expression of several genes (Urao et al., 1993; Abe et al., 1997). Individual genes of the MYC and MYB families in *Arabidopsis* as well as being induced by abiotic stresses are also induced by bacterial infection and during the hypersensitive response (Abe et al., 1997; Kranz et al., 1998; Daniel et al., 1999; Menssen and Hermeking, 2002). Putative W-box motifs were also identified in the promoter region of

AP2.76. These elements mediate transcriptional responses to pathogen-derived elicitors and are present in the promoters of many plant genes that are associated with (Maleck and Dietrich, 1999; Dong et al., 2003). A putative G-box element was found in the *AP2.72* promoter, an element known to mediate transcriptional responses to various stresses (Menkens et al., 1995). The presence of MYB, MYC, W-box and G-box *cis*-elements in the promoters of *AP2.76* and *AP2.72* might further support a defense- or stress-related role for these transcription factors.

Chapter 4

Functional Analysis of *AP2.76* and *AP2.72*

Part 1: Isolation of *AP2.76* and *AP2.72* knockout lines

4.1 Introduction

The availability of databases containing complete genome sequences and expressed sequence tags (ESTs) confronts molecular biologists with the problem of understanding the function of thousands of previously unknown genes. Computer studies can assign putative functions by relatedness to known genes, but only the biological characterisation of mutants can provide definitive answers (Miklos and Rubin, 1996). The process of identifying a gene's function subsequent to establishing its DNA sequence is known as reverse genetics, for which a number of methods have been explored. In plants, these methods include homologous recombination, gene silencing, and insertional mutagenesis (Tissier et al., 1999). In plants unlike yeast, targeted gene disruption is laborious and inefficient, involving the generation of hundreds of thousands of transgenic plants for every gene assayed (Kempin et al., 1997). Gene silencing, via sense or antisense suppression also has been widely used to gain insight into the function of plant genes (Baulcombe, 1996). However, essential genes cannot be down-regulated in this way because suppression would lead to dominant lethal phenotypes that cannot be maintained. Also, gene silencing may affect entire gene families, making it difficult to pinpoint the role of individual members of the family.

On the other hand insertional mutagens, primarily transposons or T-DNA are well suited for large-scale reverse genetics and offer alternative, more versatile methods for assessing gene function (Martienssen, 1998).

4.1.1 T-DNA and transposon insertion mutagenesis in *Arabidopsis*

T-DNA and transposable elements can alter gene function upon insertion into coding or regulatory sequences (Martienssen, 1998). Many thousands of *Arabidopsis* lines have been generated that harbour either T-DNA or transposon insertions (Sundaresan et al., 1995; Martienssen, 1998; Meissner et al., 1999; Tissier et al., 1999). Individual lines carrying insertions within a gene of interest can be identified by PCR by using a gene-specific primer in combination with a primer complementary to border sequences of the insertion element (Meissner et al., 1999). T-DNA insertions, however, are often complex, characterised by multiple inverted or tandem copies or truncated T-DNA, making molecular analysis difficult (Gheysen et al., 1990). Transposons offer several advantages in insertional mutagenesis as compared with T-DNA. For example, they can be remobilised germinally, thereby producing reverants that can confirm the phenotypic consequences of the insertion, or somatically, thereby permitting mosaic analysis. Plant transposons show a marked preference for insertion into genetically linked sites (Bancroft and Dean, 1993). This property can be used to isolate new mutant alleles or to perform local mutagenesis in a particular region of interest.

4.1.2 The maize *Activator/Dissociation* (*Ac/Ds*) system in *Arabidopsis*

Maize transposons can be engineered to transpose at sufficiently high rates in *Arabidopsis* to permit effective gene tagging (Bancroft et al., 1993). A system based on the maize *Activator/Dissociation* (*Ac/Ds*) element established a transposon system that generated several hundred independent gene trap or enhancer trap insertions in *Arabidopsis* (Sundaresan et al., 1995). These transposon insertions, available from Cold Spring Harbour Laboratory (CSHL), can be used for the identification and characterisation of genes that are expressed during development. The insertion lines also provide specific markers for different tissues, organs, and cell types that should be valuable for characterising the effects of regulatory mutations, and for identification of novel cell types and sub-types in higher plants. This two-component system is based on transactivation of the defective transposon *Ds* in one *Arabidopsis* line by crossing it with a second line carrying an immobilised *Ac* element. Two *Ds* derivatives, *DsG* and *DsE* are available for gene trapping and enhancer trapping strategies,

respectively. Gene traps and enhancer traps vectors carry a reporter gene that is not normally expressed unless it is integrated near or within a host chromosomal gene. In the case of enhancer traps, a minimal promoter that can respond to nearby enhancers controls expression of the reporter (typically GUS), while gene traps carry a splice acceptor site so that integration within introns leads to readthrough transcription and splicing (O'Kane and Gehring, 1987; Wilson et al., 1990; Skarnes, 1993). High-level transposition of DsE and DsG is driven by a CaMV35S promoter-*Ac* transposase gene fusion (Scofield et al., 1993).

4.1.3 Sainsbury Laboratory *Arabidopsis* Transposants (SLAT) collection

An alternative insertional mutagenesis system, based on the maize *Enhancer/Suppressor-mutator* (*En/Spm*) comprises a single T-DNA construct carrying a defective *Spm* (*dSpm*) element with a phosphinothricin herbicide resistance (*BAR*) gene, a transposase expression cassette, and the *SUI* gene as a counterselectable gene (The *SUI* gene from *Streptomyces griseolus* confers sensitivity to a sulfonyleurea proherbicide from Du Pont (O'Keefe et al., 1994)). Unlike the system based on the maize *Activator/Dissociation* (*Ac/Ds*) element described above, the positive and negative selections can be applied to soil grown plants, rather than seedlings grown *in vitro*. These characteristics facilitate the rapid assembly of a large collection of inserts to be used for reverse and forward genetic screens. The resulting lines have been called SLAT lines (for Sainsbury Laboratory *Arabidopsis thaliana* Transposants). A sequenced insertion site (SINS) database of more than 7000 published sequences has been created by sequencing the insertion sites and genomic DNA junction points. The complete database is available at <http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/SINS-database/>.

Part 1 of this chapter describes the isolation and characterization of an *AP2.76* enhancer trap insertion line (*AP2.76-DsE*), identified in the CSHL *DsE*-tagged collection and an *AP2.72* insertion line (*AP2.72-dSpm*) identified in the *dSpm*-tagged SLAT collection.

4.2 Materials and methods

4.2.1 DNA manipulations

General materials and DNA manipulations have been described in Chapter 5.

4.2.2 Plant material

4.2.2.1 The *AP2.76* knockout line

Arabidopsis seeds heterozygous for an enhancer trap element (DsE) insertion in *AP2.76* (*AP2.76-DsE*) (stock number ET 6942) were obtained from CSHL. A *DsE* starter line (DsE1) which carried the untransposed *DsE* and *Arabidopsis* ecotype Landsberg *erecta* were used as controls.

4.2.2.2 The *AP2.72* knockout line

Seeds of the *Arabidopsis* SINS 05-30-94 line (stock number: N55110), which were heterozygous for a *dSpm* insertion in *AP2.72*, were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) as was the *Arabidopsis* ecotype Columbia-0 wild-type line (Col-0: stock number N1092) used as a control for the *AP2.72* knockout line.

4.2.3 Oligonucleotide primers

- a) For PCR and sequencing analysis of *AP2.76-DsE* knockout line.

Ds302: 5'TACCGACCGTTACCGACCGTTTTTCATCC3'

AP76F:5'ATGGGTTGCGGAGATAAGATTGCCGCA3'

Ds502:5'CGGTTATACGATAACGGTCGGTACGGGAT3'

AC066689K:5'GGGGTACCTTAATTGGCAAGAACTTC3'

AP76T:5'AGGGACCACTTTAGGCATATGGTCGAGAC3'

- b) To generate the DIG-*AP2.76*(2) probe for Southern blot analysis of the *AP2.76-DsE* knockout line.

AC066689A:5'GGGGTACCTCAGAAATCAGAGCATCTC3'

AC066689B:5'GCTCTAGAAAGCGGCGGCTGGAGACCC3'

c) To generate the DIG-AP2.76 probe for northern analysis of the *AP2.76-DsE* knockout line.

AP76P1: 5'CGAGTGAGCTACTCGGACTCGGAGAT3'

AP76P2:5'CTTCCCAAATCAGCTCTGGATCGAAC3'

d) For PCR and sequencing analysis of the *AP2.72-dSpm* knockout line.

dSpm1: 5'CTTATTTTCAGTAAGAGTGTCGGGTTTTGG3'

dSpm11:5'GGTGCAGCAAAACCCACACTTTTACTTC3'

Ap72T1:5'GAACCACCGATCAGCATACAACGTGTC3'

Ap72T3:5'GGCAATCAAACCGAAGTTCAAGTATGTGG3'

Ap72T2:5' ATCCTCGTCAATTCAATGTTTTGTCCCAA3'

E) To generate the single-stranded DIG-AP2.72 probe for northern and the DIG-AP2.72(2) probe for Southern analysis of the *AP2.72-dSpm* knockout line.

AL163491A: 5'CGGGATCCAAGGCCCTACTACCACCAC3'

AL163491B:5'GGGGTACCTTCTCTTCTTCTTCACGCTG3'

4.2.4 High salinity and dehydration treatments

Arabidopsis plants (*AP2.76-DsE*, *AP2.72-dSpm* and wild-type) were grown in soil as described in section 5.1.4. Plants were treated and examined 3 weeks after seed germination. Detached *Arabidopsis* leaves were placed in Petri dishes filled with 200mM NaCl and dehydrated in Petri dishes on Whatman 3MM paper at 60% relative humidity and 22°C under dim light. Leaves were examined every 24hrs for a period of 96hrs by comparing phenotype in transgenic and wild type leaves.

4.2.5 Growth and developmental analysis

Twelve T3 seeds from a homozygous plant of *AP2.76-DsE* or *AP2.72-dSpm* plants were grown together with seeds of wild type *Arabidopsis* ecotype Columbia O or Landsberg in individual 3 x 8 cm square pots. Prior to their transfer to the greenhouse, seeds were sown in

the soil and stratified for 3 days at 4°C to synchronise seed germination. Plants were then grown under green house conditions as described in section 5.1.4. The parameters measured were percentage of germination, number of leaves, maximum and minimum rosette radius, time of first bolt, and time of the anthesis of the first flower. Measurements were initiated four days after transfer to the greenhouse and then made every two days until the first flower open was observed.

4.2 Results

4.3.1 Isolation of the *AP2.76-DsE* line by PCR

4.3.1.1 Identification of the *AP2.76-DsE* line

By using the *AP2.76* genomic sequence as a query in a BLASTN-based search, a transposant line heterozygous for an enhancer trap element (*DsE*) insertion in *AP2.76* was identified in the CSHL *DsE* insertion sequence database (Sundaresan et al., 1995). The *DsE* element was inserted at nucleotide 827 bp in the intron-less ORF of the *AP2.76* genomic sequence (Figure 4.1 (C)).

In order to isolate plants harbouring the *DsE* insertion in *AP2.76*, seeds were sterilised by the Vapour-phase method (section 5.2.12), germinated on MS+ supplemented with km (50µg/ml) and resistant seedlings were isolated. DNA from individual seedlings was analysed by PCR using an *AP2.76* gene specific primer AP76F (located 436 bp downstream (3') from the ATG in the genomic sequence) and Ds302, a primer located at the 3' end of *DsE* element (as shown in Figure 4.1 (C) and Figure 4.2 (A)). Six plants showed the presence of the expected PCR product of approximately 407 bp (Figure 4.2 (A) and 4.3 (A)). A PCR analysis was also carried out with a primer located 478 bp downstream of the stop codon of *AP2.76* (AP76T) and Ds502, a primer located at the 5' end of the *DsE* element (as shown in Figure 4.1 (C) and Figure 4.2 (A)). Six plants showed the presence of the expected PCR product of 664 bp (Figure 4.2 (A) and 4.3 (B)). These results indicate that there was no deletion at either end of the *DsE* element at its insertion site at nucleotide 827 bp in the *AP2.76* ORF. The expected PCR products from both the 5' and 3' ends of the insertion were sequenced and as is usual for transposon insertions, a short duplication of the target sequence was observed at both ends of the *DsE* insertion (5' GGGATGAA3').

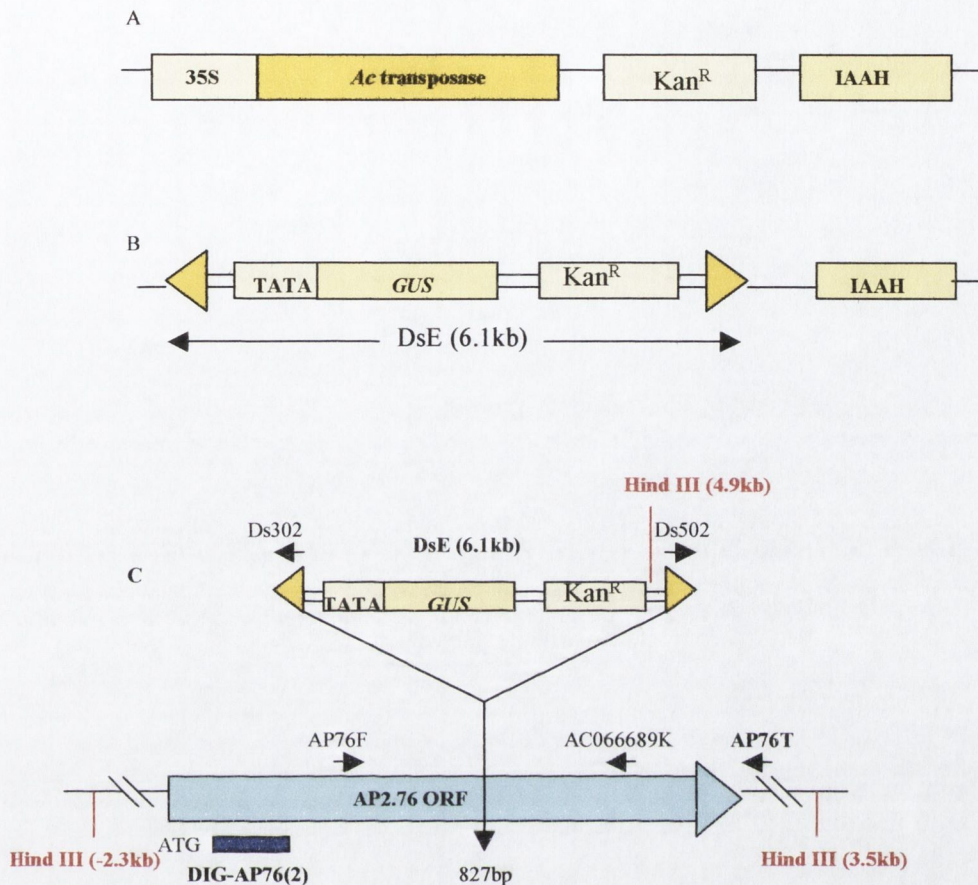


Figure 4.1. Diagram showing *AP2.76* –*DsE* insertion line.

A) *Ac* element in T-DNA vector Sandersan et al., 1995.

B) *DsE* element in T-DNA vector Sandersan et al., 1995.

(IAAH) Indole acetic acid hydrolase gene conferring sensitivity to NAM; (TATA) minimal promoter; (Kan^R) Kanamycin resistance. Triangles represent the terminal inverted repeats of the *Ds* element.

C) Transposition of *DsE* element from T-DNA into *AP2.76*.

Diagram showing the structure of *AP2.76* with a *DsE* insertion. Restriction site *HindIII* used for Southern blot analysis are shown in red and distance of *HindIII* sites relative to ATG are specified. Primers used for PCR analysis are also shown (arrowed lines)

The *AP2.76* specific probe DIG-AP76(2) used for Southern analysis is also shown.

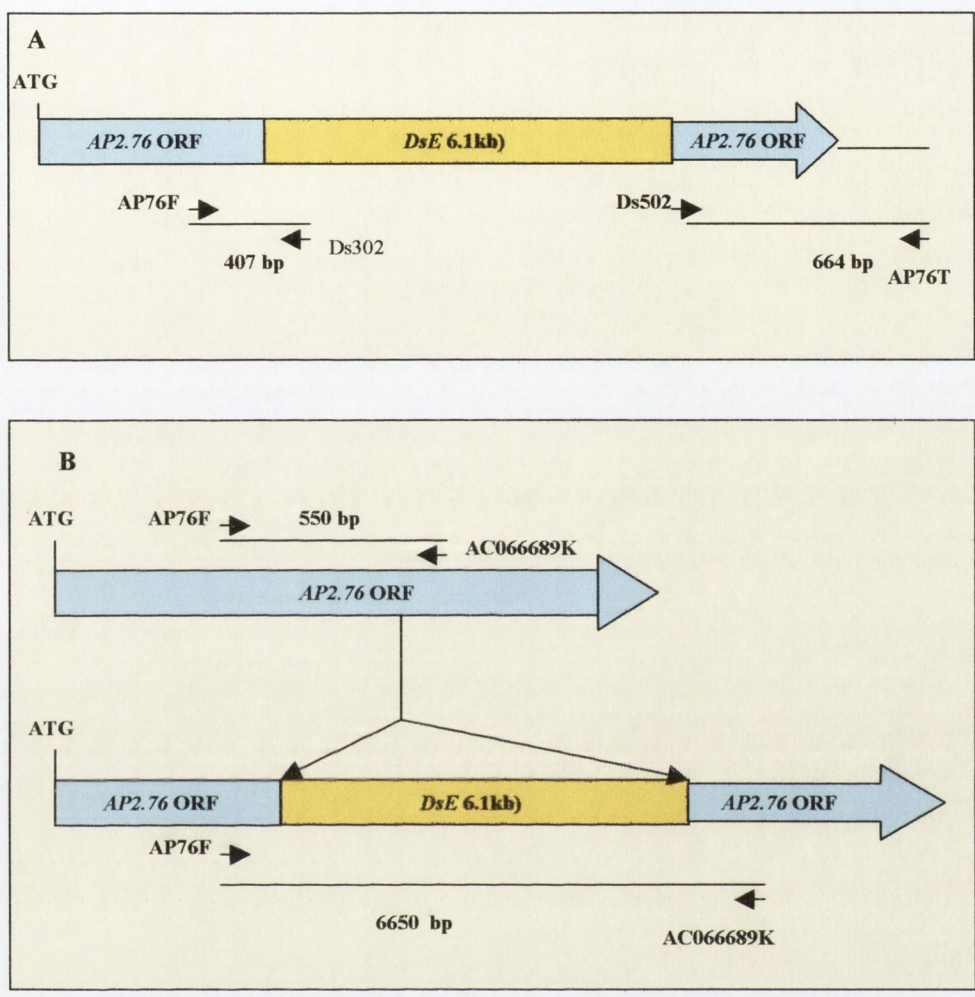


Figure 4.2. Diagrams showing predicted PCR products generated by different primers to identify insertional lines.

A) PCR using the primer pairs AP76F and Ds302, and Ds502 and AC066689K to identify the position of the *DsE* element in *AP2.76*.

B) PCR using the AP76F and AC066689K primers to identify the heterozygous and homozygous lines.

4.3.1.2 Identification of homozygous *AP2.76-dSpm* lines

PCR analysis was performed using DNA from six plants thought to contain the *DsE* insertion in *AP2.76* using the primer pair AP76F and AC066689K which flank the insertion site (Figure 4.1(A) and 4.2(B)). Only wild-type Columbia O, Landsberg *erecta* DNA and 1 plant thought to contain the *DsE* insertion in *AP2.76*, showed the presence of the predicted 550 bp PCR fragment that corresponds to the wild-type *AP2.76* allele. The *DsE* insertion is approximately 6.1 kb and was too large for amplification by *Taq* polymerase-based PCR using this primer combination. The absence of the 600 bp PCR fragment in five of the plants analysed suggested that they might be homozygous for the insertion (Figure 4.3(C)).

4.3.2 Southern blot analysis of *AP2.76-DsE* insertion line

In order to confirm the PCR-based results described above, a Southern blot analysis was carried out on DNA obtained from three plants thought to contain the *DsE* insertion in *AP2.76* (detected as homozygous above), and from a plant carrying the untransposed *DsE* element and from wild-type Landsberg *erecta*. Hybridisation was performed using a digoxigenin-labelled (DIG) *AP2.76* fragment of 0.3 kb (DIG-AP76(2)). Results presented in Figure 4.4 show that in the 3 candidate plants analysed a single band of approximately 8 kb hybridised with the probe (lanes 2, 3 & 4); whereas the plant carrying the untransposed *DsE* element and the Landsberg control showed a band of 5.8 kb corresponding to the wild-type genomic sequence (lanes 5 & 6, respectively). Thus the insertion lines are homozygous for the *AP2.76-DsE* allele.

4.3.3 Northern blot analysis of the *AP2.76-DsE* insertion line

AP2.76 transcript accumulation has been shown to be inducible by ABA (section 3.3.4). In order to determine if lines homozygous for the *AP2.76-DsE* allele were complete knockout lines, wild-type Landsberg *erecta* seedlings and homozygous *AP2.76-DsE* seedlings were treated with ABA and a northern blot analysis was performed using the DIG-AP2.76(2) probe, generated by PCR using the primers AP76P1 and AP76P2. As shown in Figure 4.5

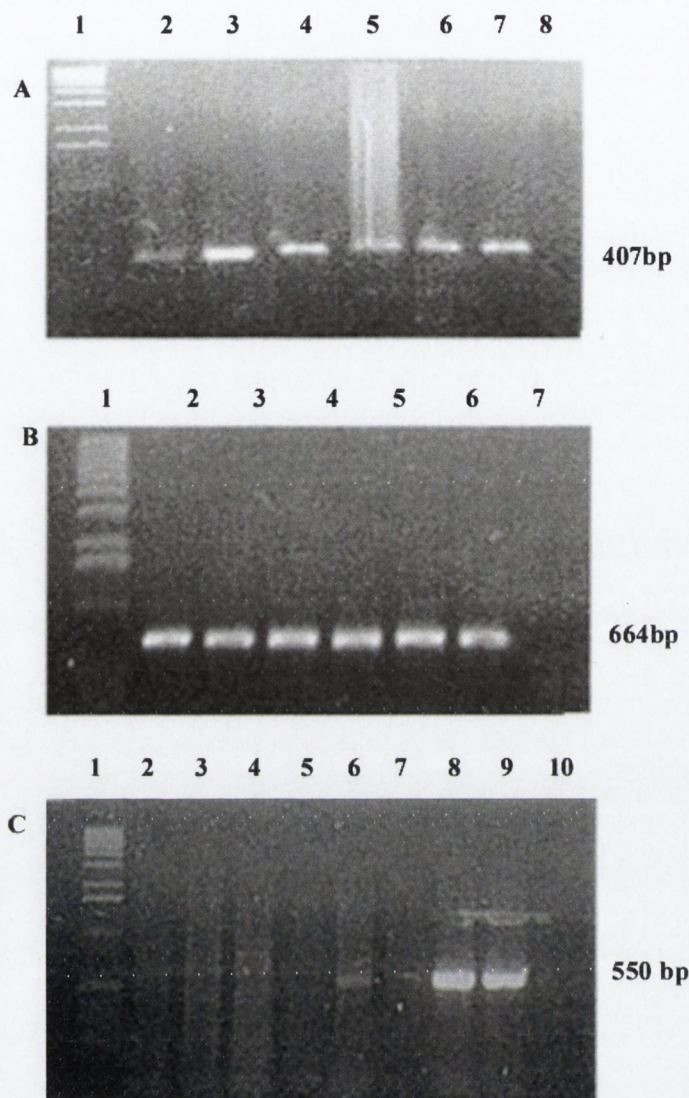


Figure 4.3 PCR analysis of *AP2.76-DsE* insertion line.

A) PCR using the Ds302 and AP76F primers to identify the 5' insertion site. Lane 1 contains the 1kb ladder; lanes 2-7 contain individual plants with the *AP2.76-DsE* insertion; lane 8 contains Landsberg (as a negative control)

B) PCR using the Ds502 and AP76T primers to identify the 3' insertion site. Lane 1 contains the 1kb ladder; lanes 2-7 contain individual plants with the *AP2.76-DsE* insertion; lane 8 contains Landsberg (as a negative control)

C) PCR using the AP76F and AC066689K primers to identify the heterozygous and homozygous lines.

Lane 1 contains the 1kb ladder; lanes 2-7 contain individual plants with the *AP2.76-DsE* insertion; lane 8 contains Landsberg (as a positive control); lane 9 contains Columbia (as a positive control); lane 10 contains a negative control (water).

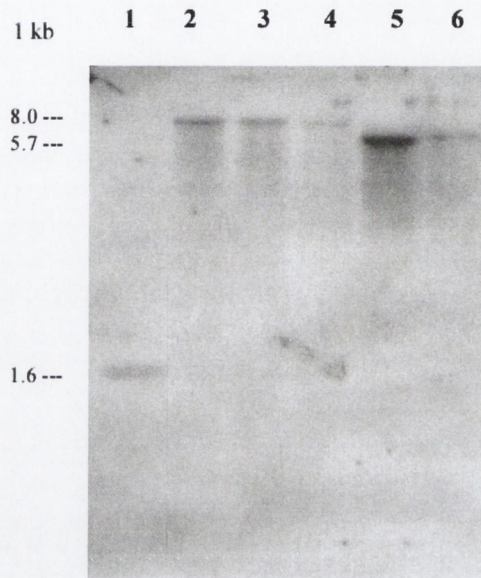


Figure 4.4. Identification of homozygous plants of *AP2.76-DsE* insertion lines by Southern blot analysis. Total DNA of an untransformed plant as negative control (lane 6) and three *AP2.76-DsE* plants (lanes 2 to 4) and one plant carrying the untransposed *DsE* element (lane 5), were digested with *Hind III*. Hybridisation was performed using the DIG-AP76(2) probe. Bands of approximately 8kb and 5.7kb are indicated, 1kb ladder (lane 1).

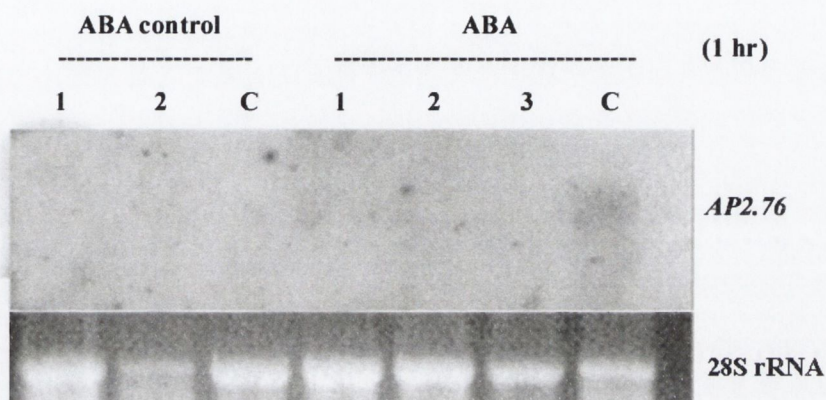


Figure 4.5. Northern blot analysis of expression of *AP2.76* in *AP2.76-DsE* insertion lines.

Total RNA was also isolated from three lines homozygous for the *AP2.76-DsE* insertion (1, 2 & 3) and from wild-type Landsberg *erecta* plants (C) that had been treated with 100 μ M ABA or a control solution lacking ABA for 1 hr. The RNA gel blot was hybridised with a *AP2.76* specific probe as indicated at right. Ethidium bromide staining of the rRNA gel was used to show equal loading.

AP2.76 mRNA accumulated in the control Landsberg *erecta*, but not in the 3 homozygous *AP2.76-DsE* insertion lines after treatment with ABA for 1 hour. No accumulation of *AP2.76* mRNA was apparent in control untreated seedlings.

This result shows that ABA is not capable of inducing *AP2.76* transcripts in seedlings homozygous for the *DsE* insertion in *AP2.76* indicating that these lines are indeed *AP2.76* knockout lines.

4.3.4 GUS expression analysis of homozygous *AP2.76-DsE* insertion lines

The enhancer trap element *DsE* is designed so that expression of the element-localized GUS reporter gene can be driven by any enhancer-like elements fortuitously located close to its point of insertion in chromosomal DNA (Sundaresan et al., 1995). For this reason the pattern of GUS expression in plants of the *AP2.76-DsE* insertion line was examined histochemically as described in section 5.3.2.

The three *AP2.76-DsE* insertion lines that were homozygous for the *DsE* insertion in *AP2.76* showed GUS activity in the pollen grains of flowers (Figure 4.6 (A) & (B)) and in the main root (Figure 4.6 (C)). In contrast, a *DsE* starter line which contained the untransposed *DsE* element showed that *GUS* was expressed ubiquitously throughout the flower, and in the stems and siliques (Figure 4.6 (D)) indicating that the *GUS* expression pattern observed in the *AP2.76-DsE* insertion line is unique and may be driven by upstream *AP2.76* promoter sequences.

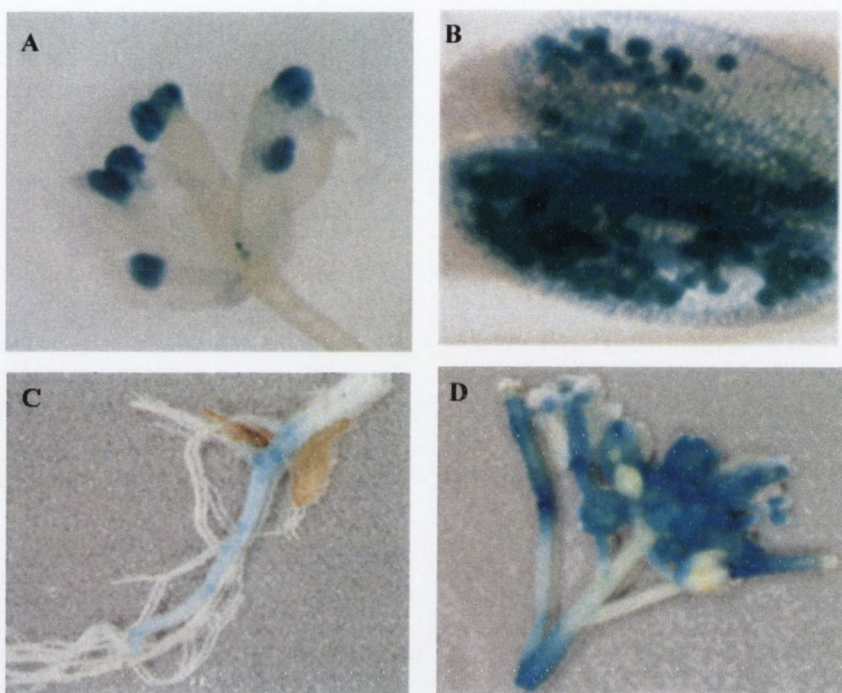


Figure 4.6. Histochemical analysis of GUS activity in *AP2.76-DsE* insertion line.

Homozygous plants of the *AP2.76-DsE* insertion line grown in the green house showed GUS activity in and pollen grains (A) & (B) and in the main root (C). A plant carrying the untransposed *DsE* element showed GUS activity throughout the stems and floral organs (D).

4.3.5 Isolation of the *AP2.72-dSpm* line by PCR

4.3.6.1 Identification of the *AP2.72-dSpm* line

The complete genomic sequence of *AP2.72* plus 500 bp upstream of its start codon (from 68685 -71262/Ac: AL163491) was used to search the SINS database (<http://www.jic.bbsrc.ac.uk/Sainsbury-Lab/jonathan-jones/SINS-database/sins.htm>) The BLASTN results showed that a *dSpm* element was inserted at nucleotide 767 bp in the single intron in *AP2.72* (Figure 4.7 (B)).

In order to isolate plants harbouring the *dSpm* insertion in *AP2.72* seeds were germinated on soil. One week old seedlings were sprayed with *BASTA* (100 mg/ml) every second day for 10 days, and resistant plants were then isolated and screened for the insertion by PCR analysis using an *AP2.72* gene specific primer *AP72T1* (located 345 bp downstream of the ATG in the genomic sequence) and *dSpm11*, a primer located at the 5' end of *dSpm* (as shown in Figure 4.7 (B) and 4.8(A)). Six plants showed the presence of the expected PCR product of approximately 480 bp (Figure 4.8(A) & 4.9 (A)). A PCR analysis was also carried out with the primer *AP72T3* (located 1204 bp downstream of the ATG in the genomic sequence) and *dSpm1*, a primer located at the 3' end of *dSpm* (as shown in Figure 4.7 (B) & 4.8(A)). Five plants showed the presence of the expected PCR product of 500 bp (Figure 4.8(A) & 4.9(B)). These results indicate sequences have not been deleted at either the 5' or 3' ends of the insertion site. A PCR product flanking both the 5' and 3' junctions of the insertion was sequenced and as expected for transposon insertions, a short duplication of the target sequence was observed at both ends of the *dSpm* insertion (5' TAGTGTTTT3').

4.3.6.2 Identification of homozygous and heterozygous *AP2.72-dSpm* lines

An additional PCR analysis of DNA from the six insertion plants described above using the primers *AP72T1* and *AP72T2* which flank the *dSpm* insertion site (Figure 4.7 (B) & 4.8(B)) showed that 4 of the plants contained the predicted 500 bp PCR fragment (Figure 4.9 (lanes 3, 4, 5 & 6)) diagnostic of the wild-type *AP2.72* allele. The *dSpm* insertion is approximately 5.5 kb in length and is therefore too large for amplification by conventional

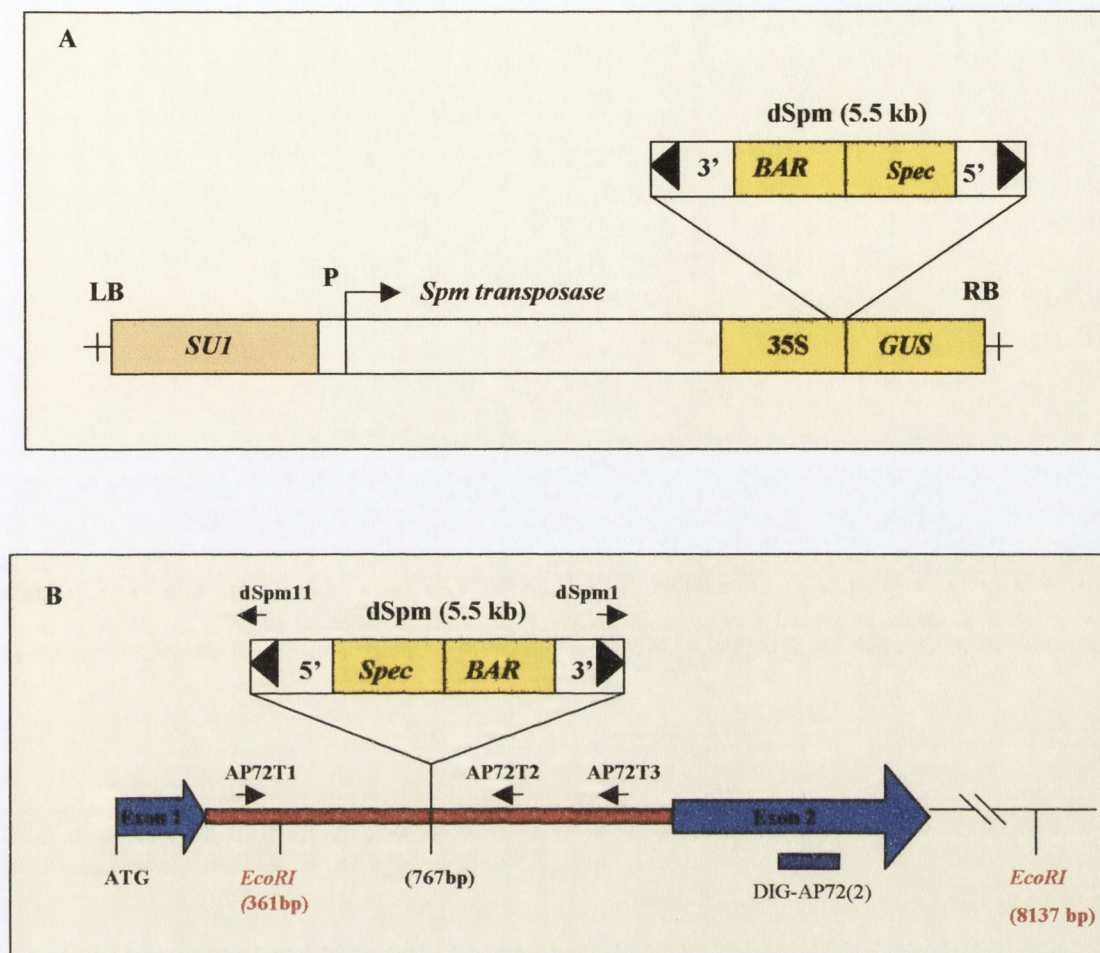


Figure 4.7. Diagram showing *AP2.72-dSpm* insertion line.

- A) Schematic illustration of the T-DNA construct used to generate a stable *dSpm* transposition in the *AP2.72* gene. LB and RB, left and right borders, respectively; P, promoter driving the expression of the transposase; Spec, spectinomycin resistance gene for selection in bacteria; *SUI*, sulfonyleurea proherbicide sensitive counterselectable gene; *BAR*, phosphinothricin (PPR) resistance gene; 35S, cauliflower mosaic virus 35S promoter; *GUS*, β -glucuronidase gene (Tissier et al., 1999).
- B) Structure of genomic *AP2.72* with a *dSpm* insertion. Exons are represented by arrowed blue bars, Introns are represented by narrow red bars. Restriction enzyme site *EcoRI* used for Southern blot analysis is shown in red and the distance of the *EcoRI* sites relative to the ATG of *AP2.72* are indicated. Primers used for PCR analysis are shown. The *AP2.72* specific probe DIG-AP72(2) used for Southern analysis is also shown.

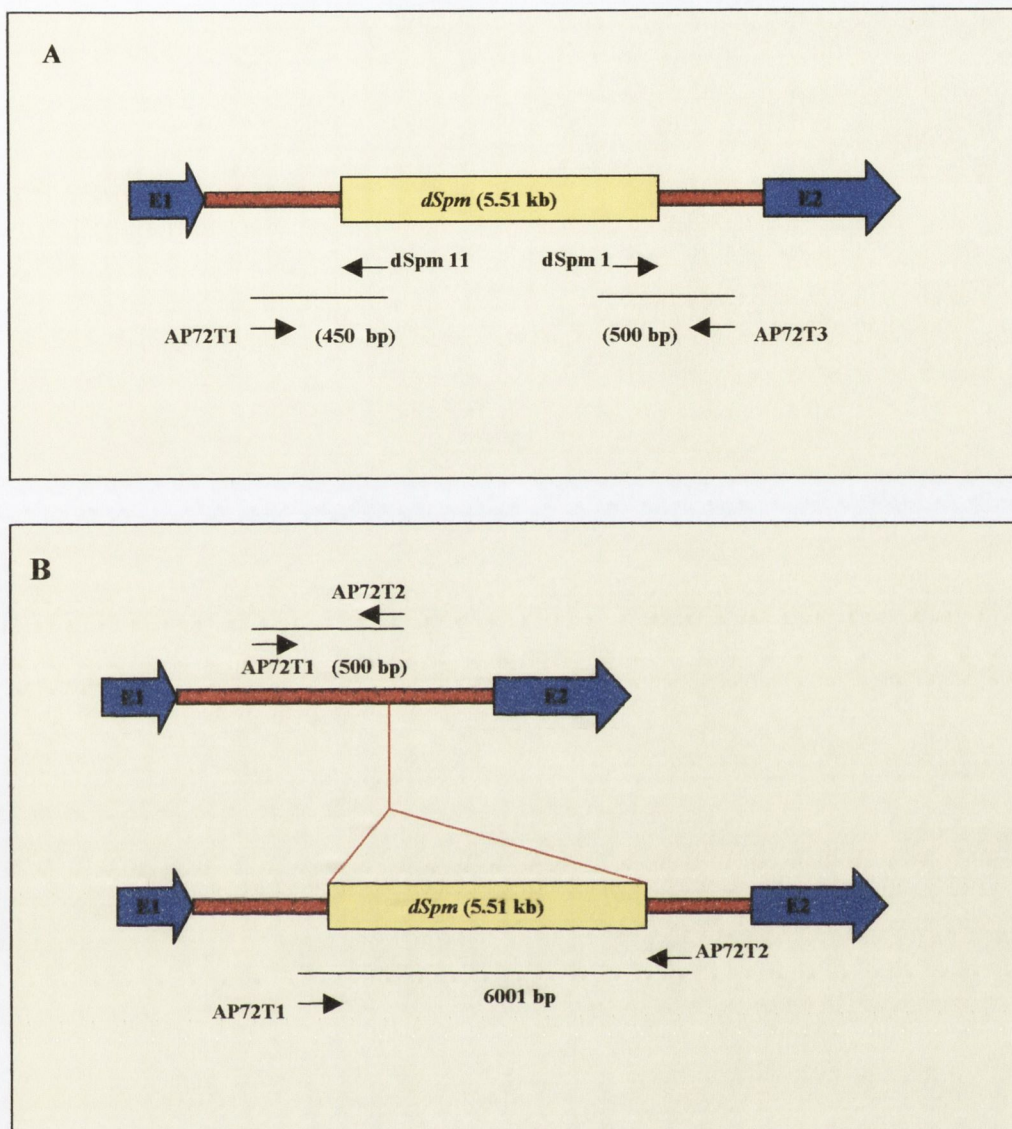


Figure 4.8. Diagrams showing predicted PCR products generated by different primers to identify insertional lines.

A) PCR using the primer pairs AP72T1 and dSpm11, and dSpm1 and AP76T3 to identify the position of the *DsE* element in *AP2.76*.

B) PCR using the AP72T1 and AP72T2 primers to identify the heterozygous and homozygous lines of the *AP2.72-dSpm* insertion line.

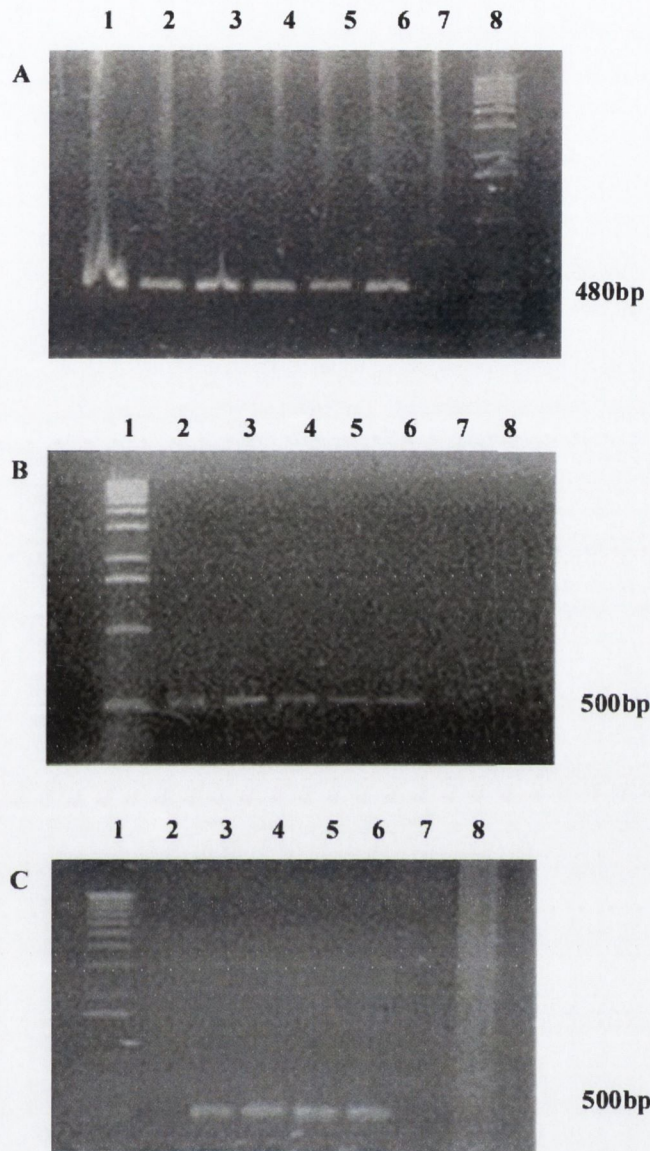


Figure 4.9 PCR analysis of *AP2.72-dSpm* insertion line.

A) PCR using the *dSpm11* and *AP72T1* primers to identify the 5' insertion site. Lane 1-6 contain individual plants with the *AP2.72-dSpm* insertion; lane 7 contains *Col O* (as a negative control) and lane 8 contains the 1kb ladder.

B) PCR using the *dSpm1* and *AP72T3* primers to identify the 3' insertion site. Lane 1 contains the 1kb ladder; lanes 2-7 contain individual plants with the *AP2.72-dSpm* insertion; lane 8 contains *Col O* (as a negative control).

C) PCR using the *AP72T1* and *AP72T3* primers to identify the heterozygous and homozygous lines.

Lane 1 contains the 1kb ladder; lanes 2-7 contain individual plants with the *AP2.72-dSpm* insertion; lane 8 contains a negative control (water).

PCR. The absence of the 500 bp PCR fragment in two of the plants analysed (Figure 4.9 (lanes 2 & 7)) suggested that they might be homozygous for the *dSpm* insertion.

4.3.7 Southern blot analysis of *AP2.72-dSpm* insertion line

A Southern blot analysis was carried out on DNA obtained from the 2 plants shown above to be putatively homozygous for the *dSpm* insertion and a single plant thought to be heterozygous for the insertion (Figure 4.9(C)) using the probe DIG-*AP2.72*(2) (generated by PCR using the primer pair AL163491A and AL163491B). Results presented in Figure 4.10 confirm the homozygosity of the two plants for the *dSpm* insertion in *AP2.72* since only one band of approximately 13.3 kb hybridised with the probe (lanes 2 & 3). In contrast, the heterozygous line contains two bands of approximately 13.3 kb and 7.7 kb (lane 4). The Columbia O control also showed a band of 7.7 kb which therefore corresponds to the wild-type *AP2.72* allele (Figure 4.10 (lane 5)).

4.3.8 Northern blot analysis of the *AP2.72-dSpm* insertion line

AP2.72 expression in response to induction by SA was analysed in seedlings homozygous for the *dSpm* insertion allele and in wild-type Columbia O seedlings using the DIG-*AP2.72* single stranded probe (generated by PCR using the *AP2.72*-specific primers AL163491A and AL163491B). A single-stranded probe was used to avoid nonspecific hybridisation. As shown in Figure 4.11, *AP2.72* mRNA accumulated to high levels in the control Columbia O after treatment with SA for 5 hours. No accumulation of *AP2.72* mRNA was apparent in the 2 homozygous *AP2.72-dSpm* insertion lines (H(1) & H(2)) whereas a slight accumulation of *AP2.72* transcripts was seen in the heterozygous (Ht(1)) insertion line. No significant accumulation of *AP2.72* transcripts was apparent in seedlings treated with H₂O as a control.

This result suggests that SA is not capable of inducing *AP2.72* transcripts in seedlings homozygous for the *dSpm* insertion in *AP2.72* indicating that these lines are indeed *AP2.72* knockout lines.

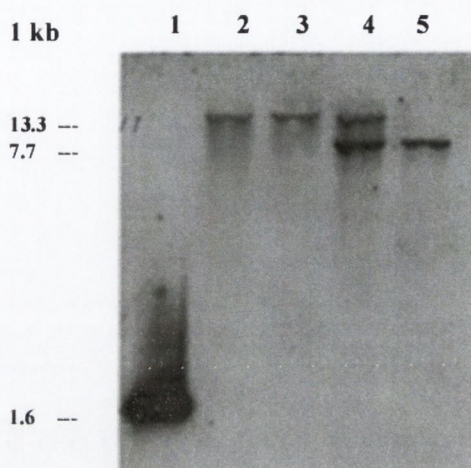


Figure 4.10. Identification of homozygous and heterozygous plants of *AP2.72-dSpm* lines by Southern blot analysis.

Total DNA of an untransformed plant as negative control (lane 5) and three *AP2.72-dSpm* expressing plants (lanes 2 to 4) were digested with *EcoRI*. Hybridisation was performed using the *AP2-72* specific probe DIG-*AP72(2)*. Bands of approximately 13.3kb and 7.7kb are indicated. 1kb ladder (lane 1).

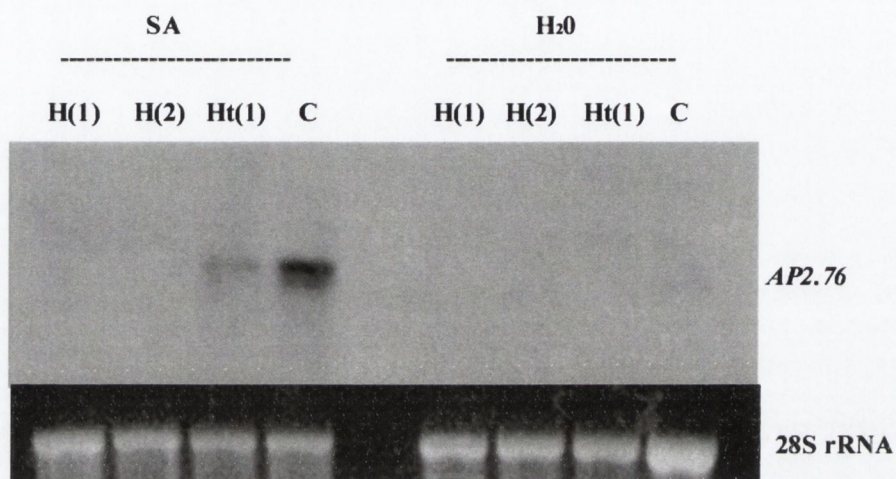


Figure 4.11. Northern blot analysis of *AP2.72* expression in *AP2.72-dSpm* insertion lines.

Total RNA was isolated from 2 lines homozygous for the *AP2.72-dSpm* insertion (H(1) & H(2)), from 1 line heterozygous for the insertion (Ht(1)) and from wild-type Columbia 0 seedlings (C) that had been treated with 2mM SA for 5 hrs or with H₂O as a control. RNA gel blots were hybridised with a *AP2.72* gene specific probe as indicated at the right. Ethidium bromide staining of the RNA gel was used to show equal loading.

4.3.9 Phenotypic analysis of the *AP2.72-dSpm* and *AP2.76-DsE* insertion lines

Seeds from homozygous *AP2.76-DsE* and *AP2.72-dSpm* insertion lines with *Arabidopsis* ecotype Landsberg and Columbia O respectively as wild type controls were grown under green house conditions as described in section 5.1.4.2. Plants were analysed for growth parameters such as germination, number of leaves, flowering time, and rosette radius as described in section 4.2.5. No obvious differences between wild type and *AP2.72* knockout plants (*AP2.72-dSpm*) or between wild type and *AP2.76* knockout plants (*AP2.76-DsE*) were observed (data not shown).

This study previously demonstrated that *AP2.76* and *AP2.72* transcripts were induced by several abiotic stresses including high salinity (*AP2.76* and *AP2.72*) and dehydration (*AP2.76*) (Chapter 3). These observations strongly suggested that both *AP2.76* and *AP2.72* may play a role in regulating gene expression in response to abiotic stress. In addition accumulation of several genes known to be involved in dehydration and high salinity stresses were up-regulated in transgenic plants over-expressing the *AP2.76* gene (Chapter 4, Part 3).

To investigate whether the *AP2.76* and *AP2.72* knockout plants were more susceptible to high salinity and dehydration stress, leaf discs from homozygous *AP2.76-DsE* and *AP2.72-dSpm* were subjected to high salinity and dehydration stresses as outlined in section 4.2.4. Leaves were examined by comparing phenotype in transgenic to wild type leaves every 24hrs for a period of 96hrs. No obvious differences in susceptibility to high salinity and dehydration (e.g rate of chlorophyll loss and rate of dessication) were observed (data not shown).

Part 2: Generation of *AP2.76* and *AP2.72* over-expression lines

4.4 Introduction

Although in general, knockout and knockdown mutation strategies have provided a wealth of information about gene function, many of the mutants generated do not show a discernible phenotype (Zhang, 2003).

Over-expression (or ectopic expression) offers an alternative and complementary strategy to knockout/knockdown analysis that is less affected by functional redundancy. In yeast, the large scale over-expression of random genes has proven to be useful in identifying numerous genes that are involved in controlling the cell cycle (Stevenson et al., 2001). The functions of these genes have been difficult or impossible to uncover using the recessive knockout approach (Zhang, 2003). In plants, there are several recent examples in which knockouts, specifically knockouts of transcription factor genes, failed to generate informative phenotypes. Gene functions were revealed only when the corresponding genes were over-expressed (Baima et al., 2001; Fan and Dong, 2002). Even when the knockout mutant shows a clearly informative phenotype, over-expression can still be valuable because it can generate completely unexpected phenotypes and thus shed light on aspects of transcription factor function that would otherwise be missed (Schellmann et al., 2002; Wada et al., 2002).

4.4.1 Over-expression of transcription factor ORFs using a strong constitutive promoter

In the most common strategy for generating gain-of-function alleles, transcription factor ORFs are over-expressed from a strong constitutive promoter, such as the CaMV 35S promoter. The majority of over-expression studies published recently have utilised this promoter (Zhang, 2003). This strategy has been used successfully in numerous cases in which the over-expression phenotypes, together with other complementary data, are unambiguous in assigning transcription factor function. Over-expression using this strategy often creates hypermorphic alleles, and thus the phenotype directly implies the endogenous function of the gene (Zhang, 2003). For example, when over-expressed, many transcription factors are

4.5 Materials and methods

4.5.1 DNA manipulations

General materials and DNA manipulations are described in Chapter 5.

4.5.2 Plasmids

AV441496, (Asamizu et al., 2000), and see Chapter 2 (section 2.2.2).

N97284, (Newman et al., 1994), and see Chapter 2 (section 2.2.2).

pROK219, contains an expression cassette comprising the CaMV35S promoter and the nopaline synthase terminator (3'NOS) separated by a multiple cloning site, in pUC19 (Figure 4.12).

pROK2, is an *Agrobacterium* binary vector containing an expression cassette comprising the CaMV35S promoter and the nopaline synthase terminator (3'NOS) separated by a multiple cloning site. Two neomycin phosphotransferase genes confer kanamycin resistance as the selectable marker in *E. coli* and in plant cells (Figure 4.12).

4.5.3 Plant material

Wild-type *Arabidopsis thaliana* Columbia seeds were grown in the greenhouse at approximately 25°C and a 16 hours light, 8 hours dark photoperiod. Plants were also grown under *in vitro* conditions at 25°C and 12 hours light, 12 hours dark cycle.

4.5.4 Oligonucleotide primers

A) To amplify the AP2.76 ORF:

AC066689B: 5'CGGGATCCATGGAAGAAAGCAATGAT3'

AC066689K:5'GGGGTACCTTAATTGGCAAGACTTC3'

B) To amplify the AP2.72 ORF:

AL163491X: 5'GCTCTAGATGGTCTCCGCTCTCAGCC3'

AL163491K:5'GGGGTACCTATTCTCTTGGGTAGTT3'

where bold indicates bases that were added in order to create restriction enzyme sites.

C) For sequence verification of constructs

CAMV1: 5'CCTCCTCGGATTCCATTGCCCAGCTA3'

D) For PCR analysis of plants transformed with the pROK-AP2.76 construct.

CAMV1: as described above

AP76ORF:5'GCCGCTTGGAGCATTAGCTCGGAAG3'

E) For PCR analysis of plants transformed with the pROK-AP2.72 construct.

CAMV1: as described above

AP72ORF:5'GCCTTGGACCCGTTTCAGGGAAGTTCAGT3'

F) To generate the DIG-CaMV probe

CaMVF:5'CCCACTGAATCAAAGGCCATGGAGT3'

CaMVR:5'GCGTCATCCCTTACGTCAGTGGAGAT3'

E) To generate the DIG-AP2.76 probe

AP76P1: 5'CGAGTGAGCTACTCGGACTCGGAGAT3'

AP76P2:5'CTTCCCAAATCAGCTCTGGATCGAAC3'

F) To generate the DIG-AP2.72 probe

AL163491A: 5'CGGGATCCAAGGCCCTACTACCACCAC3'

AL163491B:5'GGGGTACCTTCTCTTCTTCTTCACGCTG3'

4.5.5 Phenotypic analysis of AP2.76 and AP2.72 over-expression plants

AP2.76 and AP2.72 over-expression and wild type plants were grown in soil as described in section 5.1.4. Plants were treated and examined 3 weeks after seed germination. Detached *Arabidopsis* leaves were exposed to high salinity and dehydration treatments as described in section 4.2.4.

Twelve T3 seeds from AP2.76 and AP2.72 over-expression plants were grown together with seeds of wild type *Arabidopsis* ecotype Columbia in individual 3 x 8 cm square pots. Growth and development analysis were carried out as described in section 4.2.5.

4.6 Results

4.6.1 Generation of *AP2.76* and *AP2.72* over-expression constructs

In order to over-express *AP2.76* and *AP2.72*, their respective ORFs were amplified by PCR and cloned between the *CaMV35S* promoter and the 3'NOS terminator in *pROK219* as detailed in Figure 4.12. The integrity of the overexpression constructs, called *pROK219-AP2.76* and *pROK219-AP2.72* was verified by restriction digestion (Figure 4.13 A & B) and by sequencing with the *CaMV1* primer.

The expression cassettes were subcloned from *pROK219* into the *pROK2* binary vector and again verified by restriction digestion (Figure 4.13 (C) and (D)). The resulting constructs were called *pROK-AP2.76* and *pROK-AP2.72*. These were introduced into *Agrobacterium* strain AGL1 by electroporation and used to transform *Arabidopsis* Columbia wild-type plants via the “Floral Dip” method (section 5.2.12).

4.6.2 Screening for primary transgenic over-expression lines

4.6.2.1 Identification of putative transgenic plants by PCR

Following selection on MS+ medium containing km (50µg/ml), putative transgenic plants were analysed for the presence of the *35S-AP2.76* and the *35S-AP2.72* fusions by PCR analysis using primers specific for the *AP2.76* and *AP2.72* ORFs and for the *35S CaMV* promoter. This analysis identified 5 plants containing the expected 635 bp fragment corresponding to the *35S-AP2.76* cassette (Figure 4.14A) and 6 plants the expected 600 bp fragment corresponding to the *35S-AP2.72* cassette (Figure 4.14B).

4.6.2.2 Southern blot analysis of plants containing the *35S-AP2.76* fusion

In order to show that the five plants shown by PCR to contain the *35S-AP2.76* cassette represent individual transformations, a Southern gel-blot analysis was carried out on total DNA digested with *HindIII* (which cuts once only in the T-DNA containing the over-

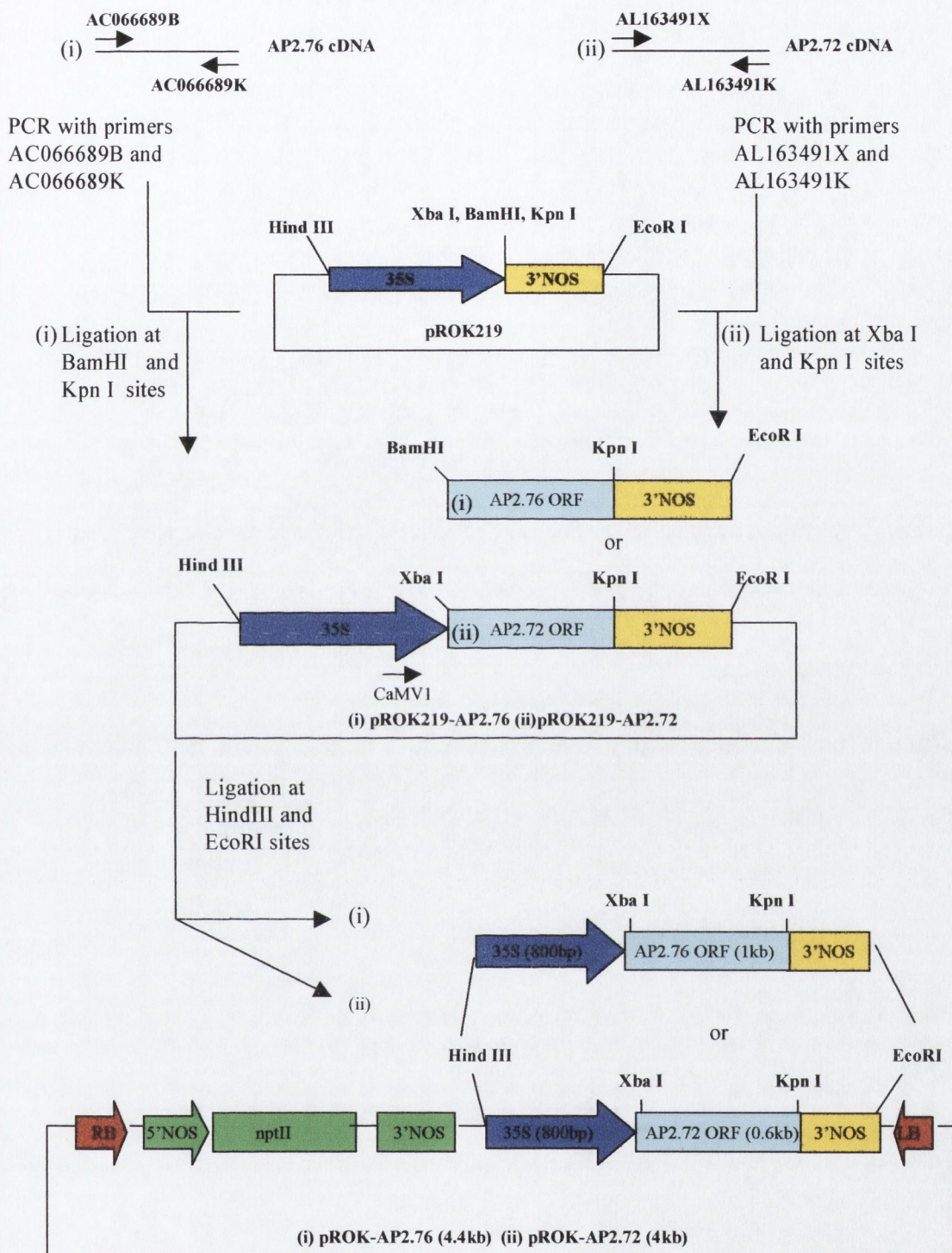


Figure 4.12. Construction of pROK-AP2.76 and pROK-AP2.72 which contain 35S-AP2.76 and 35S-AP2.72 fusions respectively. Construction of (i) AP2.76 and (ii) AP2.72 ORFs under the control of the cauliflower mosaic virus 35S promoter in a binary vector. The restriction sites shown were used for cloning the fragments into the corresponding vector.

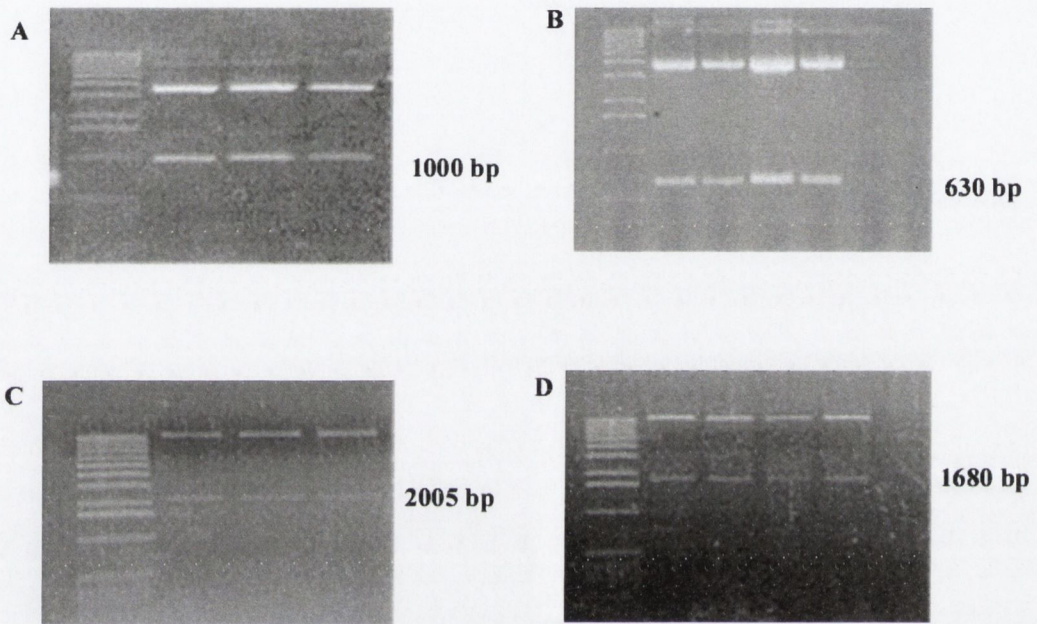


Figure 4.13. Verification of the structure of 35S-AP2.76 and 35S-AP2.72 fusion constructs.

Restriction digest of transformant clones containing (A) pROK219-AP2.76 vector with *Bam*HI/*Kpn*I restriction enzymes and (B) pROK219-AP2.72 vector with *Xba*I/*Kpn*I restriction enzymes.

Restriction digest of transformant clones containing (C) pROK-AP2.76 vector with *Hind*III/*Eco*RI restriction enzymes and (D) pROK2-AP2.72 vector with *Hind*III/*Eco*RI restriction enzymes.

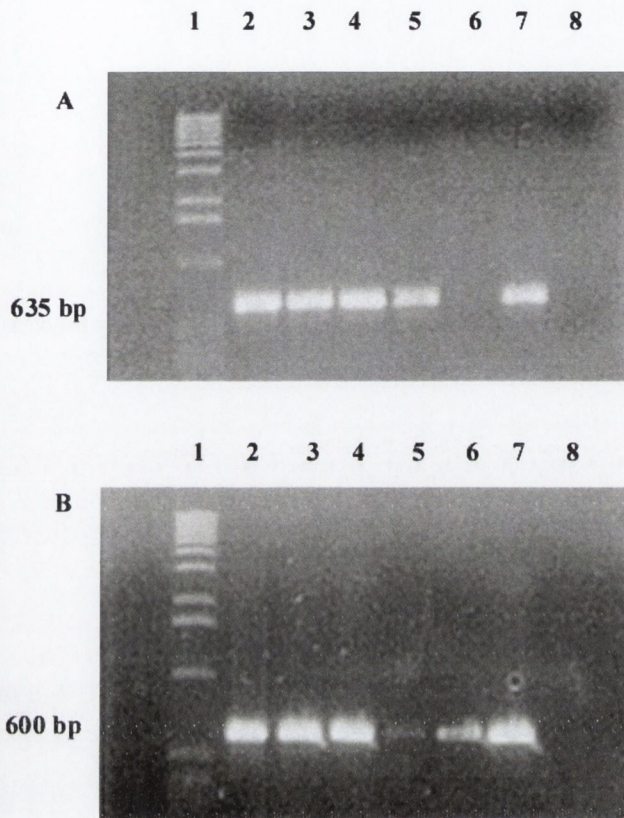


Figure 4.14. PCR analysis of transgenic plants expressing the *CaMV-AP2.76* and *CaMV-AP2.72* fusions.

A) PCR analysis of transgenic plants expressing the *CaMV-AP2.76* fusion, that were transformed with the pROK-AP2.76 plasmid. Primers specific for the *CaMV-AP2.76* fusion (AP76ORF and CaMV1) amplified a 635 bp fragment.

B) PCR analysis of transgenic plants expressing the *CaMV-AP2.72* fusion, that were transformed with the pROK-AP2.72 plasmid. Primers specific for the *CaMV-AP2.72* fusion (AP72ORF and CaMV1) amplified a 600 bp fragment.

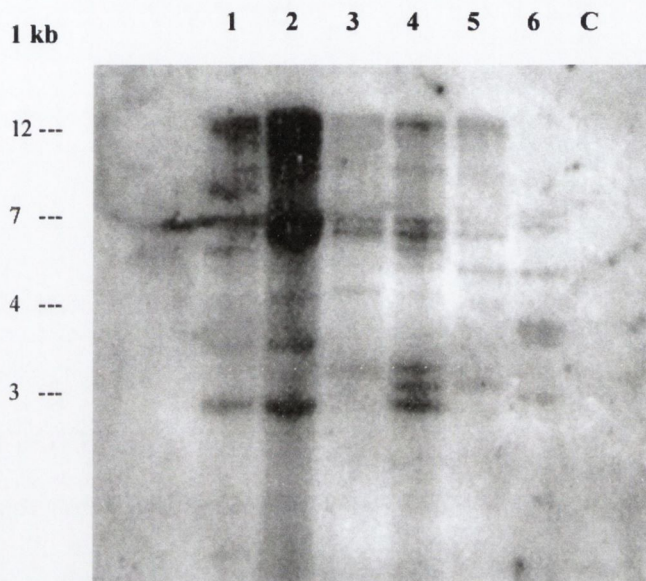


Figure 4.15. Southern blot analysis of plants expressing the 35S-AP2.76 fusion.

Total DNA of an untransformed plant as a negative control (C) and of six transgenic plants (lanes 1 to 6) were digested with *HindIII*. Hybridisation was performed using a *CaMV* probe that had been DIG labelled by PCR. Six transgenic plants (lanes 1, 2, 3, 4, 5 and 6) show the presence of the *CaMV* promoter at different copy numbers. 1 kb (1 kb ladder).

expression cassette) using a DIG-labelled CaMV35S promoter probe fragment of 453 bp (DIG-CaMV), generated using the CaMVF and CaMVR primers. The results presented in Figure 4.15 show the presence of several band of different size in each plant indicating the various lines represent independent transformations.

4.6.2.3 Identification of over-expression lines containing the 35S-AP2.76 and 35S-AP2.72 gene fusions

At least six progeny from each primary transgenic line were germinated on soil and allowed to set seed. The seeds (approximately 100) were then germinated on MS+ agar plates supplemented with km (50µg/ml). Three 35S-AP2.76 over-expression lines (OE76.1, OE76.2 and OE76.3) and three 35S-AP2.72 over-expression lines (OE72.1, OE72.2 and OE72.3) representing independent transformation events and showing 100% kanamycin resistance were selected for further analysis.

4.6.2.4 Phenotypic analysis of the OE72 and OE76 over-expression lines

Seeds from AP2.76 over-expression (OE76.3) and AP2.72 overexpression (OE72.1) lines (lines were chosen for strongest level of over-expression) with *Arabidopsis* ecotype Columbia as wild type control were grown under green house conditions as described in section 5.1.4.2. Plants were analysed for growth parameters such as germination, number of leaves, flowering time, and rosette radius as described in section 4.5.5. No obvious differences between wild type and AP2.72 over-expression plants (OE72.1) or between wild type and AP2.76 over-expression (OE76.3) were observed (data not shown).

This study strongly suggests that both AP2.76 and AP2.72 may play a role in regulating gene expression in response to abiotic stresses such as high salinity and dehydration. AP2.76 and AP2.72 transcripts were demonstrated to be induced by several abiotic stresses including high salinity (AP2.76 and AP2.72) and dehydration (AP2.76) (Chapter 3). In addition several genes known to be involved in dehydration and high salinity stresses were up-regulated in transgenic plants over-expressing the AP2.76 gene (Chapter 4, Part 3).

To investigate whether *AP2.76* and *AP2.72* over-expression led to enhanced salt tolerance or enhanced dehydration tolerance leaf discs from *AP2.76* over-expression plants (OE76.3) and from *AP2.72* over-expression plants (OE72.1) and were subjected to high salinity and dehydration stresses as outlined in section 4.5.5. Leaves were examined by comparing phenotype in transgenic to wild type leaves every 24hrs a 96hr period. No obvious differences in susceptibility to high salinity and dehydration were observed (data not shown).

4.6.3 Northern blot analysis of OE72 and OE76 over-expression lines

A northern blot analysis, using the DIG-AP76 probe showed the *AP2.76* transgene was expressed at high level in the three OE76 lines, contrasting with the endogenous *AP2.76* transcript which was not detectable in wild-type Columbia seedlings (Figure 4.16 (A)).

The *AP2.72* transgene was also expressed at high level (detected using the single-stranded DIG-AP72 probe; Figure 4.16 (B)) in the three OE72 lines analysed while the endogenous *AP2.72* transcript showed no significant accumulation in wild-type Columbia seedlings.

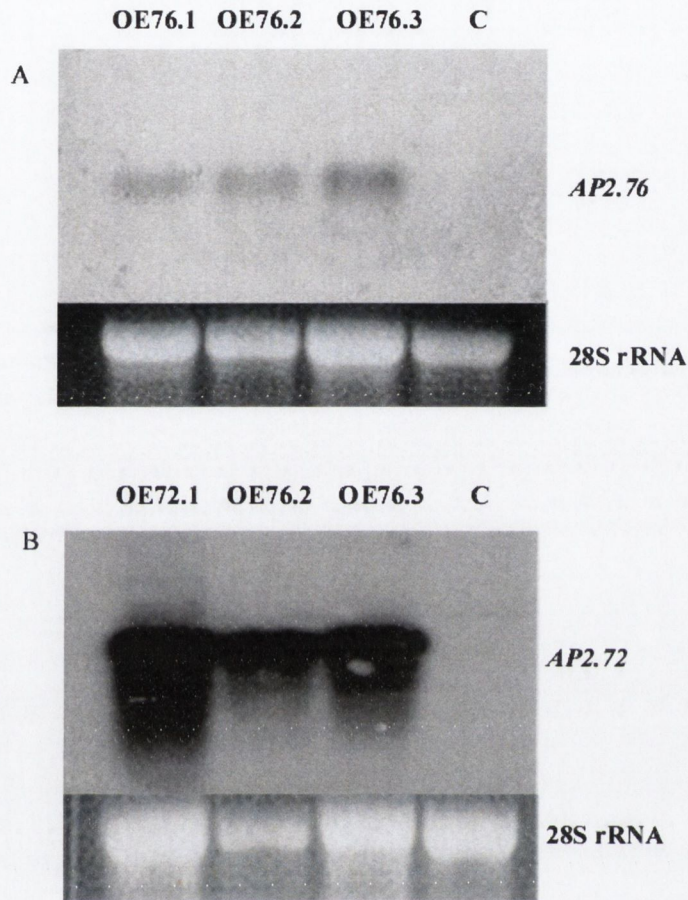


Figure 4.16 Accumulation of the *AP2.76* and *AP2.76* transcripts in transgenic plants expressing *35S-AP2.76* and *35S-AP2.72* fusions respectively.

A) Total RNA was isolated from three to four week old *35S-AP2.76* transgenic *Arabidopsis* seedlings (OE76.1, OE76.2 and OE76.3) and from wild-type seedlings (C). Hybridisation was performed with an *AP2.76* gene specific probe as indicated at right.

B) Total RNA was isolated from three to four week old *35S-AP2.72* transgenic *Arabidopsis* seedlings (OE72.1, OE72.2, OE72.3) and from wild-type seedlings (C). Hybridisation was performed with an *AP2.72* gene specific probe as indicated at right.

Part 3: Identification of putative regulatory targets of AP2.72 and AP2.76 by analysing gene expression in over-expression and knockout lines

4.7 Introduction

4.7.1 Target genes mediating defense responses in *Arabidopsis*

Activation of defense pathways by the signalling molecules, SA, JA and ET ultimately leads to the expression of a large number of target genes whose products participate directly in defending the plant against biotic and abiotic stress (Kunkel and Brooks, 2002). SA, for example, induces the expression of genes encoding acidic antimicrobial PR proteins including PR1 (encoding a protein of unknown function), PR2 (a β -1,3-glucanase) and PR5 (a thaumatin-like protein) (Uknes et al., 1992). The *cpr1*, *cpr5* and *cpr6* mutations cause elevated SA levels, constitutive expression of *PR1*, *PR2* and *PR5* and resistance to biotrophic pathogens *P. syringae* and *P. parasitica* (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998). Likewise, plants that carry mutations in *NPR1*, a gene that encodes a signalling component that functions downstream of SA, fail to induce the SAR marker genes *PR1*, *PR2* and *PR5*, and exhibit enhanced susceptibility to these pathogens (Glazebrook et al., 1996; Reuber et al., 1998).

JA and ET co-regulate a subset of PR genes encoding basic antimicrobial proteins such as PR3 (a chitinase) and PR4 (a hevin-like protein), PDF1.2 (a plant defensin) and Thi2.1 (an anti-fungal thionin) independently of SA. Indeed these genes are commonly used to monitor JA-dependent defense responses (Reymond and Farmer, 1998). *Arabidopsis* mutants that are impaired in JA perception e.g. *coi1* and *jar1* and the ethylene signalling mutant *ein2*, exhibit enhanced susceptibility to a variety of pathogens, including the necrotrophic fungal pathogens *A. brassiciola*, *B. cinerea* and *E. carotovvora* (Glazebrook et al., 1996; Reuber et al., 1998; Feys and Parker, 2000).

4.7.2 Molecular and genetic aspects of plant responses to environmental stresses

Plants respond to osmotic stress, imposed by high-salinity and drought by modulating gene expression which eventually leads to the restoration of cellular homeostasis, detoxification of toxins and recovery of growth (Xiong and Zhu, 2002). Two major groups of genes are induced by abiotic stresses (Shinozaki et al., 2003). The first group includes proteins that probably function in protecting cells from dehydration, such as enzymes required for the biosynthesis of various osmoprotectants, late embryogenesis abundant (LEA) proteins, antifreeze proteins, chaperones and detoxification enzymes (Seki et al., 2003). The second group contains protein factors involved in the further regulation of signal transduction and gene expression that probably function in the stress response: protein kinases, transcription factors and enzymes involved in phospholipid metabolism (Shinozaki and Yamaguchi-Shinozaki, 1997). Several different sets of *cis*- and *trans*-acting factors are known to be involved in stress-responsive transcription. Some of them are controlled by ABA but others are not, indicating the involvement of both ABA-dependent and independent regulatory systems for stress-responsive gene expression.

4.7.3 Microarray analyses of the *Arabidopsis* stress and defense transcriptome

DNA chip-based microarray technology is increasingly being used to monitor global changes in gene expression in *Arabidopsis* in response to abiotic and biotic stress. The abiotic stress transcriptome responding to drought, cold and high-salinity stresses has been investigated in four major studies: (Seki et al., 2001; Chen et al., 2002; Kreps et al., 2002; Oono et al., 2003). Microarray studies characterising the *Arabidopsis* defense transcriptome include: (Schenk et al., 2000; Cheong et al., 2002; Glazebrook et al., 2003; Mahalingam et al., 2003). These studies (a) facilitate the identification of hitherto unsuspected genes that are regulatory targets of the various signalling pathways, (b) the complexity of the respective pathways and (c) the extent of the overlap between them.

In order to identify putative regulatory targets of AP2.76, a cDNA microarray analysis of gene expression differences between the overexpression line OE76.3 and wild-type *Arabidopsis* was undertaken; this was followed by northern blot verification of candidate up-regulated genes. In addition, a comparative northern blot analysis of putative target gene expression levels in the overexpression lines (OE72.1, OE72.2 and OE72.3) versus wild-type plants was carried out in order to identify regulatory targets of AP2.72.

4.8 Materials and Methods

4.8.1 DNA manipulations

General materials and DNA manipulations have been described in chapter 5.

4.8.2 Plasmids

37F10 contains the *PDF1.2* cDNA cloned into the *Sall/NotI* sites of the pZL 1 vector; (Penninckx et al., 1996); Accession no: T04323 and was obtained from the Arabidopsis Biological Resource Centre (ABRC).

138H14 contains a thaumatin-like protein (Accession no: T46212) cDNA cloned into the *Sall/NotI* sites of the pZL 1 vector and was obtained from ABRC.

PR1cDNA contains the *PR1* (Accession no: AY117187) cDNA cloned into the *HindIII* and *BamHI* sites of the PGEM-T vector (Uknes et al., 1992) and was donated by Dr. Thomas Newman of the Department of Plant Physiology of The Pennsylvania State University

PR5cDNA contains the *PR5* (Accession no:CB185581) cDNA cloned into the *HindIII* and *BamHI* sites of the PGEM-T vector (Uknes et al., 1992) and was donated by Dr. Thomas Newman of the Department of Plant Physiology of The Pennsylvania State University.

U16022 contains the *PR3* (Accession no: AY001519) cDNA cloned into the pENTR/SD-dTopo vector and was obtained from ABRC.

U15591 contains the *PR4* (Accession no: BT000046) cDNA cloned into the pENTR/SD-dTopo vector and was obtained from ABRC.

Thi2.1cDNA contains the *Thi2.1* cDNA cloned into the *EcoRI* site of pUC19 (Epple et al., 1995);(Accession no: L41244) and was donated by Dr. Epple of the Swiss Federal Institute of Technology, Zurich.

JR1cDNA contains the *JR1* cDNA cloned into the *Sall/NotI* sites of the pZL 1 vector and was donated by Dr. Sanchez-Serrano of the Department of Plant Molecular Genetics of the National Centre for Biotechnology Madrid.

JR3cDNA contains the *JR3* cDNA cloned into the *NotI* site of pUC19 and was donated by Dr. Sanchez-Serrano of the Department of Plant Molecular Genetics of the National Centre for Biotechnology Madrid.

RAFL07-11-M21 contains the *rd29A* cDNA that was cloned in pBluescript and was obtained from RIKEN Genomic Sciences Centre (Accession no:AY054465).

RAFL05-04-C07 contains the *ERD10* cDNA that was cloned in pBluescript and was obtained from RIKEN Genomic Sciences Centre (Accession no: AY048208).

CAB.cDNA contains chlorophyll A/B binding protein (CAB) cDNA (Accession no: AI994859) and was obtained from Incyte Genomics.

VSP.cDNA contains vegetative storage protein (VSP) cDNA (Accession no: AI999152) and was obtained from Incyte Genomics.

4.8.3 Oligonucleotide primers

Primers used to generate gene-specific probes for expression analysis in *35S-AP2.76* and *35S-AP2.72* over-expression lines and in *AP2.76-DsE* and *AP2.72-dSpm* knockout lines are described in Table 1.

Probe name	Primers (5'-3')
DIG-PDF	PDFA: 5'CTAAGTTTGCTTCCATCATCACCTTATA3' PDFB: 5'ATGTAAAATACACACGATTTAGCACCAAAG3'
DIG-Th	ThA: 5'CTCCACAAGGAGGCTCGGGTTTAAACTG3' ThB: 5'GGTACTACTCTGACAACCGTAACCGTACTG3'
DIG-PR1	PR1A: 5'CAAGATAGCCCACAAGATTATCTAAGGG3' PR1B: 5'GCATGATCACATCATTACTTCATTAGTAGTG3'
DIG-PR5	PR5A: 5'CACTCTAAGGAACAATTGCCCTACCACCG3' PR5B: 5'GTGATTTCTGAGTTAGCTCCGGTACAAGTG3'
DIG-PR3	PR3A: 5'CTACAGGTGGATGGGCTACAGCACCG3' PR3B: 5'ACTAAATAGCAGCTTCGAGGAGGCC3'
DIG-PR4	PR4A: 5'GGACAACAATGCGGTCTGCAAGGCG3' PR4B: 5'GCATGTTTCTGGAATCAGGCTGCCC3'
DIG-Thi	ThiA: 5'GCTCATAATGAGTCTGGTCATGGCACAAGT3' ThiB: 5'CAGTTTAGGCGGCCAGGTGGGACTACATA3'
DIG-JR1	JR1F: 5'GTCACCGATAGAGATCGTCACAGCTC3' JR1R: 5'CAAACCTCTCTGCTCCAAGCAATGTCT3'
DIG-JR3	JR3F: 5'GTTATCACAAGGCAAGCGAGCGTG3' JR3R: 5'TCAGAGTAGAAGAATAGGAGAAGCCAATG3'
DIG-rd29	Rd29F: 5'CTCTGCTTTCTGGAACAGAGGATGTAACG3' Rd29R: 5'CAGCTCCTGATTCACTACCAAAGCCCATC3'
DIG-Erd	ErdF: 5'CGTTCAGAGCAGGAGACCCCTAAGGT3' ErdR: 5'TGCTGTTTCAACCAGCGGTGTGGTGT3'
DIG-VSP	VSPF: 5'GCTGGCACCTTGGTGTGAGACCTCTAA3' VSPR: 5'ACGCCAGCAGCTTCGAGATTGTTCGAG3'
DIG-CAB	CABF: 5'CCGTCCCAAATACCTAGGACCATTTTCC3' CABR: 5'TCCAAACATTGAGAACATAGCAAGACGACC3'

Table 1: Primers used to generate specific probes for various pathogen-inducible genes (*PDF1.2*, *PR1*, *PR5*, *PR3*, *PR4* and *Thi2.1*); dehydration-inducible genes (*rd29A* and *ERD10*); various wound-inducible genes (*JR1* and *JR3*) and genes identified as *AP2.76*-inducible genes by the cDNA microarray analysis (*CAB*, *VSP*).

4.8.4 Northern blot analyses

Total RNA was extracted from unstressed seedlings of the over-expression 35S-*AP2.76* and 35S-*AP2.72* lines and from unstressed seedlings of the homozygous knockout lines *AP2.76-DsE* and *AP2.72-dSpm*. Seedlings were germinated on MS+ medium and maintained as described in section 5.1.4.1. Specific probes for several pathogen inducible genes (*PDF1.2*, *PR1*, *PR2*, *PR5*, *PR3*, *PR4* and *Thi2.1*); dehydration inducible genes (*rd29A* and *ERD10*); a gene associated with light signalling (*CAB*) and various wound inducible genes (*JR1*, *JR3* and *VSP*) were generated by PCR using the primers described in Table 1 and the respective cDNA clones described in section 4.9.3 as templates, using the DIG probe synthesis kit. Northern hybridizations were carried out as described in section 5.2.15.

4.8.5 Microarray analysis

Total RNA was extracted from seedlings of OE76.3 line and from wild-type *Arabidopsis* ecotype Columbia and submitted to the Flanders Interuniversity Institute for Biotechnology (VIB) Microarray Facility. The array consists of approximately 4,600 unique cDNA clones from Incyte Genomics and 2000 genes encoding transcription factors that were supplied by REGIA. Microarray preparation, hybridisation and scanning was performed as described by VIB (<http://www.microarrays.be/Home.htm>). Hybridisations were performed twice using a dye-swap replication (Wild-type RNAs labelled with CyS(red), mutant or over-expression RNAs labelled with Cy3(Green) on the first hybridisation, and vice versa for the second one). Because each cDNA was printed in duplicate on the array, four data points for every gene were obtained. Data and statistical analysis were performed also by VIB according to the method of (Kerr and Churchill, 2001). The induction/repression fold was expressed as the base 2 logarithm ratio of the hybridisation signal relative to the background signal in *AP2.76* over-expression plants, to the hybridisation signal relative to the background signal in wild-type plants.

The sequence corresponding to the promoter region of each gene that was found to be up-regulated more than 2 fold in *AP2.76* over-expressing plants was deduced from genomic sequences. From these intergenic sequences, up to 500 bp upstream of the ATG start codon

were extracted and subjected to motif searches to retrieve *cis*- acting elements. Both the position and frequency of occurrence were determined with the publicly available MatInspector (<http://www.genomatrix.de/cgi-bin/matinspector/matinspector.pl>).

4.9 Results

4.9.1 Over-expression of *AP2.76* in *Arabidopsis* causes upregulation of PR genes

Since our previous findings suggested that *AP2.76* might play a role in the regulation of response to pathogens, the expression of several different classes of PR genes was examined in the *AP2.76* over-expression lines OE76.1, OE76.2 and OE76.3. As shown in Figure 4.17, over-expression of *AP2.76* caused a significant increase in the steady state abundance of *PR3*, *PR4* & *PDF1.2* transcripts, each of which is known to (a) be induced by JA and ET; (b) contain a GCC box element in their promoters and (c) encode a protein with known antimicrobial properties (Thomma et al., 1998; Schenk et al., 2000). The effect of the over-expression of *AP2.76* on the expression of *Thi2.1*, which encodes the potent anti-fungal defense protein thionin, was also examined (Epple et al., 1995, 1997). Figure 4.17 (E) shows that the transcript abundance of *Thi2.1* was not increased in transgenic plants over-expressing *AP2.76*.

These results confirm that *AP2.76* functions as a *trans*-acting factor in the JA/ET mediated signalling transduction cascade that leads to the activation of the basic PR genes (*PR3* & *PR4*) and genes encoding plant defensins and thionins (Penninckx et al., 1996; Thomma et al., 1998; Pieterse and van Loon, 1999).

4.9.2 Microarray analysis

4.9.2.1 Over-expression of *AP2.76* in *Arabidopsis* induced the expression of genes associated with dehydration, high-salinity and wounding stresses

To identify genes which are regulated by *AP2.76*, putative target gene expression in transgenic line OE76.3 over-expressing *AP2.76* was compared with that in wild-type *Arabidopsis* (ecotype Columbia) using a VIB cDNA microarray,

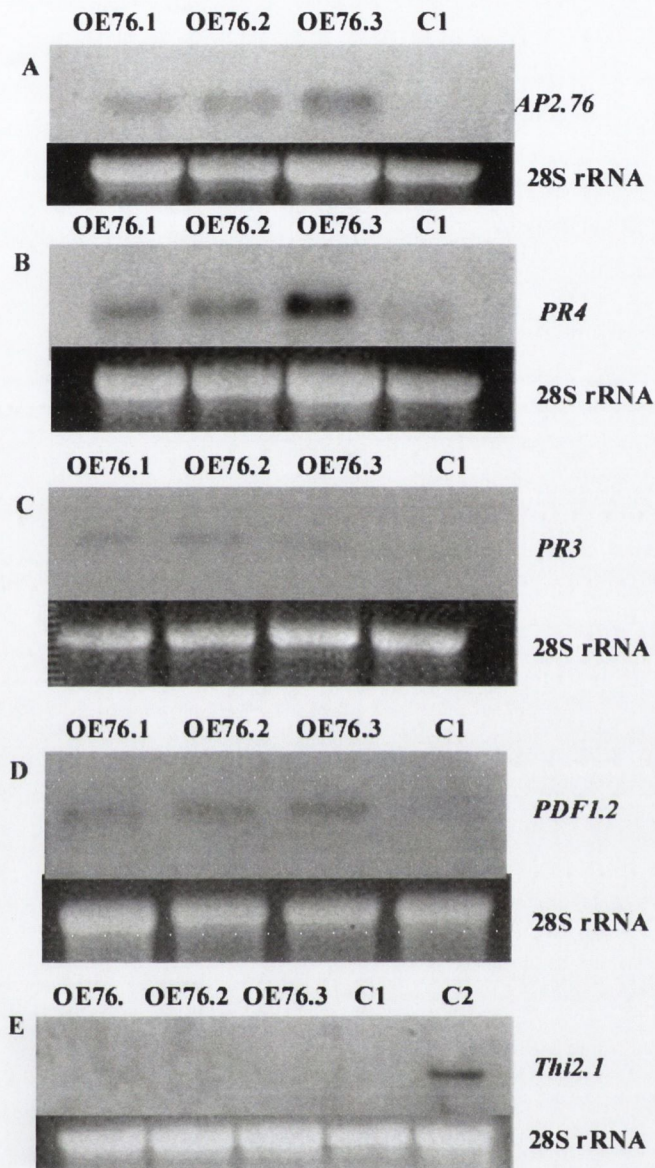


Figure 4.17. Overexpression of *AP2.76* in *Arabidopsis* upregulates PR genes. Expression of pathogen inducible genes in *35S-AP2.76* (OE76.1, OE76.2 & OE76.3); in control wild-type seedlings under normal conditions (negative control (C1)) and in wild-type seedlings treated with 100 μ M jasmonic acid (positive control (C2)). Total RNA was isolated from 3-4 week old seedlings and analysed by RNA gel blot hybridisation using the DIG-*AP2.76* probe as an *AP2.76* gene specific probe. RNA gel blotting also was conducted to measure the amount of *PR4*, *PR3*, *PDF1.2* and *Thi2.1* mRNA in control and transgenic plants carrying the *35S-AP2.76* fusion. Numbers indicate independent homozygous lines of *35S-AP2.76* transgenic plants.

Functional category	Gene accession	ORF Name	Description	Fold Induction
Lipid-transfer protein	AI995855	At5g59320	Lipid-transfer protein-like	7.9
Detoxification enzyme	AI993444	At4g25100	Superoxide dismutase	2.7
Transport proteins	AI996898	At2g29650	Na ⁺ -dependent inorganic phosphate transporter	2.1
	AI994399	At5g26240	CLC-d chloride channel protein	2.1
Wound responsive	AI999546	At5g24780	Vegetative storage protein VSP1	2.4
	AI999152	At5g24770	Vegetative storage protein2 VSP2	2.8
Stress responsive	AI996273	At5g25610	RD22, responsive to dehydration, mediated by ABA	2.8
	AI997801	At5g67030	Similar to stress inducible gene in drought tolerant cowpea	2.7
	AI995008	At1g06040	Salt tolerance protein	2.4
Protease Inhibitor	AI995265	At1g73330	Protease inhibitor	2.2
Carbohydrate metabolism	AI994456	At4g26530	Fructose-biphosphate aldolase	3.8
	AI993488	At5g37940	NADP-dependent oxidoreductase	2.0
Transcription factors	AI995298	At1g71030	Myb family transcription factor	2.4
	AI994179	At5g57660	Zinc finger family transcription factor	2.1
	AI994172	At3g59060	bHLH-related transcription factor	2.1
	AW004110	At3g53530	Heavy metal associated domain-containing TF	2.6
	AI997323	At2g30600	POZ domain family TF	2.0
Photosynthesis	AI994859	At3g27690	Light harvesting chlorophyll <i>a/b</i> binding protein	6.2
	AI996279	At1g61520	Chlorophyll <i>a/b</i> binding protein	2.8
	AI996962	At1g03130	Photosystem I subunit III protein	2.6
	AI997939	At5g64040	Photosystem I subunit precursor	2.6
Others	AI996702	At5g19140	Aluminium induced protein	2.4
	AI999399	At5g13630	Cobalamin biosynthesis protein	2.4
	AI994485	At3g59680	Unknown protein	2.2
	AI997421	At1g08580	Expressed protein	2.2
	AW004448	At3g15620	Unknown protein	2.2
	AI998286	At3g10020	Unknown protein	2.2
	AI997281	At1g08380	Expressed protein	2.1
	AI994637	At5g24490	Expressed protein	2.0
	AI998176	At2g42610	Expressed protein	2.9

Table 2. *Arabidopsis* genes up-regulated twofold or more in AP76 over-expression plants sorted according to functional category.

containing approximately 6600 *Arabidopsis* genes. Of these genes, only 30 exhibited a greater than 2-fold increase in expression in the over-expression line when compared with the wild-type. Many of the genes that were identified have been implicated in responses to abiotic stresses such as dehydration, wounding and high-salinity. The microarray data (Table 2) revealed a 7.9 fold induction of a lipid transfer protein in the over-expression line. Lipid transfer proteins may function in the repair of stress-induced damage in membranes or changes in the lipid composition of membranes, perhaps to regulate permeability to toxic ions and the fluidity of the membrane (Torres-Schumann et al., 1992; Seki et al., 2002); several microarray studies have identified lipid transfer proteins as dehydration and high-salinity inducible proteins (Seki et al., 2001; Seki et al., 2002).

Detoxification enzymes such as superoxide dismutases are thought to be involved in the elimination of reactive oxygen species (ROS) whose levels increase when plants are exposed to biotic or abiotic stress conditions (Xiong and Zhu, 2002). Several reports have shown that over-expression of superoxide dismutases leads to increased tolerance to abiotic stresses such as dehydration and high-salinity (Bohnert and Sheveleva, 1998). As shown in Table 2, the over-expression of *AP2.76* increased the expression of a superoxide dismutase gene 2.7-fold, consistent with a putative role for *AP2.76* in the regulation of dehydration and high-salinity gene expression.

Transport proteins such as Na⁺ dependent phosphate co-transporter have previously been shown to be induced by dehydration, cold and high-salinity stresses (Seki et al., 2002).

Several studies have shown that vegetative storage proteins (VSPs) are induced by the bacterial necrotroph *E. carotovora*, wounding, jasmonic acid, dehydration and high-salinity stresses (Staswick, 1990; Zhu, 2001; Guerineau et al., 2003). Table 2 shows two VSP genes (*VSP1* and *VSP2*) were significantly up-regulated in transgenic plants over-expressing *AP2.76* consistent with our data suggesting a role for *AP2.76* in the wound and dehydration response pathways.

The microarray analysis presented in Table 2 also showed up-regulation of a protease inhibitor in OE76.3 transgenic plants and several published microarrays have shown that dehydration and high-salinity cause an up-regulation of several protease inhibitors (Kawasaki et al., 2001; Seki et al., 2001; Seki et al., 2002). Table 2 also showed that other genes associated with abiotic stress such as *rd22* (a dehydration-responsive gene that is induced by

ABA (Abe et al., 1997)), a gene similar to a stress-inducible gene in drought tolerant cowpea and a salt tolerance protein were also significantly up-regulated. Several genes encoding transcription factors were upregulated e.g. member of the basic helix-loop-helix (bHLH) family, the bZIP family and the MYB family.

Taken together these results complement our previous findings showing *AP2.76* was induced by dehydration, high-salinity and wounding stresses and suggest a role for *AP2.76* in the regulation of target genes mediating the plant's response to abiotic stress.

4.9.2.2 Promoter analysis of genes determined by microarray analysis to be *AP2.76* inducible genes

Microarray analysis identified 30 genes that were up-regulated in response to constitutive overexpression of *AP2.76*. The nucleotide sequence corresponding to the promoter regions of 25 of these genes was deduced from the corresponding genomic sequence. Approximately 500 bp upstream of the ATG start codon was extracted and subjected to motif searches. Table 3 summarises the occurrence of ABRE, W-box, DRE core (CCGAC) and GCC-box sequences in the promoter regions.

At least one W-box motif (TTGAC(C/T)) was identified in the promoter regions of 13 genes, significantly higher than the 9 occurrences expected in a set of 25 random promoters as determined by Chakravarthy et al. (2003). Seven genes contained the ABRE element (PyACGTG(T/G)C) in their promoters, suggesting they are ABA-inducible and may be controlled by an ABA-dependent pathway. In addition, 5 genes contained the DRE/CRT-related CCGAC core motif in their promoters. In total, of the 25 promoters analysed in detail, 7 contained none of the *cis*-elements that were analysed (ABRE, W-box, DRE-box or GCC-box). These were examined further but no single *cis*-element that was shared by a majority of these promoters was identified.

Gene accession	Description	ABRE (PyACGTG(T/G)C)	DRE core (CCGAC)	W box (TTGAC)	GCC core (GCCGCC)
AI995855	Lipid-transfer protein-like			TTGAC (-230)	
AI993444	Superoxide dismutase			TTGAC (-410)	
AI996898	Na ⁺ -dependent inorganic phosphate transporter				
AI994399	CLC-d chloride channel protein				
AI999546	Vegetative storage protein VSP1			TTGAC (-460)	
AI999152	Vegetative storage protein2 VSP2			TTGAC (-496)	
AI996273	RD22, responsive to dehydration, mediated by ABA			TTGAC (-446) TTGAC (-314)	
AI997801	Similar to stress inducible gene in drought tolerant cowpea	GACGTGTC (-445)	CACCGACGA (-13)	TTGAC (-230) TTGAC (-440)	
AI995008	Salt tolerance protein			TTGAC (-460) TTGAC (-478)	
AI995265	Protease inhibitor		TTCCGACAA (-33)		
AI994456	Fructose-biphosphate aldolase	TACGTGTC (-140)	AACCGAGAA (-419)		
AI993488	NADP-dependent oxidoreductase				
AI995298	Myb family transcription factor			TTGAC (-120)	
AI994179	Zinc finger family transcription factor	CACGTGTC (-180)	TACCGACAT (-420)	TTGAC (-224)	
AI994172	bHLH family transcription factor				
AW004110	Heavy metal associated domain-containing protein				
AI997323	POZ domain family transcription factor				
AI994859	Light harvesting chlorophyll <i>a/b</i> binding protein	GACGTGTC (-130)			
AI996279	Chlorophyll <i>a/b</i> binding protein	CACGTGGC (-195)			
AI996962	Photosystem I subunit III protein			TTGAC (-73) TTGAC (-442)	GCCGCC (-475)
AI996702	Aluminium induced protein				
AI999399	Cobalamin biosynthesis protein	CACGTGTC (-203)	ATCCGACAA (-260) ACCCGACGA (-488)	TTGAC (-380)	
AI997421	Expressed protein			TTGAC (-221)	
AI998286	Unknown protein	AACGTGTC (-455)		TTGAC (-225) TTGAC (-352) TTGAC (-425) TTGAC (-460)	
AI998176	Expressed protein			TTGAC (-166)	

Table 3. ABRE, CCGAC core sequences, W-box motif and GCC-box sequences observed in the promoter regions of the *AP2.76*-inducible genes identified by microarray analysis.

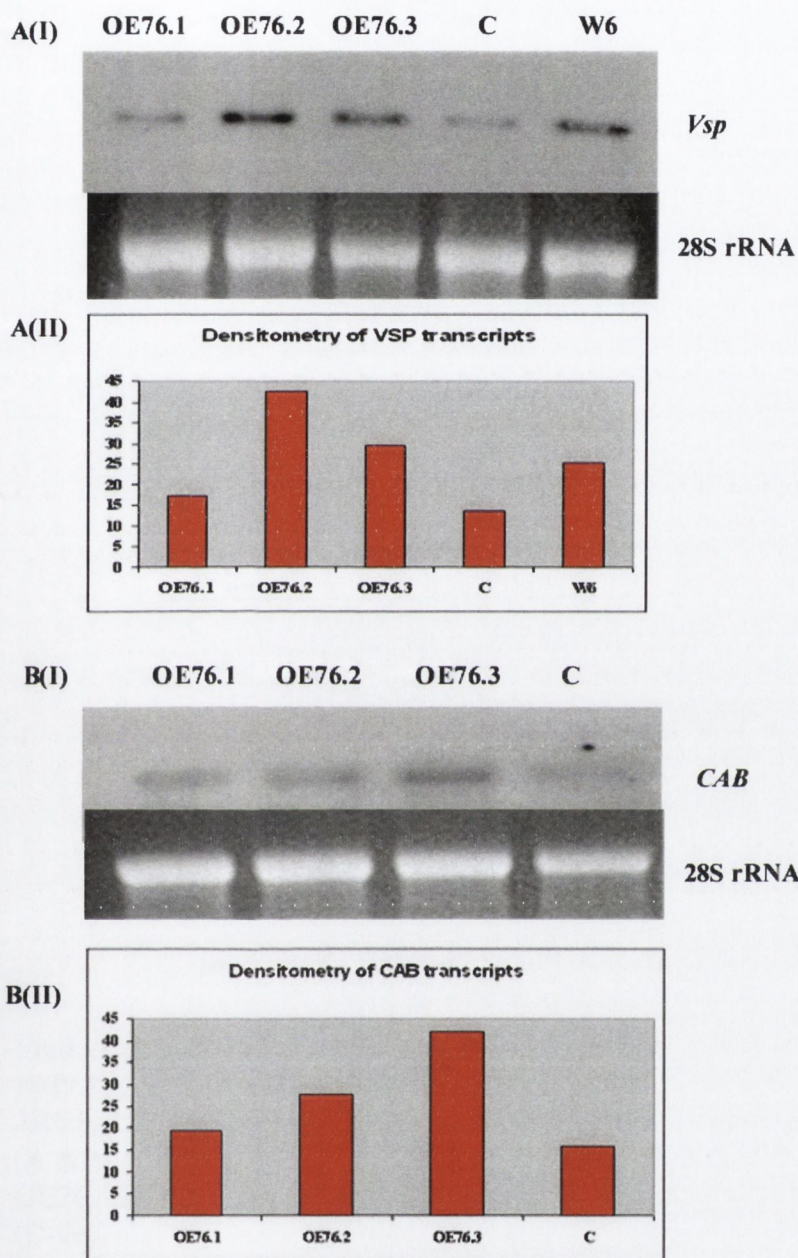


Figure 4.18. Expression of *Vsp* and *CAB* in 35S-AP2.76 transgenic plants that over-express AP2.76.

A(I) Expression of *Vsp* in OE76.1, OE76.2 & OE76.3 lines; in wild-type Columbia plants (C); and in wild-type Columbia plants that were subjected to wounding treatment 6 hours before RNA extraction (W6).

A(II) *Vsp* relative transcript accumulation as determined by densitometry.

B(I) Expression of *CAB* in OE76.1, OE76.2 & OE76.3 lines; and in wild-type Columbia plants (C).

B(II) *CAB* relative transcript accumulation as determined by densitometry.

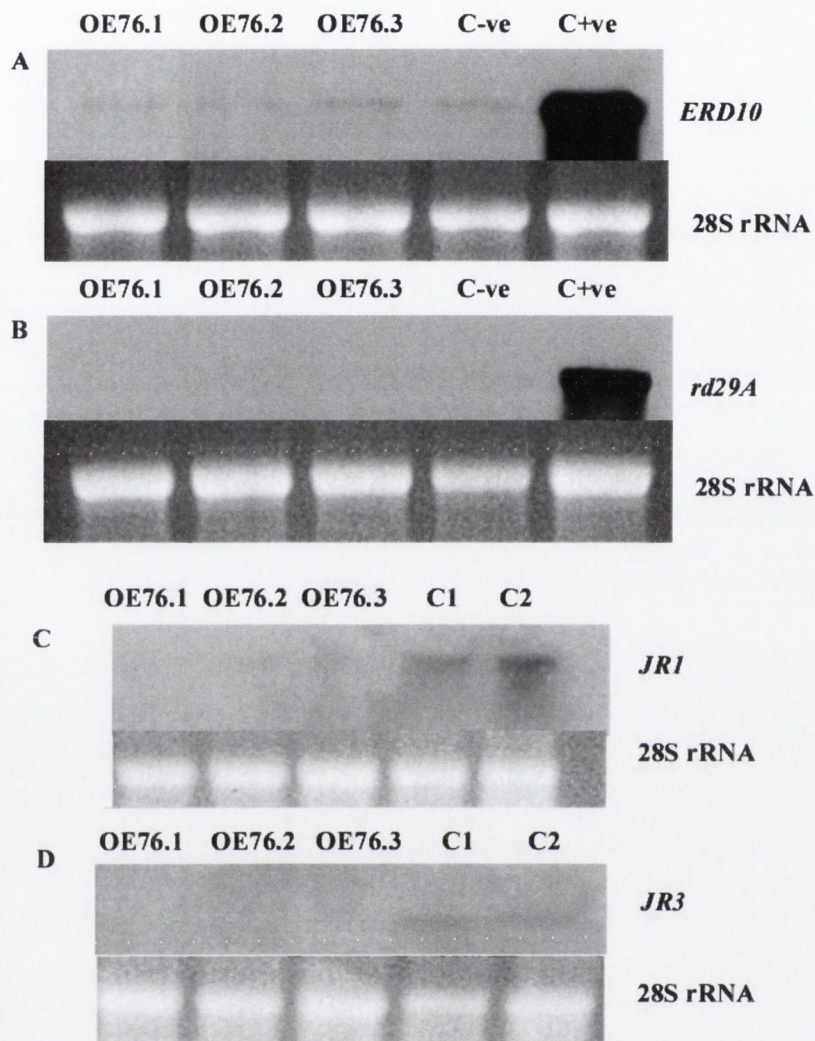


Figure 4.19. Expression of the dehydration-responsive genes *rd29A* and *ERD10* and the wound-responsive genes *JR1* and *JR3* in transgenic *Arabidopsis* plants overexpressing *AP2.76*. (A & B) Expression of *ERD10* and *rd29A* in OE76.1, OE76.2 and OE76.3 lines; in unstressed wild-type seedlings (negative control (C-ve)) and in wild-type seedlings that were exposed to dehydration for 2 hours (positive control (C+ve)). Total RNA was isolated from 3-4 week old seedlings and analysed by RNA gel blot hybridisation using *ERD10* and *rd29A* gene specific probes, as indicated on right. (C & D) Expression of *JR1* and *JR3* in OE76.1, OE76.2 & OE76.3 lines and in wild-type seedlings that were wounded for 1 hour (C1) and 5 hours (C2) as positive controls. Total RNA was isolated from 3-4 week old seedlings and analysed by RNA gel blot hybridisation using *JR1* and *JR3* gene specific probes, as indicated on right. Ethidium bromide staining of the RNA gel was used to show equal loading.

was examined in the OE76 lines. As shown in Figure 4.19, over-expression of *AP2.76* did not cause an increase in the abundance of *JR1* or *JR3* transcripts.

4.9.4 Expression of *AP2.72* and various pathogen-related target genes in overexpression and knockout lines

Our previous studies suggested that the SA-inducible *AP2.72* might play a role in SAR and therefore regulate the *PR* genes. For this reason, the expression of *PR1* and *PR5* (whose expression levels serve as convenient markers for monitoring SAR (Dong, 1998)) was examined in the overexpression lines OE72.1, OE72.2 and OE72.3 and in homozygous and heterozygous *AP2.72* knockout lines (*AP2.72-dSpm*).

AP2.72 transcripts accumulated at high levels in all three OE72 lines compared with wild-type plants. In contrast, even following induction with SA, *AP2.72* transcripts were undetectable in plants homozygous for the *AP2.72-dSpm* insertion. However, SA-treated plants heterozygous for the *AP2.72-dSpm* insertion showed a slight accumulation and wild-type Columbia O plants strong accumulation of *AP2.72* transcripts (Figure 4.20 (A)).

A northern blot analysis using a *PR1*-specific probe (DIG-PR1) showed that over-expression of *AP2.72* did not increase *PR1* transcript abundance (Figure 4.20 (B)). Moreover, the level of accumulation of *PR1* transcripts in *AP2.72-dSpm* knockout plants that had been treated with SA for 5 hours was similar to the level found in wild-type control Columbia O plants. These results suggest that *AP2.72* is not involved in the direct *trans*-activation or repression of *PR1* transcription. Figure 4.20 (C) shows a northern blot carried out using a *PR5*-specific probe (DIG-PR5). No significant accumulation of *PR5* transcripts was detected in the OE72 lines or in wild-type Columbia plants.

SAR is believed to be a result of the concerted activation of pathogenesis-related (*PR*) gene transcription. Since the expression levels of *PR1* and *PR5* were not altered in the OE72 lines or *AP2.72* knockout mutants, we think it unlikely that *AP2.72* is directly involved in the development of SAR.

Our previous analysis of the expression of *AP2.72* suggested that it might also be involved in a defense response pathway that is distinct from the SA-mediated SAR pathway but requires JA and ET as signalling molecules which induce expression of other *PR* genes

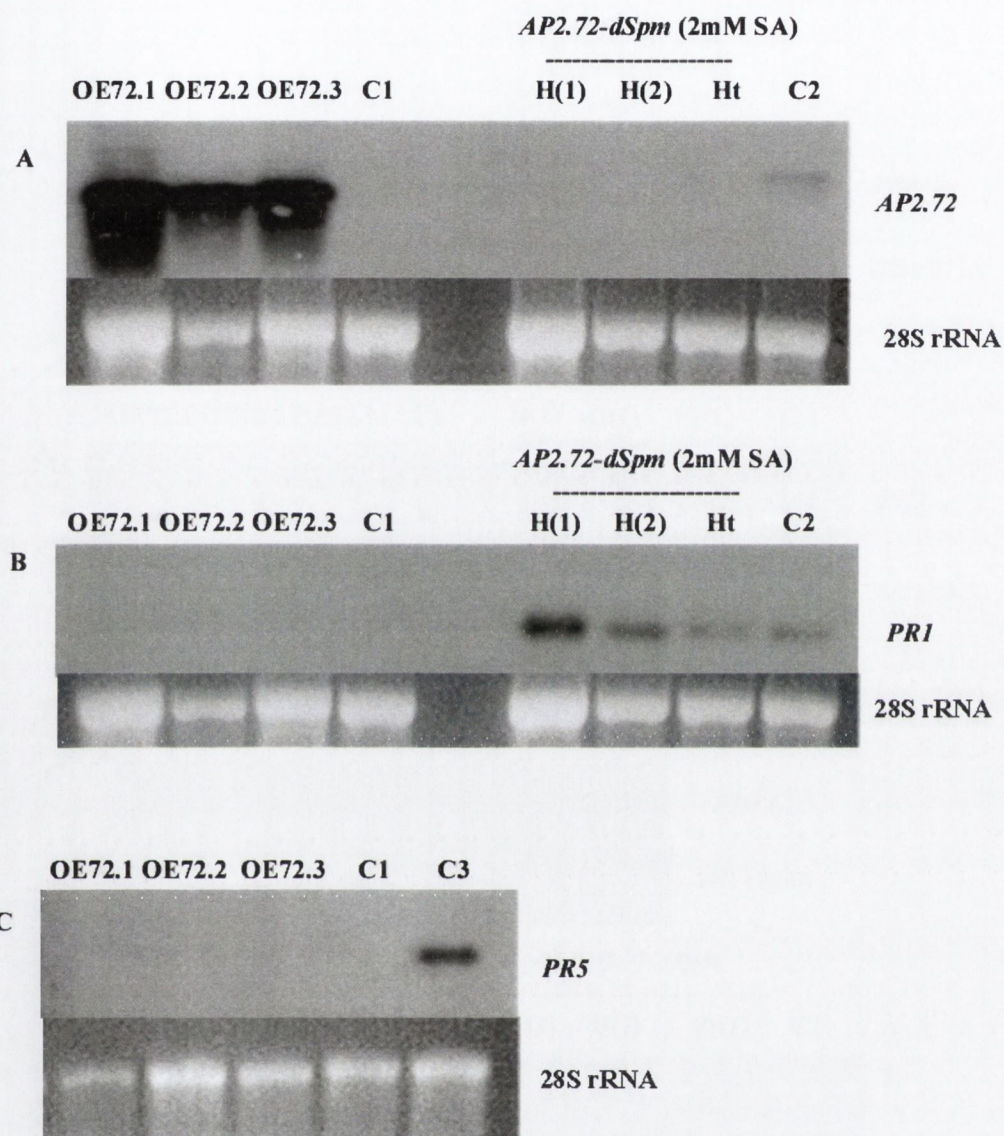


Figure 4.20. Expression of the SAR marker genes in transgenic *Arabidopsis* plants over-expressing *AP2.72* and in *AP2.72* knockout mutants.

Expression of *AP2.72* (A), *PR1* (B) and *PR5* (C) in lines OE72.1, OE72.2 and OE72.3 and in control wild-type plants (C1); in homozygous and heterozygous (H(1), H(2) & Ht(1)) *dSpm-AP2.72* knockout mutant plants and in control wild-type plants (C2) that had been treated with 2mM SA for 5 hours; in wild-type plants that had been treated with 2mM salicylic acid for 7 hours as a positive control for *PR5* (C3).

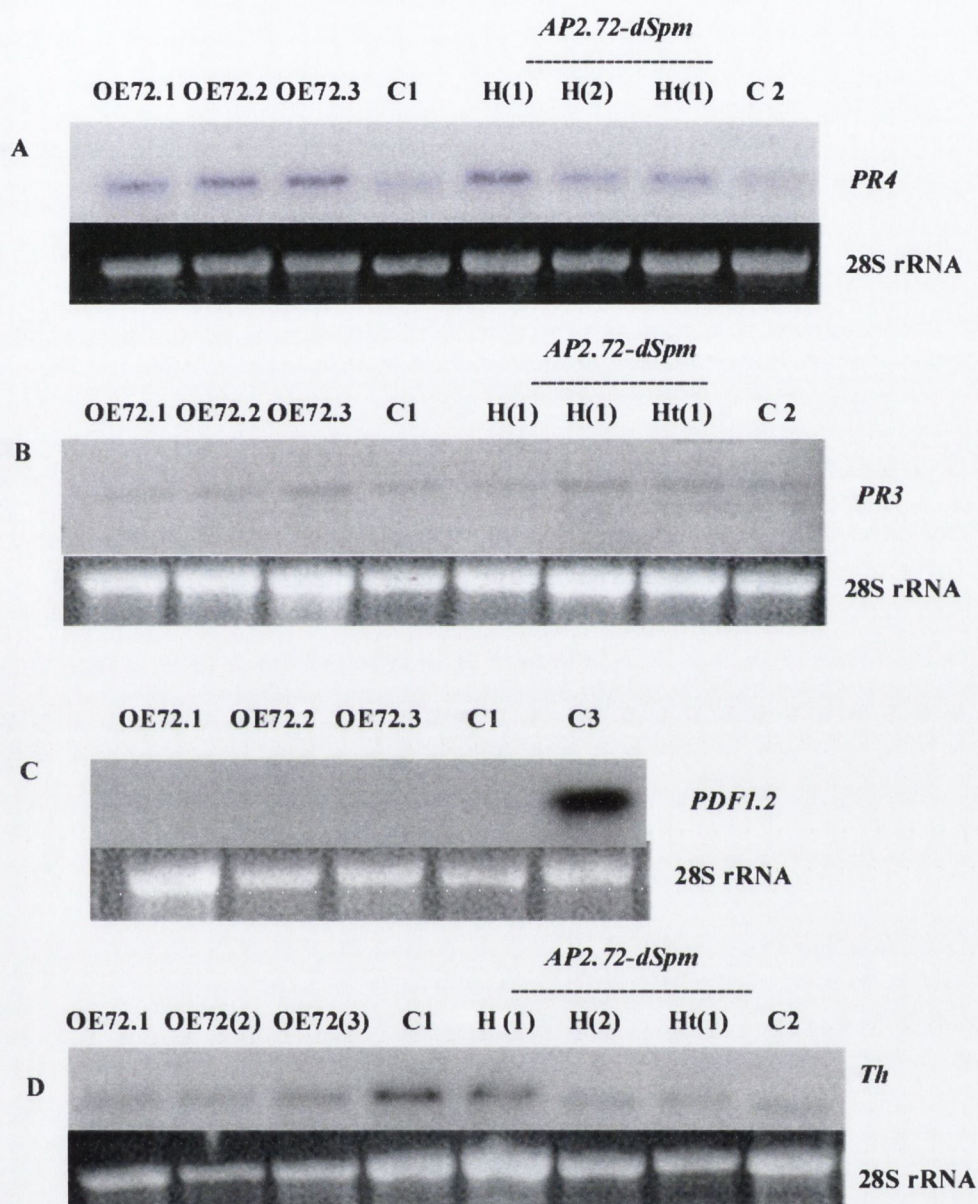


Figure 4.21. Expression of various pathogen inducible genes in transgenic *Arabidopsis* plants over-expressing *AP2.72* and in *AP2.72* knockout mutants. Expression of *PR4* (A), *PR3* (B), *PDF1.2* (C) and (D) *Th* in OE72.1, OE72.2 and OE72.3 lines and in control wild-type plants (C1); in homozygous and heterozygous (H(1), H(2) and H(Ht) *dSpm-AP2.72* knockout mutant plants and in control wild-type plants (C2); and in wild-type plants that had been treated with 100μM jasmonic acid as a positive control (C3) for *PDF1.2*.

(e.g. *PR3* & *PR4*) and plant defensin and thionin genes (*PDF1.2* and *Thi2.1*) (Penninckx et al., 1996; Thomma et al., 1998; Pieterse and van Loon, 1999). To investigate this hypothesis, the expression of *PR3*, *PR4* and *PDF1.2* was examined in OE72 and in *dSpm-AP2* lines by northern blot analysis.

As shown in Figure 4.21, *AP2.72* overexpression did not cause an increase in the accumulation of *PR3*, *PR4*, *PDF1.2* or *Th*. Neither were *PR3*, *PR4* or *Th* transcript levels affected in the *dSpm-AP2.72* knockout plants, compared with wild-type. These results indicate that *AP2.72* does not appear to act as a direct *trans*-acting factor in the JA/ET-mediated defense pathways.

4.10 Discussion

4.10.1 Activation of JA/ET-regulated genes by *AP2.76*

The anti-microbial PR proteins are highly induced and accumulate to high levels in leaves following infection by viruses, bacteria and fungi (Bowles, 1990). Analysis of PR gene promoters has led to the identification of an 11-bp *cis*-acting element, TAAGAGCCGCC (Hart et al., 1993; Ohme-Takagi and Shinshi, 1995; Sessa et al., 1995), referred to as the GCC box (Ohme-Takagi and Shinshi, 1995). Several members of the ERF/AP2 transcription factor subfamily bind specifically to the GCC box and mediate the high level expression of genes such as *PDF1.2*, *PR3* and *PR4* (Fujimoto et al., 2000; Park et al., 2001; Gu et al., 2002). Our previous data, based on sequence comparisons (Chapter 2), led to the hypothesis that *AP2.76*, might be involved in GCC-box mediated transcription. The induced expression of GCC-box containing genes such as *PDF1.2*, *PR3* and *PR4* in *AP2.76* over-expression plants support this prediction and suggest that *AP2.76* may bind the GCC-box specifically. Clearly, further investigation into the DNA binding preference of *AP2.76* is needed to confirm this hypothesis.

4.10.2 Expression of *AP2.76* altered the transcript accumulation of several genes associated with stress- and defense- responses

The experiments described in Chapter 3 showed that in addition to its induction by inducers of the JA/ET-dependent signalling pathway(s), *AP2.76* transcription was also induced by ABA, dehydration and high-salinity suggesting it may also function in the regulation of the ABA-independent DRE/CRT-box mediated gene expression as well as ABA-dependent abiotic stress pathways.

Thirty *AP2.76*-inducible genes (exhibiting a greater than 2-fold expression in OE76.3 plants, compared with wild-type plants) were identified by a cDNA microarray analysis. The identities of many of these genes is consistent with their involvement in stress- and defense-related responses. For example *rd22* is a dehydration responsive gene (Abe et al., 1997); lipid transfer proteins and superoxide dismutases are known to play roles in repairing damaged cells (Torres-Schumann et al., 1992; Seki et al., 2002; Xiong and Zhu, 2002) and *VSP1* and *VSP2*

are known to be induced by wounding in a JA/ET-regulated pathway (Rojo et al., 1999) and are induced in response to *Erwina carotovora* exoenzyme elicitors in *Arabidopsis* (Norman-Setterblad et al., 2000). In addition, transcription factors belonging to families with well-characterised roles in stress- and defense-regulated gene expression, such as bZIP and MYB family members (Abe et al., 1997; Dong et al., 2003; Li et al., 2004) were identified as *AP2.76*-inducible genes. Several *AP2.76*-inducible genes, however, had no obvious role in defense- or stress-related responses.

Four photosynthesis-related genes were identified as *AP2.76*-inducible by microarray analysis. Several studies have reported that various biotic and abiotic stresses cause a general down-regulation in photosynthesis related genes (Mysore et al., 2002; Seki et al., 2002; Chakravarthy et al., 2003). However several lines of evidence indicate that light signal transduction pathways are positively connected to pathogenesis-related and stress-signalling pathways. Seki et al. (2001) identified a putative photosystem I reactor subunit precursor as a drought- and cold-inducible gene and also as a target gene of *DREB1A*, a member of the ERF/AP2 transcription factor family. Cold-induced gene expression via the *DRE/CRT cis*-acting element is greatly enhanced by a light signalling pathway mediated by phytochrome B that induces expression of chlorophyll A/B-binding proteins (CAB), indicating cross-talk between abiotic-stress and light-signalling pathways (Kim et al., 2002). The SA-mediated signalling pathway is known to interact with the phytochrome A-mediated pathway that induces CAB gene expression (Genoud et al., 1998). Mutants lacking detectable phytochrome A and B proteins show a strong reduction in SA-mediated expression of PR and resistance to *P. syringae* pv. *maculicola* is significantly reduced (Genoud and Metraux, 1999) in these mutants. This suggests that the light signal transduction and pathogenesis-related gene-signalling pathways are inter-connected (Genoud and Metraux, 1999). Induction of genes encoding CAB proteins by SA treatment is another example of such coordinate responses (Schenk et al., 2000).

The expectation that the promoters of the *AP2.76* inducible genes identified by the array would contain the *DRE/CRT*-box or the *GCC*-box *cis*-acting elements was not realised: only a minority of the promoters examined contained these elements. Promoter analysis of 25 significantly up-regulated genes indicated that 13 contained one or more W-box motifs and 7 contained an ABRE element in their promoter regions. This suggests both a direct and a potentially indirect role for *AP2.76* in the regulation of gene expression. The ABA-responsive

element (ABRE) functions in ABA-dependent stress-induced gene expression (Grill and Himmelbach, 1998). Several bZIP transcription factors can bind the ABRE motif and activate expression of many ABA-inducible genes (Choi et al., 2000; Uno et al., 2000). The plant-specific WRKY transcription factors specifically recognise the W-box element which is found in the promoter regions of a large number of defense related genes and are necessary for the inducible expression of these genes (Rushton et al., 1996; Eulgem et al., 1999). The involvement of WRKY factors in plant defense is well documented; some of these factors have been shown to trigger expression of defense-related genes in SAR (Eulgem et al., 1999; Maleck et al., 2000; Robatzek and Somssich, 2001, 2002), control *Arabidopsis* innate immunity activated by bacterial flagellin (Asai et al., 2002) and confer disease resistance in transgenic plants (Deslandes et al., 2002). The presence of the ABRE and W-box *cis*-acting elements in the promoters of many of the *AP2.76* inducible genes might further support a stress- and defense-related role for these genes and raises the possibility that *AP2.76* may regulate gene expression synergistically with transcription factors that bind these elements.

On the basis of results reported in Chapter 3, *AP2.76* was proposed to play a role in regulating ABA-independent DRE/CRT-box mediated gene expression in response to dehydration and high-salinity. Consequently, it was expected that the over-expression of *AP2.76* might induce the stress-responsive *rd29A* and *erd10* genes which contain a DRE/CRT *cis*-element (Seki et al., 2001). However neither *rd29A* nor *erd10* mRNA accumulated in *35S-AP2.76* transgenic plants under normal conditions as determined by northern blot analysis. There are at least two possible explanations for this. One is that *AP2.76* does not have a function in *rd29A* and *erd10* gene expression. The other is that *AP2.76* may require synergistic interactions with other transcription factors to regulate the expression of *rd29A* and *erd10* in response to dehydration or high-salinity stress. We also postulated that *AP2.76* might be involved in the JA/ET-regulated wound response pathway thereby regulating the expression of the wound-inducible genes. This in fact was confirmed by both microarray and northern blot analyses which revealed substantial up-regulation of *VSP1* and *VSP2*.

However *AP2.76* did not appear to regulate *JR* gene expression, as *JR1* or *JR3* mRNA levels were not altered in *35S-AP2.76* transgenic plants under normal conditions as determined by northern blot analysis.

4.10.3 Putative mechanisms by which *AP2.76* might regulate the expression of target genes

Several putative mechanistic possibilities for the regulation of gene expression by *AP2.76* are shown in Figure 4.22. In model A, *AP2.76* binds the GCC-box or the DRE-box or an as yet undiscovered *cis*-acting element to directly activate the expression of target defense- and stress-related genes respectively. Examples of this might be the defense-related genes that encode PDF1.2, PR3 and PR4, which were shown to be up-regulated by *AP2.76* over-expression. However, because the GCC-box or the DRE-box *cis*-elements were not present in many of the promoters of the *AP2.76*-inducible genes identified by microarray analysis, it is possible that another novel element, yet to be determined is bound by *AP2.76*.

In a second possibility, shown as model B, *AP2.76* binds a GCC-box or DRE-box or an as yet undiscovered *cis*-acting element in the promoter of a transcription factor gene (TF 'X'), which in turn modulates expression of the target genes. Indeed, the microarray analysis identified five transcription factor genes which were upregulated in the OE76.3 line. These included a zinc finger transcription factor gene containing a DRE-box element, a bHLH-containing transcription factor, a MYB transcription factor, a POZ domain transcription factor and a Heavy metal associated domain containing transcription factor.

In model C (Figure 4.22), a scenario is depicted in which non GCC-box or non DRE-box containing *AP2.76*-inducible promoters associate with *AP2.76* indirectly via a protein:protein interaction with a transcription factor that binds a stress-related *cis*-acting element in promoters of target genes. A bHLH-containing transcription factor (a domain present in MYC proteins) and a MYB transcription factor gene were identified as *AP2.76* inducible genes. Interestingly, *rd22* whose expression is activated by the cooperative action of a MYC and a MYB transcription factor was also identified as an *AP2.76*-inducible gene. Perhaps *AP2.76* can interact physically with a MYB or a MYC factor to alter the expression of some *AP2.76*-inducible genes such as *rd22*. One precedent for such a model is the interaction between a MYC-like protein and an EREBP protein involved in the regulation of the rice *Wx* gene (Zhu et al., 2003). Several members of the ERF/AP2 family have been reported to interact with other transcription factors. For example the *Arabidopsis* protein AtEBP has been reported to interact with OBF1, a bZIP protein in *Arabidopsis* (Buttner and Singh, 1997) and AREBs and DRE-box binding proteins have been shown to function as interactive

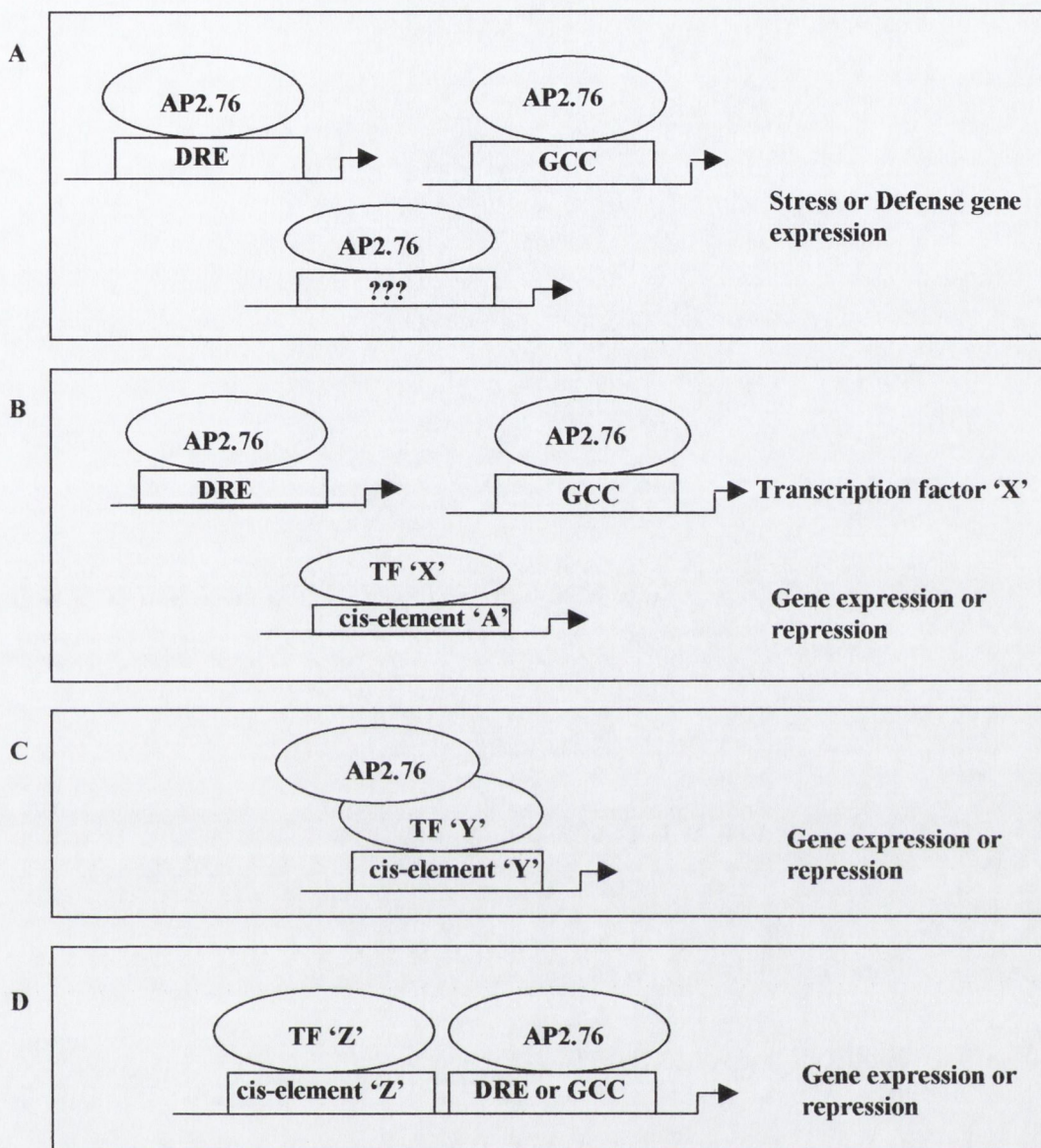


Figure 4.22. Models for AP2.76-Mediated Gene Expression.

AP2.76 is postulated to regulate the expression of stress and defense genes directly via the GCC-box or DRE-box (or a non-GCC and non-DRE-box element) and indirectly via other transcription factors. In model A, AP2.76 interacts directly with the GCC-box, the DRE-box or an as yet undiscovered *cis*-acting element to directly activate the expression of various defense- or stress- related genes. Model B shows that AP2.76 might bind a GCC-box or DRE-box or an as yet undiscovered *cis*-acting element in the promoter of a transcription factor gene (TF X) which in turn modulates expression of the target genes. In model C AP2.76 interacts physically with another transcription factor (TF Y), and this interaction facilitates the expression of genes whose promoter contains a *cis*-element bound directly by TF Y. Finally, in the “tandem binding” model D, gene expression occurs upon binding of the GCC-box or the DRE-box by AP2.76 and of another non-GCC and non-DRE box (*cis*-element Z) by a non-ERF/AP2 transcription factor (TF Z). See discussion for further details.

transcriptional activators during induction of the *rd29A* gene by dehydration, high-salinity, and ABA treatments (Narusaka et al., 2003).

Finally model D depicts a mechanism in which the promoters of some *AP2.76* inducible genes are regulated by the synergistic binding of both *AP2.76* and another defense-related transcription factor (TF 'Z'). Several *AP2.76*-inducible genes were identified that contain both DRE-box and ABRE *cis*-acting elements in their promoters. Furthermore Narusaka et al. (2003) showed that the DRE-box *cis*-element functions as a coupling element for the ABRE *cis*-element in ABA-dependent expression of *Arabidopsis* gene *rd29A* in response to dehydration and high-salinity stresses.

Determining the DNA binding specificity of *AP2.76* would give valuable insights into the mechanisms by which *AP2.76* regulates gene expression. Investigating protein-protein interactions perhaps by using the yeast two-hybrid system might help determine whether *AP2.76* interacts with other transcription factors such as AREBs or MYBs, and thus help to unravel the signalling pathways in which *AP2.76* functions.

4.10.4 Over-expression of *AP2.72* did not alter expression of either JA/ET-regulated, or SA-regulated PR genes

Although *AP2.72* was found to be induced by SA in an *NPR1*-dependent manner (Chapter 3), *AP2.72* overexpression did not result in up-regulation of the SA-regulated genes *PR1* and *PR5* known to be involved in SAR (Uknes et al., 1992). Moreover, the SA-induced expression of *PR1* was unaffected in *AP2.72* knockout lines. Taken together these results suggest *AP2.72* is neither a direct *trans*-activator nor a repressor or SAR-associated genes. However it may also be that *AP2.72*, which shows nucleo-cytoplasmic partitioning (Chapter 2), requires post-translational modification or a putative cofactor to function in response to pathogen attack. Many experiments have shown that certain types of transcription factor require cofactor(s) or post-translational modification for activation of their DNA binding function in plants (Despres et al., 2000; Gu et al., 2000). The *NPR1* protein enhances the DNA binding activity of members of the TGA family of bZIP transcription factors that are essential for inducing PR gene expression (Kinkema et al., 2000). Since the transcriptional activation of *AP2.72* by SA is dependent on *NPR1*, it is tempting to speculate that *AP2.72* may also require

activators of SAR, or an interaction with NPR1 in order to activate the expression of target genes. Clearly elucidating more precisely the subcellular localisation of AP2.72 during the activation of SAR might provide a better understanding of its function.

Several *cpr* mutants (for constitutive expressor of PR genes) such as *cpr1*, *cpr5* and *cpr6* show elevated SA levels, constitutive expression of *PR1*, *PR2* and *PR5* and enhanced resistance to *P. syringae* and *P. parasitica* (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998). Observing the expression of AP2.72 in these mutant backgrounds might give clues that would help resolve the role of AP2.72 in pathogen defense.

Expression of the JA/ET regulated genes *PR3*, *PR4* or *PDF1.2* was unaltered in both AP2.72 knock-out and over-expression lines. It is therefore unlikely that AP2.72 acts as a trans-activator or repressor in JA/ET-dependent pathway(s) mediating defense against pathogens. The experiments reported in Chapter 3 showed that SA-induced expression of AP2.72 was dependent on *NPR1*. Studies in *N. tabacum* and *Arabidopsis* reveal that JA- and SA-mediated defense responses can be mutually antagonistic (Norman-Setterblad et al., 2000). Moreover new evidence indicates that the cellular localisation of NPR1 might play a crucial role in modulating the SA-mediated suppression of JA-responsive genes (Spoel et al., 2003). JA signalling is important for resistance to *A. brassiciola*, *B. cinerea* and *E. carotovora* as evidenced by *coi1* and *jar1* mutants which show enhanced susceptibility (Thomma et al., 1998; Vijayan et al., 1998; Norman-Setterblad et al., 2000; Stintzi et al., 2001), and ET signalling has been shown to be important for resistance to *B. cinerea* and *E. carotovora* on the basis that *ein2* mutants show enhanced susceptibility (Thomma et al., 1999; Norman-Setterblad et al., 2000). Therefore, monitoring the expression of AP2.72 in wild-type plants, in mutants that are impaired in JA perception (e.g. *coi1* and *jar1*) and in the *ein2* mutant, in response to infection with the fungal pathogens *A. brassiciola* and *B. cinerea* and the bacterial pathogen *E. carotovora* might help to define the role of AP2.72 in JA/ET-dependent defense responses.

Unlike SAR which is regulated by SA, ISR triggered in roots by nonpathogenic rhizosphere-colonising *Pseudomonas* is regulated by JA and ET, but remarkably also by *NPR1* (van Wees et al., 2000; Ton et al., 2001). Furthermore, ISR and SAR can be activated simultaneously, resulting in an additive level of protection against *P. syringae* (van Wees et al., 2000). It is therefore possible that AP2.72 may act as a *trans*-acting factor in ISR as well as SAR. Unfortunately no diagnostic changes in gene expression specifically associated with ISR

have been detected (Pieterse et al., 1998), making it difficult to monitor its induction. Rhizobacteria-mediated ISR has been studied extensively in *Arabidopsis* using the non-pathogenic rhizobacterium *P. fluorescens* strain WCS417r as the inducing agent and *P. syringae* pv. *tomato* (Pst) as the challenging pathogen (van Wees et al., 2000; Ton et al., 2001). Monitoring the expression of *AP2.72* in response to WCS417r in SA-nonaccumulating *nahG* transgenic plants which fail to express SAR (Lawton et al., 1995) but show normal levels of ISR (Pieterse et al., 1996), would help in evaluating the putative role of *AP2.72* in ISR. It may also be useful to evaluate the JA and ET-mediated induction of *AP2.72* in the *npr1-1* and *npr1-2* mutants, as this might help to establish whether *AP2.72* functions in ISR.

4.10.5 Absence of phenotypic changes in *AP2.76* or *AP2.72* overexpression and knockout mutants

No phenotypic changes were observed in *35S-AP2.76* or in *35S-AP2.72* overexpression lines or in *DsE-AP2.76* or *dSpm-AP2.76* knockout mutants. In addition no visible retardation of salt-induced or dehydration-induced senescence was observed when *AP2.76* overexpression plants were exposed to dehydration or high-salinity treatments (data not shown). However, since *AP2.76* appears to activate expression of genes involved in JA/ET-regulated pathogen defense responses, infecting *AP2.76* knockout mutants and *AP2.76* overexpression lines with a necrotrophic pathogen such as *A. brassiciola* or *B. cinerea*, might reveal enhanced susceptibility and enhanced resistance phenotypes, respectively, in respect of these pathogens.

It is becoming increasingly clear that combinatorial control of gene expression, which allows the creation of regulatory diversity by a limited number of transcription factors, is a common control mechanism in *Arabidopsis*. We have postulated that *AP2.76* and *AP2.72* may regulate gene expression in combination with other transcription factors. The fact that *AP2.76* and *AP2.72* may function interdependently rather than alone is possibly one of the major reasons why the over-expression of *AP2.76* or *AP2.72* or the lack of expression in knockout mutants did not result in an altered phenotype. Therefore to obtain an instructive phenotype, it may be necessary to over-express an additional transcription factor together with *AP2.76* or *AP2.72* in the same plant.

In addition based on the high protein sequence similarity of *AP2.76* to *RAP2.4* (Chapter 2) it may be possible that both genes might have a partially redundant function. Thus engineering plants which over-express both the *AP2.76* and *RAP2.4* genes or plants with reduced levels of both the *AP2.76* and *RAP2.4* genes might yield an informative phenotype. The *AP2.72* gene also showed a high level of protein sequence similarity to *RAP2.6* (Chapter 2) and therefore it may be possible that both these genes also have a partially redundant function. Engineering plants which over-express both the *AP2.72* and *RAP2.6* genes or plants with reduced levels of both the *AP2.72* and *RAP2.6* genes might yield an informative phenotype.

Chapter 5

Materials and Methods

5.1 Materials

5.1.1 Bacterial strains

Escherichia coli strain DH5 α (*E. coli*); genotype $\phi 80dlacZ\Delta M15$, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r_k^- , m_k^+), *supE44*, *relA1*, *deoR*, $\Delta lacZYA-argF$) U169 (Hanahan, 1983).

Agrobacterium tumefaciens strain AGL1 (*A. tumefaciens*); genotype C58, RecA, PTiBo542 Δ T – DNA (Lazo et al., 1991)

5.1.2 Bacterial growth media

LB (Luria-Bertani) medium: For *E. coli*: 10g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10g/L NaCl; pH7.0 (For LB agar plates, 1.5% w/v agar was added).

YM medium: For *Agrobacterium*: 0.04% yeast extract, 10g/L mannitol, 1.7mM NaCl, 0.8mM MgSO₄, 2.2mM K₂HPO₄, pH7.0 (For YM agar plates, 1.5% w/v agar was added).

5.1.3 Plant growth media

MS (Murashige-Skoog) media: For seedlings: 4.3 g/L MS sales, pH 5.7 (For MS agar plates, 0.8% of agar was added) (Murashige and Skoog, 1962). For MS+ agar plates, 2% sucrose and 0.8% of agar was added.

MS-Gamborg media: For roots: 4.3 g/L MS sales, 1 X Gamborg's vitamin solution (100X-Sigma), 2% sucrose, pH5.7.

5.1.4 Plant Material

5.1.4.1 *In vitro* plant material

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were maintained under sterile conditions in MS medium in a growth room under a 16 hour light/8hour dark cycle at 23-25°C.

Arabidopsis thaliana (ecotype Columbia) seedlings were germinated on MS medium and maintained under sterile conditions in a growth room under a 16 hour/8hour dark cycle at 23-25°C.

5.1.4.2 *In vivo* plant material

Arabidopsis plants were grown in Erin peat-based potting compost under green house conditions at 22°C with a day length of 16hours.

5.1.5 Restriction enzymes

All restriction enzymes and buffers were obtained from New England Biolabs, SIGMA or Roche Molecular Biochemicals.

5.2 DNA manipulations

General DNA manipulations such as restriction enzyme digestions and ligations were carried out as described by (Sambrook et al., 1989)

5.2.1 Agarose gel electrophoresis

For routine sizing of PCR products, analysis of restriction digestions, and estimating genomic and plasmid DNA concentrations, samples were separated in 0.8% w/v agarose gels containing 0.5 X TBE buffer (45mM Tris-borate and 1mM EDTA) and ethidium bromide (0.5ug/ml) by electrophoresis in 0.5 X TBE and visualized with a longwave UV transilluminator (UVP Inc.).

5.2.2 Miniprep plasmid DNA isolation

Plasmid DNA isolation was carried out using the Jetquick Plasmid Miniprep Spin Kit/250 (Genomed), and by following the recommended protocol.

5.2.3 Purification of DNA fragments

DNA fragments were excised from an agarose gel and purified using the Jetquick Gel Extraction Spin Kit/250 (Genomed), and by following the recommended protocol.

5.2.4 Preparation and transformation of *E. coli* DH5 α CaCl₂-competent cells

E. coli strain DH5 α was streaked from a glycerol stock on LB agar plates and incubated overnight at 37°C. A single colony was picked and grown overnight in 5ml LB liquid medium, then the overnight culture was diluted 1/50 in fresh LB liquid medium and grown at 37°C in an orbital incubator (shaken at 200 rpm) until the culture reached an OD₆₀₀ of 0.5-0.7. Cells were pelleted by centrifugation at 4000 rpm for 10 mins at 4°C and resuspended in ice-cold 0.1M CaCl₂. The cells were incubated on ice for 15 minutes and recovered by recentrifugation at 3000 rpm for 5 minutes. Cells were resuspended in 0.1M CaCl₂ containing 15% glycerol, aliquoted into individual Eppendorf tubes and stored at -70°C.

For transformation, 70 μ l of competent cells were added to 10 μ l ligation mixture and incubated on ice for 20 minutes. The solutions were heat shocked at 42°C for 1 minute and 500 μ l of fresh LB medium added. Samples were incubated at 37°C for 1 hour, after which

cells were centrifuged at 8000 rpm for 1 minute at room temperature. Approximately 400µl of the supernatant was discarded before plating the remainder on LB agar medium supplemented with specific antibiotics and incubated at 37°C overnight.

5.2.5 Dephosphorylation of DNA

Digested plasmid vector DNA was dephosphorylated by treatment with calf intestinal alkaline phosphatase (CIAP) (Roche Molecular Biochemicals) before using in ligation reactions. After CIAP treatment the sample was purified as described in section 5.2.7.

5.2.6 Re-purification of DNA following modification by enzymes

DNA samples were purified by extraction with an equal volume of TE buffer-saturated phenol, vortexed for 1 minute and centrifuged at 12000 rpm for 4 minutes. The aqueous phase was recovered. Sepharose CL-6B columns were routinely used to change the DNA buffer or to remove impurities including residual phenol.

The Sepharose CL-6B spin-dialysis method described by (Murphy and Kavanagh, 1988) was used as follows: a tiny hole was made at the bottom of a 500µl microcentrifuge tube using a fine needle, and approximately 60µl of glass beads that had been sterilized by autoclaving in TE (10 mM Tris-HCL, 1 mM EDTA; pH 8.0) buffer was added followed by approximately 900µl of a Sepharose CL-6B suspension. The column was placed inside a 1.5ml microcentrifuge tube with the bottom removed and the system was centrifuged at low speed (2000 rpm) for 3 minutes. Finally the column was washed in 70µl H₂O and centrifuged again as described. DNA-containing samples (approximately 50µl) were applied to the spin-dried Sepharose CL-6B column and recovered in an intact 1.5ml tube following column centrifugation.

5.2.7 DNA sequencing

All sequencing reactions were carried out by MWG Biotech. Approximately 1.5µg of each template for sequencing was sent away in pellet form. For each reaction 20µl of 10pmol/µl primer solution was also provided. Sequencing results were viewed on www.MWG.biotech.com

5.2.8 Plant Genomic DNA extraction and purification

Approximately 500mg of leaf tissue was homogenized in 500µl of plant DNA extraction buffer (0.1M Tris-HCl pH8.0, 50mM EDTA, 50mM NaCl, 2% SDS, 0.75% β-mercaptoethanol) using a pestle and mortar. The resulting homogenate was subjected to two phenol, and one chloroform extraction. The supernatant was collected by centrifugations at 10,000 rpm for 4 minutes. A DNAase-free RNaseA treatment was performed for 20 minutes at 37°C followed by another phenol and chloroform extraction. Percipitation was carried out with 1/10 volume of 3 M NaAc pH 5.2 and 2.5 volumes of ethanol at -20°C for at least 1 hour. The pellet was collected by centrifugation at 12000 rpm for 15 minutes, washed with 75% ethanol and suspended in 50µl TE buffer.

DNA concentration was determined by measuring the absorbance of the sample at 260nm using a Shimadzu UV-VIS spectrophotometer, model UV mini 1240. In addition, the integrity of the DNA was assessed by agarose gel electrophoresis.

5.2.9 Preparation of *Agrobacterium* AGLI electro-competent cells

The *Agrobacterium* strain AGLI was streaked from a glycerol stock onto LB agar plates containing rif (50µg/ml) and amp(100µg/ml) and incubated at 28°C for 48hrs. A single colony was inoculated into 10ml YM liquid medium containing selective antibiotics and shaken overnight at 200rpm at 28°C. 1ml of overnight culture was diluted into 100ml of fresh YM liquid medium, shaken at 220rpm, at 28°C until the culture reached an OD₆₀₀ ~ 0.5-0.7. Cells were collected by centrifugation at 7000 rpm for 10 minutes at 4°C, resuspended gently in 10 ml cold 1mM HEPES pH 7.0 and centrifuged again under the same conditions. Cells were suspended in 10ml of cold 10% glycerol, 1mM HEPES pH7.0, and the cells were

collected by centrifugation under the same conditions. Finally cells were gently suspended in 0.5ml of cold 10% glycerol, 1mM HEPES pH7.0, and 60µl aliquots stored in Eppendorf tubes at -70°C. Electroporation was done using 20ul of competent cells that had been prepared as described above, and 1µl of binary vector (~50ng of DNA). The electroporation was carried out using a GIBCO cell-porator system following the manufacturer's instructions. Cells were plated on YM agar plates supplemented with specific antibiotics and incubated for 48 hours at 28°C.

5.2.10 PCR amplifications

All PCR reactions were carried out either on a MJ Research Inc. Programmable Thermal Controller PTC-100 without mineral oil, or on a Minicicler TM for reactions with mineral oil.

5.2.10.1 General PCR conditions

All PCR reactions comprised a 50µl volume containing approximately 1-50ng DNA template, 20mM MgSO₄, 0.2mM dNTPs, ~ 100ng of each specific primer and 2.5U of *Pfu* DNA polymerase (Stratagene Cloning Systems). The cycle conditions used for a standard PCR were as follows: 94°C for 1 minute, 35 cycles of 94°C for 45 seconds, 58°C - 62°C for 1 minute and 72°C for 2 minutes; plus an extension of 72°C for 2 minutes.

5.2.10.2 Generation of DIG-labelled probes

DNA fragments were digoxigenin-labelled (DIG) using a DIG Probe synthesis kit (Roche Molecular Biochemicals) according to instructions in the kit manual. For double-stranded probes, approximately 5-50pg of cloned plasmid DNA was used as the template. For single-stranded probes 1-10ng of plasmid DNA, and 0.01µM of forward and 1µM of reverse primers were used.

5.2.11 Transformation of *Arabidopsis* plants

After confirming the presence of the binary vector in *Agrobacterium* by plasmid isolation and restriction digestion analysis, a colony containing the verified binary vector construct was used to transform *Arabidopsis* ecotype Columbia. Plants that had been grown in a green house under normal conditions (25°C and 16 hours photoperiod) were transformed by the “Floral Dip” method (Clough and Bent, 1998) as follows: *Arabidopsis* seeds were germinated in small pots of potting compost covered with a mesh. Once plants started flowering (after approximately 4 weeks) the first bolts were clipped to encourage proliferation of many secondary bolts. Plants were ready for transformation at roughly 5-7 days after clipping. *Agrobacterium* strain AGL1 carrying the binary vector construct was grown in 10-15ml of YM liquid medium for 48 hours at 28°C. This preculture was then diluted in 400-600ml of LB liquid medium containing selective antibiotics, grown until an OD₆₀₀ of 1.0-1.8. *Agrobacterium* cells were centrifuged at 6000 rpm for 30 minutes and suspended to obtain an OD₆₀₀ of 0.8 in 5% sucrose solution. Approximately 200ml of *Agrobacterium* solution was used for dipping three pots of *Arabidopsis*. Before dipping, Silwet L-77 was added to the culture at a concentration of 0.05% (500µl/L) and mixed well. Above-ground parts of the plant were dipped in this *Agrobacterium* solution for 30 seconds, with gentle agitation. Dipped plants were then placed in a growth chamber overnight to maintain high humidity and to avoid direct light. The next day the excess sucrose solution on the plants was washed off with water, and plants were allowed to set seeds.

5.2.12 Vapour-phase sterilization of *Arabidopsis* seeds

The vapour-phase sterilization of *Arabidopsis* seeds protocol was adapted from Maud Hinchey (Ye et al., 1999) as follows: seeds in vials or Eppendorf tubes were placed inside a dessicator jar in the presence of a 250ml beaker containing 50ml of commercial bleach and 3ml HCL concentrate for 4-8 hours. Seeds were then sprinkled on plant growth medium containing appropriate antibiotics to select for transgenic seedlings, incubated at 4°C for 2 days and germinated under *in vitro* conditions (25°C and a 12 hours light/day cycle).

5.2.13 RNA extraction protocol

Total RNA was extracted from leaves, flowers, stems and green siliques of mature *Arabidopsis* plants grown under greenhouse conditions (25°C and a 16 hours light:8 hours dark photoperiod). Roots were grown in MS-Gamborg liquid medium in a flask shaken at 100 rpm in total darkness, and RNA was isolated after 2 weeks. Seedlings were grown on MS+ agar plates under *in vitro* conditions, and RNA was isolated after one week. The RNA extraction protocol was adapted from (Eggermont et al., 1996) as follows: Approximately 500mg of tissue was ground to a fine powder in liquid nitrogen using a pestle and mortar and then homogenized in 500µl plant extraction buffer (0.1M Tris-HCL pH7.5, 50mM EDTA- Na_2 , 50mM NaCl, 2% SDS, 0.75% β -mercaptoethanol; where possible solutions were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved) and 1 volume of phenol. The supernatant was collected by centrifugation at 1000 rpm for 4 minutes followed by phenol:chloroform (1:1 v/v) and chloroform extractions. Precipitation was carried out with a 0.5 volume of 8M LiCL at -20°C for at least 1 hour. The pellet was collected by centrifugation at 12000 rpm for 15 minutes, washed with 75% ethanol and suspended in 30µl TE buffer.

RNA concentration was determined by measuring the absorbance of the sample at 260nm using an Ultraviolet-Visible spectrophotometer. The integrity of the RNA was assessed by agarose gel electrophoresis.

5.2.14 Southern blot analysis

Approximately 3µg of total DNA was used for restriction digestion and electrophoresed overnight in a 1% agarose gel in 0.5X TBE buffer (45mM Tris-borate and 1mM EDTA). Gels were denatured in 1.5M NaCl, 0.5M NaOH for 15 to 30 minutes with gentle shaking followed by neutralization in 1.5M NaCl, 0.5M Tris-HCL. pH7.5. Gels were rinsed with 20X SSC (3M NaCl, 0.3M Sodium citrate, pH7.0) before blotting overnight on Biodyne® B membrane by capillary transfer using 20X SSC according to UV light for 4 minutes on each side. Pre-hybridisation (4 hours) and hybridization (overnight) were performed at 40-42°C respectively in warm hybridization buffer (DIG Easy Hyb granules- ROCHE Molecular Biochemicals). The double-stranded probes were labeled with digoxigenin (DIG) using the PCR-DIG Probe synthesis kit at a concentration of 20ng/ml of hybridization

easy buffer. The DIG-labelled probe was diluted in approximately 70µl of DNA buffer (10mM Tris-HCL pH8.0, 50µl/ml salmon sperm DNA) and denatured at 98°C for 10 minutes. After hybridization the membrane was washed twice in turn with 2X SSC and 0.3X SSC containing 0.1% SDS at room temperature for 5 minutes and at 68°C. for 15 minutes respectively. The detection step was performed as follows: the membrane was incubated in blocking solution (0.1M maleic acid, 3M NaCl, 2% blocking reagent-ROCHE, pH7.5) for 45 minutes, and then incubated with anti-DIG-AP conjugate (diluted 1:10000 in blocking solution) for 30 minutes. Finally it was washed 4 times for 10 minutes with 0.1M maleic acid, 3M NaCl, 0.3% tween20, followed by equilibrium on detection buffer (100mM NaCl, 100mM Tris-HCL, pH9.5) for 5 minutes (twice). Detection was performed with a chemiluminescent substrate CSPD (Roche Molecular Biochemicals) and exposure with X-ray films.

5.2.15 Northern blot analysis

Where possible, all solutions were treated with 0.1% v/v DEPC at 37°C overnight, and then autoclaved. All apparatus were soaked on 0.1N NaOH for at least 2 hours and then rinsed in DEPC-treated water (DEPC-H₂O). Total RNA was extracted and its integrity assessed by agarose gel electrophoresis. For northern analysis, approximately 10µg of extracted RNA was electrophoresed in a 1% w/v agarose gel containing 1% v/v (deionized) formaldehyde and 1X NBC buffer (50mM boric acid, 5mM NaOH, 1mM sodium citrate; pH7.5). Running buffer was 1X NBC. After electrophoresis, the gels were washed for 10 minutes in 20 X SSC-DEPC (3 M NaCl, 0.3M sodium citrate, pH7.0; DEPC-treated) and blotted onto Biodyne® B membrane (GELMAN laboratory) by capillary transfer using 20X SSC-DEPC. RNAs were UV- cross-linked to the membrane. Pre-hybridisation (4hours) and hybridization (overnight) were performed at 50°C in 7% SDS-DEPC, 50% formamide, 5X SSC, 2% DIG system blocking reagent (10X blocking agent, 0.1M maleic acid, 0.15M NaCl, pH 7.5; DEPC-treated), 0.1% N-lauryl sarcosine, 50mM NaHPO₄, pH7.0. DNA probes that had been labelled with digoxigenin (DIG) using the PCR-DIG Probe synthesis kit were gel-purified (using a JetQuick kit) and used for hybridizations. Membranes were washed twice with 2 X SSC-DEPC and 0.3 X SSC-DEPC containing 0.1% SDS at room temperature for 5 minutes and at 68°C for 15 minutes respectively. The hybridization detection step was performed as described above.

5.3 Analysis of β -glucuronidase (GUS) expression in transgenic plants

Putative transgenic plants were analysed in various ways to confirm their identity. A number of GUS expression assays were performed. Primarily a quick GUS assay was carried out to determine if the GUS gene was present. DNA was extracted from a section of leaf and detection of the transgene was verified by PCR. The plants were subsequently analysed by histochemical GUS staining.

5.3.1 Quick GUS assay

Leaf discs of the same size from rosette leaves of putative transgenic plants were homogenized in Eppendorf tubes with 50 μ l GUS extraction buffer (50mM NaHPO₄ pH6.8, 10mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100) containing 1mM 4-methylumbelliferyl β -D-glucuronide (MUG) (Sigma). The samples were centrifuged at 10000 rpm for 5 minutes and the supernatant was incubated at 37°C. After 1 hour, samples were observed under a UV transilluminator.

5.3.2 Histochemical analysis of GUS expression

Plant tissue was immersed in GUS staining buffer (50mM NaHPO₄, pH6.8, 1mM EDTA, 0.06% Triton X100, 0.5mM potassium ferrocyanide, 0.5mM potassium ferricyanide and 1mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc)-Research Organics) followed by vacuum-infiltration for 20 minutes and incubation overnight at 37°C. The chlorophyll was removed with absolute ethanol or cleared in 20% glycerol/20% lactic acid (for roots) and the samples were stored in 20% ethanol at 4°C. The GUS expression pattern was observed under an Olympus BX40 (Optical CO, LTD) light microscope and Olympus SZX9 (Optical CO, LTD) stereo. Pictures were taken with an Olympus DP 10 digital camera (Optical CO, LTD).

5.4 Biolistic transformation

5.4.1 Preparation of tungsten particles for biolistic transformation

1ml of ice-cold 70% special grade ethanol was added to 30mg of accurately weighed tungsten powder (M-17), average particle size 1.1 μ m; Alpha) and the suspension was vortexed vigorously for 5 min followed by 15 min soaking on ice. The tungsten was then gently sedimented by spinning at 8000 rpm for 5 seconds in a microfuge and the ethanol was removed using a pipette. The tungsten particles were then washed three times by adding 1ml of sterile doubly distilled H₂O, vortexing vigorously for 1 minute and microfuging at 8000 rpm for 3 seconds before removing the H₂O. During the third wash a further 3 second 8000 rpm spin was performed to ensure as much as possible of the H₂O was removed. 500 μ l of sterile 50% glycerol was added and the suspension was stored at -20°C until use for a maximum period of one week.

5.4.2 Coating washed tungsten particles with DNA

The glycerol stock was vortexed vigorously for five minutes and 50 μ l of washed tungsten particles were removed to a 1.5ml eppendorf tube before the tungsten had a chance to settle. While vortexing vigorously the following were added to the tungsten aliquot in the order given: (1) 5 μ l of a 1-2 μ g/ μ l plasmid DNA solution; (2) 50 μ l of 2.5M CaCl₂ solution; (3) 20 μ l of 0.1M spermidine (free base; SIGMA tissue culture grade) solution. The plasmid DNA was microfuged for 5 minutes at 15000 rpm immediately before use to sediment residual insoluble matter. The CaCl₂ solution was prepared fresh, filter-sterilised and stored on ice before use. The spermidine solution was prepared from a 1M stock solution that had been filter sterilized and stored at -20°C for a period no longer than one month. The tungsten particles were then vortexed vigorously for 30 minutes at 4°C. After vortexing the particles were allowed to settle for 1 minute before being sedimented by microfuging for 2 seconds at 8000 rpm. The liquid was removed and discarded and 140 μ l of 100% special grade ethanol was added. The ethanol was removed without disturbing the sedimented tungsten and 48 μ l of 100% ethanol was added. The tungsten was gently resuspended by tapping the side of the tube and by low speed vortexing. The DNA coated particles were stored on ice until use, and were

used within a few hours of preparation and were resuspended by gentle vortexing before loading on the flying disc (macrocarrier).

5.4.3 Preparation of plant material for bombardment

Onion epidermal tissue was used for bombardment. This was peeled from the outer face of a concentric ring of a longitudinally quartered onion using fine forceps and placed inner surface down on support medium (MS+) in petri dishes while taking care to avoid rolling or excessive handling of the tissue. Tissue prepared in this way was used immediately.

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were also used for bombardment and were grown under sterile conditions as described in section 5.1.4.1. Healthy leaves 3-5cm in length from plants 4-5 weeks old were removed under sterile conditions and the abaxial side was bombarded. Biolistic transformation was carried out as described below.

5.4.4 Transient biolistic transformation

The BioRad biolistic chamber, model PDS-1000/He Biolistic Particle Delivery System was operated according to the manufacturer's instructions. Sufficient force for bombardment was generated by creating pressure from the helium cylinder to 1500psi and using a pump to make a vacuum in the bombardment chamber. Helium was fed into the chamber by releasing a valve up to a pressure equivalent to 28 inches of mercury. A rupture plate of 1100 psi was mounted into the chamber in front of the helium inlet. The carrier disk was inverted and pressed down into the metal support 5cm above a metallic mesh stopping disk and the entire plate slotted onto the second shelf down from the top of the chamber. The plant material to be transformed was placed in its petri dish onto the second shelf up, 6cm from the stopping disk. The chamber door was shut tightly to create a seal and a vacuum created in the chamber to 28inches of mercury. Helium entered the chamber through a valve at a pressure of up to 1500psi at which point the rupture disk burst to force the carrier plate against the stopping grid and accelerate the DNA-coated particles into the tissue.

5.4.5 Detection of GFP by fluorescence microscopy

Sections of onion tissue were mounted on a glass slide in water and covered with 1.5 mm coverslips and were viewed by fluorescence microscopy, using a Nikon Eclipse E600 microscope with a Y-LF Epi-Fluorescence attachment. Pictures were taken using a Spot RT CCD colour digital camera and images were saved as universal TIFF files for import to Adobe Photoshop for manipulation.

5.4.6 Quantitative fluorometric GUS and NAN assays

Plant material was homogenised in GUS extraction buffer (2:1 buffer: plant). The debris was removed by centrifugation at 7000 rpm for 5 mins. The supernatant containing soluble protein was transferred to a fresh tube on ice. 10 μ l aliquots of extracted soluble protein were added to GUS assay buffer (extraction buffer containing 1mM MUG) or to NAN assay buffer (extraction buffer containing 1mM MUN (4-methylumbellifery α -N-neuraminic acid)). The samples were incubated at 37°C for zero hours (control) and one hour or until fluorescence could be observed under a UV transilluminator. Reactions were stopped by the addition of 0.2M Na₂CO₃, which also enhances fluorescence. GUS and NAN activity was quantified using a spectrofluorimeter (Perkin Elmer LS-50B) with excitation set at 365nm, emission at 455nm and slit widths set at 5nm.

Chapter 6

General conclusions and future perspectives

Chapter 2, provides evidence suggesting that both AP2.76 and AP2.72 function as transcription factors: they both contain the well-conserved AP2/ERF domain, a NLS and acidic amino acid-rich regions that may act as transcriptional activator domains. Both proteins were shown to accumulate in nuclei albeit with differing efficiencies. Based on sequence comparison with other well-characterised members of the ERF/AP2 transcription factor subfamily it was proposed that AP2.76 most likely activates DRE/CRT-box mediated transcription and therefore plays a role in regulating gene expression in response to abiotic stresses, while AP2.72 was proposed to activate GCC-box mediated transcription and play a role in pathogen responses (biotic stress). Clearly, determining the DNA binding specificity of both AP2.76 and AP2.72 would be required to conclusively identify the promoter elements targeted by each protein.

Expression analysis in chapter 3 provided further evidence supporting the hypothesis that AP2.76 functions in abiotic stress signalling pathways. The expression of AP2.76 was induced by ABA, dehydration and high-salinity stresses. Interestingly, expression analysis in the ABA-deficient mutants, *aba3-1*, *aba2-4* and *aba1-5* revealed the early dehydration and high salinity-induced expression of AP2.76 to be abscisic acid-independent, suggesting that AP2.76 may function in both ABA-dependent and ABA-independent signalling pathways in response to abiotic stress. In addition to a role in abiotic stress signalling pathways, evidence was also presented to support a role for AP2.76 in biotic stress signalling pathway(s), suggesting that AP2.76 may interact specifically with both DRE/CRT-box and GCC-box *cis*-elements in target genes. The induction of AP2.76 by JA, ACC and wounding led to the hypothesis that AP2.76 may function in (i) the JA/ET-mediated signalling pathway(s) involved in pathogen defense and wounding and (ii) in the ISR pathway.

Consistent with the previous classification of AP2.72 as a GCC-box binding protein, the induced expression of AP2.72 by SA, JA and ACC, along with evidence that the induction of AP2.72 by SA was *NPR1* dependent suggested that AP2.72 functions in SA-dependent and SA-independent signal transduction pathways that mediate the response to pathogen attack. However, AP2.72 expression was also induced by ABA and high salinity suggesting it may also interact specifically with both the DRE/CRT-box and GCC-box *cis*-elements and therefore function as a transcriptional activator in two separate (abiotic and biotic stress) signal transduction pathways.

Further analysis of the expression of AP2.76 and AP2.72 in *npr1* mutants and *aba*-deficient mutants may prove vital in unravelling the precise functions of both transcription factors. Also expression analysis in other mutants compromised in disease resistance such as *cpr1*, *cpr5* and *cpr6*, *jar1*, *coil* and *ein2*, to name a few, would provide further insights into the complexity of the signalling pathways that both transcription factors are involved in and help in determining their positions in the respective signalling networks. Additional studies analysing the expression of AP2.76 and AP2.72 in response to infection by various pathogens such as *A. brassicicola*, *B. cinera*, *E. carotovora* and *P. syringae* may also help in specifying the signal transduction networks both transcription factors are involved in.

In chapter 4, the induced expression of the GCC-box containing genes *PDF1.2*, *PR3* and *PR4* in AP2.76 over-expression plants further supported a role for AP2.76 as a transcriptional activator in the JA/ET-mediated signalling pathway involved in pathogen defense. A cDNA microarray analysis revealed that the over-expression of AP2.76 induced the accumulation of transcripts for several genes associated with stress- and defense- responses. A promoter analysis of the AP2.76 inducible genes suggested both direct and indirect mechanisms by which AP2.76 might regulate gene expression.

In addition, the expression of the JA/ET-regulated or SA-regulated PR genes was unaltered in AP2.72 knock-out plants and over-expression lines. As the transcriptional activation of AP2.72 by SA was dependent on *NPR1*, it was hypothesised that like *NPR1*, AP2.72 may require activators of SAR, or an interaction with *NPR1* in order to activate the expression of target genes.

By conducting additional microarray studies, the global gene expression patterns in transgenic plants over-expressing AP2.72 or in AP2.72 knockout plants could be compared to wild-type plants and thus will play an important role in determining target genes of AP2.72.

Further characterisation by investigating possible protein-protein interactions should also provide new insight into the functions of both *AP2.76* and *AP2.72*. Epistasis analysis, such as over-expression of *AP2.76* (or *AP2.72*) in combination with another transcription factor that may work synergistically with it to regulate gene expression may help in obtaining stress tolerant or disease resistant phenotypes.

Abe, H., Yamaguchi-Shinozaki, K. (1997). Role of small RNA in gene silencing and over-regulated gene expression in Arabidopsis. *Plant Cell* 9, 1411-1421.

Abe, H., Ura, T., Ito, T., Seki, T. (2000). Arabidopsis MYC2 and MYC3 as transcriptional activators in abscisic acid signaling. *Plant Cell* 12, 1031-1043.

AGI (2000). Analysis of gene expression in Arabidopsis. *Plant Cell* 12, 1031-1043.

Allen, M.D., Yamaguchi, K., et al. (1997). A novel mode of DNA-protein interaction in the Arabidopsis thaliana MYC2 gene. *Plant Cell* 9, 1411-1421.

Artus, N.N., Dussan, M., et al. (1999). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 11, 1349-1359.

Bailly, T., Topp, G., Piatkowski, J., et al. (1999). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 11, 1349-1359.

Aranda, E., Aizawa, K., Kato, Y., and Tabata, S. (2000). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 12, 1031-1043.

Aukerman, M.J., Schuster, R. (1997). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 9, 1411-1421.

Bailly, S., Piatkowski, J., et al. (1999). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 11, 1349-1359.

Baker, S.S., Wilhelm, K.A., et al. (1999). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 11, 1349-1359.

Bancroft, I., and Dean, C. (1997). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 9, 1411-1421.

Bancroft, I., Jones, J.D., and Dean, C. (1997). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 9, 1411-1421.

Baulcombe, D.C. (1999). RNA silencing in transgenic plants. *Plant Cell* 11, 1349-1359.

Berg, D.R., Grotzer-Lewin, S. (1999). The Arabidopsis thaliana MYC2 gene encodes a transcription factor. *Plant Cell* 11, 1349-1359.

References

- Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D., and Shinozaki, K. (1997). Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* **9**, 1859-1868.
- Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* **15**, 63-78.
- AGI. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796-815.
- Allen, M.D., Yamasaki, K., Ohme-Takagi, M., Tatenno, M., and Suzuki, M. (1998). A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *Embo J* **17**, 5484-5496.
- Artus, N.N., Uemura, M., Steponkus, P.L., Gilmour, S.J., Lin, C.T., and Thomashow, M.F. (1996). Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. *PNAS* **93**, 13404-13409.
- Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* **415**, 977-983.
- Asamizu, E., Miura, K., Kucho, K., Inoue, Y., Fukuzawa, H., Ohyama, K., Nakamura, Y., and Tabata, S. (2000). Generation of expressed sequence tags from low-CO₂ and high-CO₂ adapted cells of *Chlamydomonas reinhardtii*. *DNA Res* **7**, 305-307.
- Aukerman, M.J., Schmidt, R.J., Burr, B., and Burr, F.A. (1991). An arginine to lysine substitution in the bZIP domain of an opaque-2 mutant in maize abolishes specific DNA binding. *Genes Dev* **5**, 310-320.
- Baima, S., Possenti, M., Matteucci, A., Wisman, E., Altamura, M.M., Ruberti, I., and Morelli, G. (2001). The arabidopsis ATHB-8 HD-zip protein acts as a differentiation-promoting transcription factor of the vascular meristems. *Plant Physiol* **126**, 643-655.
- Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994). The 5'-region of Arabidopsis thaliana cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol Biol* **24**, 701-713.
- Bancroft, I., and Dean, C. (1993). Transposition pattern of the maize element Ds in Arabidopsis thaliana. *Genetics* **134**, 1221-1229.
- Bancroft, I., Jones, J.D., and Dean, C. (1993). Heterologous transposon tagging of the DRL1 locus in Arabidopsis. *Plant Cell* **5**, 631-638.
- Baulcombe, D.C. (1996). RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol Biol* **32**, 79-88.
- Bergey, D.R., Orozco-Cardenas, M., de Moura, D.S., and Ryan, C.A. (1999). A wound- and systemin-inducible polygalacturonase in tomato leaves. *Proc Natl Acad Sci U S A* **96**, 1756-1760.

- Bohnert, H.J., and Sheveleva, E.** (1998). Plant stress adaptations--making metabolism move. *Curr Opin Plant Biol* **1**, 267-274.
- Bork, P.** (1993). Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally? *Proteins* **17**, 363-374.
- Bowles, D.J.** (1990). Defense-related proteins in higher plants. *Annu Rev Biochem* **59**, 873-907.
- Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X.** (1997). The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* **9**, 1573-1584.
- Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X.** (1994). A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *Plant Cell* **6**, 1845-1857.
- Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M.** (1989). Genes directing flower development in *Arabidopsis*. *Plant Cell* **1**, 37-52.
- Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M.** (1991). Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development* **112**, 1-20.
- Bray, E., Bailey-Serres, J., and Weretilnyk, E.** (2000). Responses to abiotic stresses. In *Biochemistry and Molecular Biology of Plants*, B. Buchanan, W. Gruissem, and R. Jones, R., eds (American Society of Plant Biologists), pp. 1158-1203.
- Browse, J., and Xin, Z.** (2001). Temperature sensing and cold acclimation. *Curr Opin Plant Biol* **4**, 241-246.
- Buttner, M., and Singh, K.B.** (1997). *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. *Proc Natl Acad Sci U S A* **94**, 5961-5966.
- Cao, H., Li, X., and Dong, X.** (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc Natl Acad Sci U S A* **95**, 6531-6536.
- Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X.** (1994). Characterization of an *Arabidopsis* Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. *Plant Cell* **6**, 1583-1592.
- Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X.** (1997). The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88**, 57-63.
- Carey, M.** (1998). The enhanceosome and transcriptional synergy. *Cell* **92**, 5-8.
- Carrington, J.C., and Freed, D.D.** (1990). Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. *J Virol* **64**, 1590-1597.
- Carrington, J.C., Freed, D.D., and Oh, C.S.** (1990). Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing. *Embo J* **9**, 1347-1353.
- Chakravarthy, S., Tuori, R.P., D'Ascenzo, M.D., Fobert, P.R., Despres, C., and Martin, G.B.** (2003). The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box cis elements. *Plant Cell* **15**, 3033-3050.
- Chen, C., and Chen, Z.** (2002). Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced *Arabidopsis* transcription factor. *Plant Physiol* **129**, 706-716.
- Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z., Chen, X.,**

- Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangel, J.L., Wang, X., and Zhu, T.** (2002). Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* **14**, 559-574.
- Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., and Luan, S.** (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. *Plant Physiol* **129**, 661-677.
- Choi, H., Hong, J., Ha, J., Kang, J., and Kim, S.Y.** (2000). ABFs, a family of ABA-responsive element binding factors. *J Biol Chem* **275**, 1723-1730.
- Citovsky, V., Warnick, D., and Zambryski, P.** (1994). Nuclear import of Agrobacterium VirD2 and VirE2 proteins in maize and tobacco. *Proc Natl Acad Sci U S A* **91**, 3210-3214.
- Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X.** (1998). Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis cpr6-1 mutant. *Plant Cell* **10**, 557-569.
- Clarke, J.D., Volko, S.M., Ledford, H., Ausubel, F.M., and Dong, X.** (2000). Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in arabidopsis. *Plant Cell* **12**, 2175-2190.
- Clough, S.J., and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* **16**, 735-743.
- Creelman, R.A., and Mullet, J.E.** (1997). Oligosaccharins, brassinolides, and jasmonates: nontraditional regulators of plant growth, development, and gene expression. *Plant Cell* **9**, 1211-1223.
- Daniel, X., Lacomme, C., Morel, J.B., and Roby, D.** (1999). A novel myb oncogene homologue in Arabidopsis thaliana related to hypersensitive cell death. *Plant J* **20**, 57-66.
- Davis, S.J., and Vierstra, R.D.** (1998). Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol Biol* **36**, 521-528.
- de Boer, G.J., Testerink, C., Pielage, G., Nijkamp, H.J., and Stuitje, A.R.** (1999). Sequences surrounding the transcription initiation site of the Arabidopsis enoyl-acyl carrier protein reductase gene control seed expression in transgenic tobacco. *Plant Mol Biol* **39**, 1197-1207.
- Delaney, T.P., Friedrich, L., and Ryals, J.A.** (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc Natl Acad Sci U S A* **92**, 6602-6606.
- Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D.X., Bittner-Eddy, P., Beynon, J., and Marco, Y.** (2002). Resistance to *Ralstonia solanacearum* in Arabidopsis thaliana is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. *Proc Natl Acad Sci U S A* **99**, 2404-2409.
- Despres, C., Subramaniam, R., Matton, D.P., and Brisson, N.** (1995). The Activation of the Potato PR-10a Gene Requires the Phosphorylation of the Nuclear Factor PBF-1. *Plant Cell* **7**, 589-598.
- Despres, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P.R.** (2000). The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* **12**, 279-290.
- Doares, S.H., Syrovets, T., Weiler, E.W., and Ryan, C.A.** (1995a). Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc Natl Acad Sci U S A* **92**, 4095-4098.

- Doares, S.H., Narvaez-Vasquez, J., Conconi, A., and Ryan, C.A.** (1995b). Salicylic Acid Inhibits Synthesis of Proteinase Inhibitors in Tomato Leaves Induced by Systemin and Jasmonic Acid. *Plant Physiol* **108**, 1741-1746.
- Doebley, J., and Lukens, L.** (1998). Transcriptional regulators and the evolution of plant form. *Plant Cell* **10**, 1075-1082.
- Dong, J., Chen, C., and Chen, Z.** (2003). Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. *Plant Mol Biol* **51**, 21-37.
- Dong, X.** (1998). SA, JA, ethylene, and disease resistance in plants. *Curr Opin Plant Biol* **1**, 316-323.
- Dorsett, D.** (1999). Distant liaisons: long-range enhancer-promoter interactions in *Drosophila*. *Curr Opin Genet Dev* **9**, 505-514.
- Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2003). OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J* **33**, 751-763.
- Durner, J., Shah, J. and Klessig, D.F.** (1997). Salicylic acid and disease resistance in plants. *Trends in Plant Science* **2**, 266-274.
- Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E., and Jones, J.D.** (2000). cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* **12**, 963-977.
- Ecker, J.R., and Theologis, A.** (1994). Ethylene: A unique Plant Signalling Molecule. In *Arabidopsis*, C.R. Somerville and E.M. Meyerowitz, eds (New York: Cold Spring Harbour Laboratory Press), pp. 485-522.
- Eggermont, K., Goderis, I., and Broekeart, W.F.** (1996). High-throughput RNA extraction from plant samples based on homogenisation by reciprocal shaking in the presence of a mixture of sand and glass beads. *Plant Molecular Biology Reporter* **14**, 273-279.
- Ellis, J., Dodds, P., and Pryor, T.** (2000). Structure, function and evolution of plant disease resistance genes. *Curr Opin Plant Biol* **3**, 278-284.
- Ellis, J.G., Tokuhisa, J.G., Llewellyn, D.J., Bouchez, D., Singh, K., Dennis, E.S., and Peacock, W.J.** (1993). Does the ocs-element occur as a functional component of the promoters of plant genes? *Plant J* **4**, 433-443.
- Eppe, P., Apel, K., and Bohlmann, H.** (1995). An Arabidopsis thaliana thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. *Plant Physiol* **109**, 813-820.
- Eppe, P., Apel, K., and Bohlmann, H.** (1997). Overexpression of an endogenous thionin enhances resistance of Arabidopsis against *Fusarium oxysporum*. *Plant Cell* **9**, 509-520.
- Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E.** (2000). The WRKY superfamily of plant transcription factors. *Trends Plant Sci* **5**, 199-206.
- Eulgem, T., Rushton, P.J., Schmelzer, E., Hahlbrock, K., and Somssich, I.E.** (1999). Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. *Embo J* **18**, 4689-4699.
- Fan, W., and Dong, X.** (2002). In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. *Plant Cell* **14**, 1377-1389.
- Farmer, E.E., Johnson, R.E., and Ryan, C.A.** (1992). Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiology* **98**, 995-1002.

- Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G.** (1994). Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. *Plant Cell* **6**, 751-759.
- Feys, B.J., and Parker, J.E.** (2000). Interplay of signaling pathways in plant disease resistance. *Trends Genet* **16**, 449-455.
- Fowler, S., and Thomashow, M.F.** (2002). Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14**, 1675-1690.
- Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M.** (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* **12**, 393-404.
- Galbraith, D.W., Lambert, G.M., Grebenok, R.J., and Sheen, J.** (1995). Flow cytometric analysis of transgene expression in higher plants: green-fluorescent protein. *Methods Cell Biol* **50**, 3-14.
- Genoud, T., and Metraux, J.P.** (1999). Crosstalk in plant cell signaling: structure and function of the genetic network. *Trends Plant Sci* **4**, 503-507.
- Genoud, T., Millar, A.J., Nishizawa, N., Kay, S.A., Schafer, E., Nagatani, A., and Chua, N.H.** (1998). An Arabidopsis mutant hypersensitive to red and far-red light signals. *Plant Cell* **10**, 889-904.
- Gheysen, G., Herman, L., Breynne, P., Gielen, J., Van Montagu, M., and Depicker, A.** (1990). Cloning and sequence analysis of truncated T-DNA inserts from *Nicotiana tabacum*. *Gene* **94**, 155-163.
- Gidekel, M., Jimenez, B., and Herrera-Estrella, L.** (1996). The first intron of the Arabidopsis thaliana gene coding for elongation factor 1 beta contains an enhancer-like element. *Gene* **170**, 201-206.
- Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., and Thomashow, M.F.** (2000). Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol* **124**, 1854-1865.
- Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M., and Thomashow, M.F.** (1998). Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *The Plant Journal* **16**, 433-442.
- Glazebrook, J.** (1999). Genes controlling expression of defense responses in Arabidopsis. *Curr Opin Plant Biol* **2**, 280-286.
- Glazebrook, J.** (2001). Genes controlling expression of defense responses in Arabidopsis--2001 status. *Curr Opin Plant Biol* **4**, 301-308.
- Glazebrook, J., Rogers, E.E., and Ausubel, F.M.** (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. *Genetics* **143**, 973-982.
- Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T., and Katagiri, F.** (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J* **34**, 217-228.
- Goff, S.A., Cone, K.C., and Chandler, V.L.** (1992). Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins. *Genes Dev* **6**, 864-875.
- Gorlich, D., and Mattaj, I.W.** (1996). Nucleocytoplasmic transport. *Science* **271**, 1513-1518.
- Grebenok, R.J., Pierson, E., Lambert, G.M., Gong, F.C., Afonso, C.L., Haldeman-Cahill, R., Carrington, J.C., and Galbraith, D.W.** (1997). Green-fluorescent protein fusions for efficient characterization of nuclear targeting. *Plant J* **11**, 573-586.

- Grill, E., and Himmelbach, A.** (1998). ABA signal transduction. *Curr Opin Plant Biol* **1**, 412-418.
- Gu, Y.Q., Yang, C., Thara, V.K., Zhou, J., and Martin, G.B.** (2000). Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell* **12**, 771-786.
- Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X., Han, Y., and Martin, G.B.** (2002). Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in Arabidopsis. *Plant Cell* **14**, 817-831.
- Guerineau, F., Benjdia, M., and Zhou, D.X.** (2003). A jasmonate-responsive element within the *A. thaliana* vsp1 promoter. *J Exp Bot* **54**, 1153-1162.
- Guiltinan, M.J., Marcotte, W.R., Jr., and Quatrano, R.S.** (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. *Science* **250**, 267-271.
- Guo, Y., Xiong, L., Ishitani, M., and Zhu, J.K.** (2002). An Arabidopsis mutation in translation elongation factor 2 causes superinduction of CBF/DREB1 transcription factor genes but blocks the induction of their downstream targets under low temperatures. *Proc Natl Acad Sci U S A* **99**, 7786-7791.
- Gupta, V., Willits, M.G., and Glazebrook, J.** (2000). Arabidopsis thaliana EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. *Mol Plant Microbe Interact* **13**, 503-511.
- Gutterson, N., and Reuber, T.L.** (2004). Regulation of disease resistance pathways by AP2/ERF transcription factors. *Curr Opin Plant Biol* **7**, 465-471.
- Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F., and Zhang, J.Z.** (2002). Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. *Plant Physiol* **130**, 639-648.
- Hahn, S.** (1993). Structure(?) and function of acidic transcription activators. *Cell* **72**, 481-483.
- Hammond-Kosack, K.E., and Jones, J.D.** (1996). Resistance gene-dependent plant defense responses. *Plant Cell* **8**, 1773-1791.
- Hanahan, D.** (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557-580.
- Hanna-Rose, W., and Hansen, U.** (1996). Active repression mechanisms of eukaryotic transcription repressors. *Trends Genet* **12**, 229-234.
- Hao, D., Ohme-Takagi, M., and Sarai, A.** (1998). Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *J Biol Chem* **273**, 26857-26861.
- Hao, D., Yamasaki, K., Sarai, A., and Ohme-Takagi, M.** (2002). Determinants in the sequence specific binding of two plant transcription factors, CBF1 and NtERF2, to the DRE and GCC motifs. *Biochemistry* **41**, 4202-4208.
- Hart, C.M., Nagy, F., and Meins, F., Jr.** (1993). A 61 bp enhancer element of the tobacco beta-1,3-glucanase B gene interacts with one or more regulated nuclear proteins. *Plant Mol Biol* **21**, 121-131.
- Hicks, G.R., and Raikhel, N.V.** (1995). Nuclear localization signal binding proteins in higher plant nuclei. *Proc Natl Acad Sci U S A* **92**, 734-738.
- Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T.** (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res* **27**, 297-300.
- Hildmann, T., Ebner, M., Pena-Cortes, H., Sanchez-Serrano, J.J., Willmitzer, L., and Prat, S.** (1992). General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. *Plant Cell* **4**, 1157-1170.

- Hill, A., Nantel, A., Rock, C.D., and Quatrano, R.S. (1996). A conserved domain of the viviparous-1 gene product enhances the DNA binding activity of the bZIP protein EmBP-1 and other transcription factors. *J Biol Chem* **271**, 3366-3374.
- Hilson, P., Small, I., and Kuiper, M.T. (2003). European consortia building integrated resources for Arabidopsis functional genomics. *Curr Opin Plant Biol* **6**, 426-429.
- Hoeren, F.U., Dolferus, R., Wu, Y., Peacock, W.J., and Dennis, E.S. (1998). Evidence for a role for AtMYB2 in the induction of the Arabidopsis alcohol dehydrogenase gene (ADH1) by low oxygen. *Genetics* **149**, 479-490.
- Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, T.E., and Welin, B. (2000). Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. *J Exp Bot* **51**, 177-185.
- Huang, H., Tudor, M., Su, T., Zhang, Y., Hu, Y., and Ma, H. (1996). DNA binding properties of two Arabidopsis MADS domain proteins: binding consensus and dimer formation. *Plant Cell* **8**, 81-94.
- Hurt, E.C. (1996). Importins/karyopherins meet nucleoporins. *Cell* **84**, 509-515.
- Initiative, A.G. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796-815.
- Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J.K. (1997). Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell* **9**, 1935-1949.
- Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001). Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *Plant J* **27**, 325-333.
- Jackson, A.O., and Taylor, C.B. (1996). Plant-Microbe Interactions: Life and Death at the Interface. *Plant Cell* **8**, 1651-1668.
- Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998). Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **280**, 104-106.
- Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T., and Parcy, F. (2002). bZIP transcription factors in Arabidopsis. *Trends Plant Sci* **7**, 106-111.
- Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *Embo J* **6**, 3901-3907.
- Jensen, A.B., Goday, A., Figueras, M., Jessop, A.C., and Pages, M. (1998). Phosphorylation mediates the nuclear targeting of the maize Rab17 protein. *Plant J* **13**, 691-697.
- Jin, H., and Martin, C. (1999). Multifunctionality and diversity within the plant MYB-gene family. *Plant Mol Biol* **41**, 577-585.
- Jofuku, K.D., den Boer, B.G., Van Montagu, M., and Okamura, J.K. (1994). Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. *Plant Cell* **6**, 1211-1225.
- Johnson, C., Boden, E., Desai, M., Pascuzzi, P., and Arias, J. (2001). In vivo target promoter-binding activities of a xenobiotic stress-activated TGA factor. *Plant J* **28**, 237-243.

- Kachroo, P., Shanklin, J., Shah, J., Whittle, E.J., and Klessig, D.F.** (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc Natl Acad Sci U S A* **98**, 9448-9453.
- Kagaya, Y., Ohmiya, K., and Hattori, T.** (1999). RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. *Nucleic Acids Res* **27**, 470-478.
- Kalderon, D., Richardson, W.D., Markham, A.F., and Smith, A.E.** (1984). Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311**, 33-38.
- Kang, J.Y., Choi, H.I., Im, M.Y., and Kim, S.Y.** (2002). Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell* **14**, 343-357.
- Karin, M., and Hunter, T.** (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Biol* **5**, 747-757.
- Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol* **17**, 287-291.
- Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., and Bohnert, H.J.** (2001). Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* **13**, 889-905.
- Kempin, S.A., Liljegren, S.J., Block, L.M., Rounsley, S.D., Yanofsky, M.F., and Lam, E.** (1997). Targeted disruption in Arabidopsis. *Nature* **389**, 802-803.
- Kerr, M.K., and Churchill, G.A.** (2001). Statistical design and the analysis of gene expression microarray data. *Genet Res* **77**, 123-128.
- Kim, H.J., Kim, Y.K., Park, J.Y., and Kim, J.** (2002). Light signalling mediated by phytochrome plays an important role in cold-induced gene expression through the C-repeat/dehydration responsive element (C/DRE) in Arabidopsis thaliana. *Plant J* **29**, 693-704.
- Kim, S.R., Choi, J.L., Costa, M.A., and An, G.** (2003). Identification of G-box sequence as an essential element for methyl jasmonate response of potato proteinase inhibitor II promoter. *Plant Physiology* **99**, 627-631.
- Kinkema, M., Fan, W., and Dong, X.** (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* **12**, 2339-2350.
- Kirby, J., and Kavanagh, T.A.** (2002). NAN fusions: a synthetic sialidase reporter gene as a sensitive and versatile partner for GUS. *Plant J* **32**, 391-400.
- Kirsch, C., Takamiya-Wik, M., Schmeizer, E., Hahlbrock, K., and Somssich, I.E.** (2000). A novel regulatory element involved in rapid activation of parsley *ELI7* gene family members by fungal elicitor or pathogen infection. *Molecular Plant Pathology* **1**, 243-251.
- Kishor, P., Hong, Z., Miao, G.H., Hu, C., and Verma, D.** (1995). Overexpression of [δ]-Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants. *Plant Physiol* **108**, 1387-1394.
- Kizis, D., and Pages, M.** (2002). Maize DRE-binding proteins DBF1 and DBF2 are involved in rab17 regulation through the drought-responsive element in an ABA-dependent pathway. *Plant J* **30**, 679-689.
- Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F., and Kunkel, B.N.** (2001). Resistance to *Pseudomonas syringae* conferred by an Arabidopsis thaliana coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. *Plant J* **26**, 509-522.

- Knight, H., and Knight, M.R.** (2001). Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci* **6**, 262-267.
- Kranz, H.D., Denekamp, M., Greco, R., Jin, H., Leyva, A., Meissner, R.C., Petroni, K., Urzainqui, A., Bevan, M., Martin, C., Smeeckens, S., Tonelli, C., Paz-Ares, J., and Weisshaar, B.** (1998). Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. *Plant J* **16**, 263-276.
- Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F.** (2002). Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol* **130**, 2129-2141.
- Kruse, S., Kleinedam, R.G., Roggentin, P., and Schauer, R.** (1996). Expression and purification of a recombinant "small" sialidase from *Clostridium perfringens* A99. *Protein Expr Purif* **7**, 415-422.
- Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M.** (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244-1245.
- Kunkel, B.N., and Brooks, D.M.** (2002). Cross talk between signaling pathways in pathogen defense. *Curr Opin Plant Biol* **5**, 325-331.
- Kunst, L., Klenz, J.E., Martinez-Zapater, J., and Haughn, G.W.** (1989). AP2 Gene Determines the Identity of Perianth Organs in Flowers of *Arabidopsis thaliana*. *Plant Cell* **1**, 1195-1208.
- Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J.** (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol Plant Microbe Interact* **8**, 863-870.
- Lazo, G.R., Stein, P.A., and Ludwig, R.A.** (1991). A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology (N Y)* **9**, 963-967.
- Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E.** (1998). Functional analysis of regulatory sequences controlling PR-1 gene expression in *Arabidopsis*. *Plant J* **16**, 223-233.
- Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B., and Zhu, J.K.** (2001a). The *Arabidopsis* HOS1 gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo--cytoplasmic partitioning. *Genes Dev* **15**, 912-924.
- Lee, H., Guo, Y., Ohta, M., Xiong, L., Stevenson, B., and Zhu, J.K.** (2002). LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase. *Embo J* **21**, 2692-2702.
- Lee, M.W., Qi, M., and Yang, Y.** (2001b). A novel jasmonic acid-inducible rice myb gene associates with fungal infection and host cell death. *Mol Plant Microbe Interact* **14**, 527-535.
- Leon, J., Rojo, E., and Sanchez-Serrano, J.J.** (2001). Wound signalling in plants. *J Exp Bot* **52**, 1-9.
- Leon, J., Rojo, E., Titarenko, E., and Sanchez-Serrano, J.J.** (1998). Jasmonic acid-dependent and -independent wound signal transduction pathways are differentially regulated by Ca²⁺/calmodulin in *Arabidopsis thaliana*. *Mol Gen Genet* **258**, 412-419.
- Leon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A., and Koornneef, M.** (1996). Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *Plant J* **10**, 655-661.

- Li, J., Brader, G., and Palva, E.T.** (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* **16**, 319-331.
- Li, L., Li, C., and Howe, G.A.** (2001). Genetic analysis of wound signaling in tomato. Evidence for a dual role of jasmonic acid in defense and female fertility. *Plant Physiol* **127**, 1414-1417.
- Li, X., Zhang, Y., Clarke, J.D., Li, Y., and Dong, X.** (1999). Identification and cloning of a negative regulator of systemic acquired resistance, SN11, through a screen for suppressors of *npr1-1*. *Cell* **98**, 329-339.
- Li, Z., and Thomas, T.L.** (1998). PE11, an embryo-specific zinc finger protein gene required for heart-stage embryo formation in Arabidopsis. *Plant Cell* **10**, 383-398.
- Liu, L., White, M.J., and MacRae, T.H.** (1999). Transcription factors and their genes in higher plants functional domains, evolution and regulation. *Eur J Biochem* **262**, 247-257.
- Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* **10**, 1391-1406.
- Lotan, T., Ori, N., and Fluhr, R.** (1989). Pathogenesis-related proteins are developmentally regulated in tobacco flowers. *Plant Cell* **1**, 881-887.
- Mahalingam, R., Gomez-Buitrago, A., Eckardt, N., Shah, N., Guevara-Garcia, A., Day, P., Raina, R., and Fedoroff, N.V.** (2003). Characterizing the stress/defense transcriptome of Arabidopsis. *Genome Biol* **4**, R20.
- Malamy, J., Carr, J.P., Klessig, D.F., and Raskin, I.** (1990). Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**, 1002-1004.
- Maleck, K., and Dietrich, R.A.** (1999). Defense on multiple fronts: how do plants cope with diverse enemies? *Trends Plant Sci* **4**, 215-219.
- Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A.** (2000). The transcriptome of Arabidopsis thaliana during systemic acquired resistance. *Nat Genet* **26**, 403-410.
- Martienssen, R.A.** (1998). Functional genomics: probing plant gene function and expression with transposons. *Proc Natl Acad Sci U S A* **95**, 2021-2026.
- Martin, G.B.** (1999). Functional analysis of plant disease resistance genes and their downstream effectors. *Curr Opin Plant Biol* **2**, 273-279.
- Mazarei, M., Puthoff, D.P., Hart, J.K., Rodermeil, S.R., and Baum, T.J.** (2002). Identification and characterization of a soybean ethylene-responsive element-binding protein gene whose mRNA expression changes during soybean cyst nematode infection. *Mol Plant Microbe Interact* **15**, 577-586.
- Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D., and Koornneef, M.** (1998). Arabidopsis thaliana: a model plant for genome analysis. *Science* **282**, 662, 679-682.
- Meissner, R.C., Jin, H., Cominelli, E., Denekamp, M., Fuertes, A., Greco, R., Kranz, H.D., Penfield, S., Petroni, K., Urzainqui, A., Martin, C., Paz-Ares, J., Smeeckens, S., Tonelli, C., Weisshaar, B., Baumann, E., Klimyuk, V., Marillonnet, S., Patel, K., Speulman, E., Tissier, A.F., Bouchez, D., Jones, J.J., Pereira, A., Wisman, E., and et al.** (1999). Function search in a large transcription factor gene family in

- Arabidopsis: assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. *Plant Cell* **11**, 1827-1840.
- Menke, F.L., Champion, A., Kijne, J.W., and Memelink, J.** (1999). A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *Embo J* **18**, 4455-4463.
- Menkens, A.E., Schindler, U., and Cashmore, A.R.** (1995). The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. *Trends Biochem Sci* **20**, 506-510.
- Menssen, A., and Hermeking, H.** (2002). Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc Natl Acad Sci U S A* **99**, 6274-6279.
- Meyers, B.C., Vu, T.H., Tej, S.S., Ghazal, H., Matvienko, M., Agrawal, V., Ning, J., and Haudenschild, C.D.** (2004). Analysis of the transcriptional complexity of *Arabidopsis thaliana* by massively parallel signature sequencing. *Nat Biotechnol* **22**, 1006-1011.
- Michaels, S.D., Ditta, G., Gustafson-Brown, C., Pelaz, S., Yanofsky, M., and Amasino, R.M.** (2003). *AGL24* acts as a promoter of flowering in *Arabidopsis* and is positively regulated by vernalization. *Plant J* **33**, 867-874.
- Miklos, G.L., and Rubin, G.M.** (1996). The role of the genome project in determining gene function: insights from model organisms. *Cell* **86**, 521-529.
- Mol, J., Grotewold, E., and Koes, R.** (1998). How genes paint flowers and seeds. *Trends Plant Sci* **3**, 212-217.
- Murashige, T., and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology* **15**, 473-497.
- Murphy, G., and Kavanagh, T.** (1988). Speeding-up the sequencing of double-stranded DNA. *Nucleic Acids Res* **16**, 5198.
- Murre, C., McCaw, P.S., and Baltimore, D.** (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* **56**, 777-783.
- Mysore, K.S., Crasta, O.R., Tuori, R.P., Folkerts, O., Swirsky, P.B., and Martin, G.B.** (2002). Comprehensive transcript profiling of Pto- and Prf-mediated host defense responses to infection by *Pseudomonas syringae* pv. tomato. *Plant J* **32**, 299-315.
- Nakashima, K., Kiyosue, T., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (1997). A nuclear gene, *erd1*, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in *Arabidopsis thaliana*. *Plant J* **12**, 851-861.
- Nakashima, K., Shinwari, Z.K., Sakuma, Y., Seki, M., Miura, S., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2000). Organization and expression of two *Arabidopsis* DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. *Plant Mol Biol* **42**, 657-665.
- Narusaka, Y., Nakashima, K., Shinwari, Z.K., Sakuma, Y., Furihata, T., Abe, H., Narusaka, M., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2003). Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis* rd29A gene in response to dehydration and high-salinity stresses. *Plant J* **34**, 137-148.
- Nawrath, C., and Metraux, J.P.** (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* **11**, 1393-1404.

- Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., and Dennis, E.S.** (1990). Chitinase, beta-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. *Plant Cell* **2**, 673-684.
- Newman, T., de Bruijn, F.J., Green, P., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Thomashow, M., and et al.** (1994). Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous Arabidopsis cDNA clones. *Plant Physiol* **106**, 1241-1255.
- Ni, M., Dehesh, K., Tepperman, J.M., and Quail, P.H.** (1996). GT-2: in vivo transcriptional activation activity and definition of novel twin DNA binding domains with reciprocal target sequence selectivity. *Plant Cell* **8**, 1041-1059.
- Nicholas, K.B., Nicholas, H.B.J., and II, D.D.W.** (1997). Analysis and visualisation of genetic variation. *EMBNET.News* **4**, 14.
- Niggeweg, R., Thurow, C., Kegler, C., and Gatz, C.** (2000). Tobacco transcription factor TGA2.2 is the main component of as-1-binding factor ASF-1 and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. *J Biol Chem* **275**, 19897-19905.
- Norman-Setterblad, C., Vidal, S., and Palva, E.T.** (2000). Interacting signal pathways control defense gene expression in Arabidopsis in response to cell wall-degrading enzymes from *Erwinia carotovora*. *Mol Plant Microbe Interact* **13**, 430-438.
- O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O., and Bowles, D.J.** (1996). Ethylene as a Signal Mediating the Wound Response of Tomato Plants. *Science* **274**, 1914-1917.
- Ohme-Takagi, M., and Shinshi, H.** (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* **7**, 173-182.
- Ohme-Takagi, M., Suzuki, K., and Shinshi, H.** (2000). Regulation of ethylene-induced transcription of defense genes. *Plant Cell Physiol* **41**, 1187-1192.
- Ohta, M., Ohme-Takagi, M., and Shinshi, H.** (2000). Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. *Plant J* **22**, 29-38.
- Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M.** (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* **13**, 1959-1968.
- Okamuro, J.K., Caster, B., Villarreal, R., Van Montagu, M., and Jofuku, K.D.** (1997). The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. *Proc Natl Acad Sci U S A* **94**, 7076-7081.
- O'Kane, C.J., and Gehring, W.J.** (1987). Detection in situ of genomic regulatory elements in *Drosophila*. *Proc Natl Acad Sci U S A* **84**, 9123-9127.
- O'Keefe, D.P., Tepperman, J.M., Dean, C., Leto, K.J., Erbes, D.L., and Odel, T.** (1994). Plant expression of a bacterial cytochrome P450 that catalyses activation of a sulfonylurea pro-herbicide. *Plant Physiol* **105**, 473-482.
- Oono, Y., Seki, M., Nanjo, T., Narusaka, M., Fujita, M., Satoh, R., Satou, M., Sakurai, T., Ishida, J., Akiyama, K., Iida, K., Maruyama, K., Satoh, S., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (2003). Monitoring expression profiles of Arabidopsis gene expression during rehydration process after dehydration using ca 7000 full-length cDNA microarray. *Plant J* **34**, 868-887.
- Osterlund, M.T., and Paterson, A.H.** (2002). Applied plant genomics: the secret is integration. *Curr Opin Plant Biol* **5**, 141-145.

- Park, J.M., Park, C.J., Lee, S.B., Ham, B.K., Shin, R., and Paek, K.H.** (2001). Overexpression of the tobacco Tsi1 gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell* **13**, 1035-1046.
- Peltier, J.B., Ytterberg, J., Liberles, D.A., Roepstorff, P., and van Wijk, K.J.** (2001). Identification of a 350-kDa ClpP protease complex with 10 different Clp isoforms in chloroplasts of *Arabidopsis thaliana*. *J Biol Chem* **276**, 16318-16327.
- Pena-Cortes, H., Willmitzer, L., and Sanchez-Serrano, J.J.** (1991). Absciscic Acid Mediates Wound Induction but Not Developmental-Specific Expression of the Proteinase Inhibitor II Gene Family. *Plant Cell* **3**, 963-972.
- Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F.** (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* **10**, 2103-2113.
- Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F.** (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell* **8**, 2309-2323.
- Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J.** (2000). *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell* **103**, 1111-1120.
- Pieterse, C.M., and van Loon, L.C.** (1999). Salicylic acid-independent plant defence pathways. *Trends Plant Sci* **4**, 52-58.
- Pieterse, C.M., van Wees, S.C., Hoffland, E., van Pelt, J.A., and van Loon, L.C.** (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* **8**, 1225-1237.
- Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester, M., Laan, R., Gerriets, H., Weisbeek, P.J., and van Loon, L.C.** (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* **10**, 1571-1580.
- Qiang, L., Nanming, Z., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (2000). Regulatory role of DREB transcription factors in plant drought, salt and cold tolerance. *Chinese Science Bulletin* **45**, 970-975.
- Qin, X., and Zeevaart, J.A.** (1999). The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proc Natl Acad Sci U S A* **96**, 15354-15361.
- Restrepo, M.A., Freed, D.D., and Carrington, J.C.** (1990). Nuclear transport of plant potyviral proteins. *Plant Cell* **2**, 987-998.
- Reuber, T.L., Plotnikova, J.M., Dewdney, J., Rogers, E.E., Wood, W., and Ausubel, F.M.** (1998). Correlation of defense gene induction defects with powdery mildew susceptibility in *Arabidopsis* enhanced disease susceptibility mutants. *Plant J* **16**, 473-485.
- Reymond, P., and Farmer, E.E.** (1998). Jasmonate and salicylate as global signals for defense gene expression. *Curr Opin Plant Biol* **1**, 404-411.
- Reymond, P., Weber, H., Damond, M., and Farmer, E.E.** (2000). Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* **12**, 707-720.

- Riechmann, J.L., and Meyerowitz, E.M.** (1998). The AP2/EREBP family of plant transcription factors. *Biol Chem* **379**, 633-646.
- Riechmann, J.L., and Ratcliffe, O.J.** (2000). A genomic perspective on plant transcription factors. *Curr Opin Plant Biol* **3**, 423-434.
- Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G.** (2000). Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science* **290**, 2105-2110.
- Robatzek, S., and Somssich, I.E.** (2001). A new member of the Arabidopsis WRKY transcription factor family, AtWRKY6, is associated with both senescence- and defence-related processes. *Plant J* **28**, 123-133.
- Robatzek, S., and Somssich, I.E.** (2002). Targets of AtWRKY6 regulation during plant senescence and pathogen defense. *Genes Dev* **16**, 1139-1149.
- Robbins, J., Dilworth, S.M., Laskey, R.A., and Dingwall, C.** (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615-623.
- Roggentin, P., Rothe, B., Lottspeich, F., and Schauer, R.** (1988). Cloning and sequencing of a *Clostridium perfringens* sialidase gene. *FEBS Lett* **238**, 31-34.
- Royo, E., Leon, J., and Sanchez-Serrano, J.J.** (1999). Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. *Plant J* **20**, 135-142.
- Royo, E., Titarenko, E., Leon, J., Berger, S., Vancanneyt, G., and Sanchez-Serrano, J.J.** (1998). Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathways in *Arabidopsis thaliana*. *Plant J* **13**, 153-165.
- Romero, I., Fuertes, A., Benito, M.J., Malpica, J.M., Leyva, A., and Paz-Ares, J.** (1998). More than 80R2R3-MYB regulatory genes in the genome of *Arabidopsis thaliana*. *Plant J* **14**, 273-284.
- Roth, B.A., Goff, S.A., Klein, T.M., and Fromm, M.E.** (1991). C1- and R-dependent expression of the maize Bz1 gene requires sequences with homology to mammalian myb and myc binding sites. *Plant Cell* **3**, 317-325.
- Royo, J., Vancanneyt, G., Perez, A.G., Sanz, C., Stormann, K., Rosahl, S., and Sanchez-Serrano, J.J.** (1996). Characterization of three potato lipoxygenases with distinct enzymatic activities and different organ-specific and wound-regulated expression patterns. *J Biol Chem* **271**, 21012-21019.
- Rushton, P.J., and Somssich, I.E.** (1998). Transcriptional control of plant genes responsive to pathogens. *Curr Opin Plant Biol* **1**, 311-315.
- Rushton, P.J., Reinstadler, A., Lipka, V., Lippok, B., and Somssich, I.E.** (2002). Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. *Plant Cell* **14**, 749-762.
- Rushton, P.J., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K., and Somssich, I.E.** (1996). Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *Embo J* **15**, 5690-5700.
- Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., and Hunt, M.D.** (1996). Systemic Acquired Resistance. *Plant Cell* **8**, 1809-1819.

- Sainz, M.B., Goff, S.A., and Chandler, V.L.** (1997). Extensive mutagenesis of a transcriptional activation domain identifies single hydrophobic and acidic amino acids important for activation in vivo. *Mol Cell Biol* **17**, 115-122.
- Saitou, N., and Nei, M.** (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425.
- Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2002). DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem Biophys Res Commun* **290**, 998-1009.
- Sambrook, J., Fritsch, E.F., and Maniatis, T.** (1989). *Molecular cloning. A laboratory manual.* (New York: CSHL Press).
- Sanderfoot, A.A., and Lazarowitz, S.G.** (1995). Cooperation in Viral Movement: The Geminivirus BL1 Movement Protein Interacts with BR1 and Redirects It from the Nucleus to the Cell Periphery. *Plant Cell* **7**, 1185-1194.
- Schellmann, S., Schnittger, A., Kirik, V., Wada, T., Okada, K., Beermann, A., Thumfahrt, J., Jurgens, G., and Hulskamp, M.** (2002). TRIPTYCHON and CAPRICE mediate lateral inhibition during trichome and root hair patterning in Arabidopsis. *Embo J* **21**, 5036-5046.
- Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M.** (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc Natl Acad Sci U S A* **97**, 11655-11660.
- Schwechheimer, C., Smith, C., and Bevan, M.W.** (1998). The activities of acidic and glutamine-rich transcriptional activation domains in plant cells: design of modular transcription factors for high-level expression. *Plant Mol Biol* **36**, 195-204.
- Schweizer, P., Buchala, A., Dudler, R., and Metraux, J.P.** (1998). Induced systemic resistance in wounded rice plants. *The Plant Journal* **14**, 475-481.
- Scofield, S.R., English, J.J., and Jones, J.D.** (1993). High level expression of the Activator transposase gene inhibits the excision of Dissociation in tobacco cotyledons. *Cell* **75**, 507-517.
- Seki, M., Kamei, A., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (2003). Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Curr Opin Biotechnol* **14**, 194-199.
- Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K.** (2001). Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* **13**, 61-72.
- Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K.** (2002). Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J* **31**, 279-292.
- Seo, M., and Koshiba, T.** (2002). Complex regulation of ABA biosynthesis in plants. *Trends Plant Sci* **7**, 41-48.
- Sessa, G., Meller, Y., and Fluhr, R.** (1995). A GCC element and a G-box motif participate in ethylene-induced expression of the PRB-1b gene. *Plant Mol Biol* **28**, 145-153.

- Shah, J., Tsui, F., and Klessig, D.F.** (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Mol Plant Microbe Interact* **10**, 69-78.
- Shah, J., Kachroo, P., Nandi, A., and Klessig, D.F.** (2001). A recessive mutation in the *Arabidopsis* *SSI2* gene confers SA- and NPR1-independent expression of PR genes and resistance against bacterial and oomycete pathogens. *Plant J* **25**, 563-574.
- Shannon, S., and Meeks-Wagner, D.R.** (1993). Genetic Interactions That Regulate Inflorescence Development in *Arabidopsis*. *Plant Cell* **5**, 639-655.
- Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., and Galbraith, D.W.** (1995). Green-fluorescent protein as a new vital marker in plant cells. *Plant J* **8**, 777-784.
- Shinozaki, K., and Yamaguchi-Shinozaki, K.** (1996). Molecular responses to drought and cold stress. *Curr Opin Biotechnol* **7**, 161-167.
- Shinozaki, K., and Yamaguchi-Shinozaki, K.** (1997). Gene Expression and Signal Transduction in Water-Stress Response. *Plant Physiol* **115**, 327-334.
- Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr Opin Plant Biol* **3**, 217-223.
- Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M.** (2003). Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* **6**, 410-417.
- Shinwari, Z.K., Nakashima, K., Miura, S., Kasuga, M., Seki, M., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (1998). An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. *Biochem Biophys Res Commun* **250**, 161-170.
- Silver, P.A.** (1991). How proteins enter the nucleus. In *Cell*, pp. 489-497.
- Simpson, S.D., Nakashima, K., Narusaka, Y., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2003). Two different novel cis-acting elements of *erd1*, a *clpA* homologous *Arabidopsis* gene function in induction by dehydration stress and dark-induced senescence. *Plant J* **33**, 259-270.
- Singh, K., Foley, R.C., and Onate-Sanchez, L.** (2002). Transcription factors in plant defense and stress responses. *Curr Opin Plant Biol* **5**, 430-436.
- Singh, K.B.** (1998). Transcriptional regulation in plants: the importance of combinatorial control. *Plant Physiol* **118**, 1111-1120.
- Skarnes, W.C.** (1993). The identification of new genes: gene trapping in transgenic mice. *Curr Opin Biotechnol* **4**, 684-689.
- Skriver, K., and Mundy, J.** (1990). Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* **2**, 503-512.
- Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R.** (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev* **12**, 3703-3714.
- Somerville, C., and Meyerowitz, E.** (1994). Introduction. In *Arabidopsis*, E. Meyerowitz and C. Somerville, eds (New York: Cold Spring Harbour Laboratory Press), pp. 1-20.
- Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., and Pieterse, C.M.** (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* **15**, 760-770.
- Sprenger-Haussels, M., and Weisshaar, B.** (2000). Transactivation properties of parsley proline-rich bZIP transcription factors. *Plant J* **22**, 1-8.

- Staswick, P.E.** (1990). Novel Regulation of Vegetative Storage Protein Genes. *Plant Cell* **2**, 1-6.
- Steponkus, P.L., Uemura, M., Joseph, R.A., and Thomoashow, M.F.** (1998). Mode of action of the *cor15a* gene on the freezing tolerance of *Arabidopsis thaliana*. *PNAS* **95**, 14570-14575.
- Stevenson, L.F., Kennedy, B.K., and Harlow, E.** (2001). A large-scale overexpression screen in *Saccharomyces cerevisiae* identifies previously uncharacterized cell cycle genes. *Proc Natl Acad Sci U S A* **98**, 3946-3951.
- Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E.E.** (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. *Proc Natl Acad Sci U S A* **98**, 12837-12842.
- Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F.** (1997). *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc Natl Acad Sci U S A* **94**, 1035-1040.
- Stracke, R., Werber, M., and Weisshaar, B.** (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr Opin Plant Biol* **4**, 447-456.
- Stratmann, J.W.** (2003). Long distance run in the wound response--jasmonic acid is pulling ahead. *Trends Plant Sci* **8**, 247-250.
- Sugimoto, K., Takeda, S., and Hirochika, H.** (2000). MYB-related transcription factor NtMYB2 induced by wounding and elicitors is a regulator of the tobacco retrotransposon Tto1 and defense-related genes. *Plant Cell* **12**, 2511-2528.
- Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D., Dean, C., Ma, H., and Martienssen, R.** (1995). Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev* **9**, 1797-1810.
- Suzuki, K., Suzuki, N., Ohme-Takagi, M., and Shinshi, H.** (1998). Immediate early induction of mRNAs for ethylene-responsive transcription factors in tobacco leaf strips after cutting. *The Plant Journal* **15**, 657-665.
- Suzuki, M., Kao, C.Y., and McCarty, D.R.** (1997). The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *Plant Cell* **9**, 799-807.
- Takeshita, S., Sato, M., Toba, M., Masahashi, W., and Hashimoto-Gotoh, T.** (1987). High-copy-number and low-copy-number plasmid vectors for lacZ alpha-complementation and chloramphenicol- or kanamycin-resistance selection. *Gene* **61**, 63-74.
- Thara, V.K., Tang, X., Gu, Y.Q., Martin, G.B., and Zhou, J.M.** (1999). *Pseudomonas syringae* pv tomato induces the expression of tomato EREBP-like genes *pti4* and *pti5* independent of ethylene, salicylate and jasmonate. *Plant J* **20**, 475-483.
- Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F.** (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci U S A* **95**, 15107-15111.
- Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F.** (1999). Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. *Plant Physiol* **121**, 1093-1102.
- Thomma, B.P., Penninckx, I.A., Broekaert, W.F., and Cammue, B.P.** (2001). The complexity of disease signaling in *Arabidopsis*. *Curr Opin Immunol* **13**, 63-68.

- Thomma, B.P.H.J., Eggermont, K., Broekaert, W.F., and Cammue, B.P.** (2000). Disease development of several fungi on *Arabidopsis* can be reduced by treatment with methyl jasmonate. *Plant Physiology and Biochemistry* **38**, 421-427.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G.** (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876-4882.
- Tissier, A.F., Marillonnet, S., Klimyuk, V., Patel, K., Torres, M.A., Murphy, G., and Jones, J.D.** (1999). Multiple independent defective suppressor-mutator transposon insertions in *Arabidopsis*: a tool for functional genomics. *Plant Cell* **11**, 1841-1852.
- Titarenko, E., Rojo, E., Leon, J., and Sanchez-Serrano, J.J.** (1997). Jasmonic acid-dependent and -independent signaling pathways control wound-induced gene activation in *Arabidopsis thaliana*. *Plant Physiol* **115**, 817-826.
- Ton, J., Davison, S., Van Wees, S.C., Van Loon, L., and Pieterse, C.M.** (2001). The *Arabidopsis* ISR1 locus controlling rhizobacteria-mediated induced systemic resistance is involved in ethylene signaling. *Plant Physiol* **125**, 652-661.
- Torisky, R., Fellers, J.P., and Collins, G.B.** (1996). A Focusing device for Tissue Transformation with the DuPont/Biorad PDS1000 Helium Microprojectile System. *Plant Molecular Biology Reporter* **14**, 124-133.
- Torres-Schumann, S., Godoy, J.A., and Pintor-Toro, J.A.** (1992). A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol Biol* **18**, 749-757.
- Traw, M.B., Kim, J., Enright, S., Cipollini, D.F., and Bergelson, J.** (2003). Negative cross-talk between salicylate- and jasmonate-mediated pathways in the Wassilewskija ecotype of *Arabidopsis thaliana*. *Mol Ecol* **12**, 1125-1135.
- Triezenberg, S.J.** (1995). Structure and function of transcriptional activation domains. *Curr Opin Genet Dev* **5**, 190-196.
- Uesugi, M., Nyanguile, O., Lu, H., Levine, A.J., and Verdine, G.L.** (1997). Induced alpha helix in the VP16 activation domain upon binding to a human TAF. *Science* **277**, 1310-1313.
- Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J.** (1992). Acquired resistance in *Arabidopsis*. *Plant Cell* **4**, 645-656.
- Ulmasov, T., Hagen, G., and Guilfoyle, T.** (1994). The ocs element in the soybean GH2/4 promoter is activated by both active and inactive auxin and salicylic acid analogues. *Plant Mol Biol* **26**, 1055-1064.
- Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2000). *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc Natl Acad Sci U S A* **97**, 11632-11637.
- Urao, T., Yamaguchi-Shinozaki, K., Urao, S., and Shinozaki, K.** (1993). An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell* **5**, 1529-1539.
- van Wees, S.C., de Swart, E.A., van Pelt, J.A., van Loon, L.C., and Pieterse, C.M.** (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **97**, 8711-8716.
- van Wees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., van't Westende, Y.A.M., Hartog, F., and van Loon, L.C.** (1997). Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant-Microbe Interact* **6**, 716-724.

- Vanacker, H., Lu, H., Rate, D.N., and Greenberg, J.T.** (2001). A role for salicylic acid and NPR1 in regulating cell growth in Arabidopsis. *Plant J* **28**, 209-216.
- Vidal, S., Ponce de Leon, I., Denecke, J., and E.T., P.** (1997). Salicylic acid and the plant pathogen *Erwinia carotovora* induce defense genes via antagonistic pathways. *The Plant Journal* **11**, 115-123.
- Vijayan, P., Shockey, J., Levesque, C.A., Cook, R.J., and Browse, J.** (1998). A role for jasmonate in pathogen defense of arabidopsis. *Proc Natl Acad Sci U S A* **95**, 7209-7214.
- von Arnim, A.G., and Deng, X.W.** (1994). Light inactivation of Arabidopsis photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. *Cell* **79**, 1035-1045.
- Wada, T., Tachibana, T., Shimura, Y., and Okada, K.** (1997). Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. *Science* **277**, 1113-1116.
- Wada, T., Kurata, T., Tominaga, R., Koshino-Kimura, Y., Tachibana, T., Goto, K., Marks, M.D., Shimura, Y., and Okada, K.** (2002). Role of a positive regulator of root hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. *Development* **129**, 5409-5419.
- Walbot, V.** (2000). Arabidopsis thaliana genome. A green chapter in the book of life. *Nature* **408**, 794-795.
- Wang, H., Datla, R., Georges, F., Loewen, M., and Cutler, A.J.** (1995). Promoters from kin1 and cor6.6, two homologous Arabidopsis thaliana genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum and dehydration. *Plant Mol Biol* **28**, 605-617.
- Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P., and Ryals, J.A.** (1991). Coordinate Gene Activity in Response to Agents That Induce Systemic Acquired Resistance. *Plant Cell* **3**, 1085-1094.
- Washburn, K.B., Davis, E.A., and Ackerman, S.** (1997). Coactivators and TAFs of transcription activation in wheat. *Plant Mol Biol* **35**, 1037-1043.
- Weigel, D.** (1995). The APETALA2 domain is related to a novel type of DNA binding domain. *Plant Cell* **7**, 388-389.
- Wilson, C., Bellen, H.J., and Gehring, W.J.** (1990). Position effects on eukaryotic gene expression. *Annu Rev Cell Biol* **6**, 679-714.
- Wu, K., Tian, L., Hollingworth, J., Brown, D.C., and Miki, B.** (2002). Functional analysis of tomato Pti4 in Arabidopsis. *Plant Physiol* **128**, 30-37.
- Xiong, L., and Zhu, J.K.** (2001). Abiotic stress signal transduction in plants: Molecular and genetic perspectives. *Physiol Plant* **112**, 152-166.
- Xiong, L., and Zhu, J.K.** (2002). Molecular and genetic aspects of plant responses to osmotic stress. *Plant Cell Environ* **25**, 131-139.
- Xiong, L., Schumaker, K.S., and Zhu, J.K.** (2002a). Cell signaling during cold, drought, and salt stress. *Plant Cell* **14 Suppl**, S165-183.
- Xiong, L., Ishitani, M., Lee, H., and Zhu, J.K.** (2001a). The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfuryase and modulates cold stress- and osmotic stress-responsive gene expression. *Plant Cell* **13**, 2063-2083.
- Xiong, L., Lee, H., Ishitani, M., and Zhu, J.K.** (2002b). Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis. *J Biol Chem* **277**, 8588-8596.

- Xiong, L., Lee, B., Ishitani, M., Lee, H., Zhang, C., and Zhu, J.K.** (2001b). FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. *Genes Dev* **15**, 1971-1984.
- Xu, Y., Chang, P., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A.** (1994). Plant Defense Genes Are Synergistically Induced by Ethylene and Methyl Jasmonate. *Plant Cell* **6**, 1077-1085.
- Yamaguchi-Shinozaki, K., and Shinozaki, K.** (1993). Characterization of the expression of a desiccation-responsive rd29 gene of Arabidopsis thaliana and analysis of its promoter in transgenic plants. *Mol Gen Genet* **236**, 331-340.
- Yamaguchi-Shinozaki, K., and Shinozaki, K.** (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* **6**, 251-264.
- Yamaguchi-Shinozaki, K., and Shinozaki, K.** (2001). Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. In *Rice biotechnology: improving yield, stress tolerance and grain quality*. (Wiley, Chichester: Novartis Foundation Symposium), pp. 176-189.
- Yanagisawa, S., and Sheen, J.** (1998). Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. *Plant Cell* **10**, 75-89.
- Yang, Y., Shah, J., and Klessig, D.F.** (1997). Signal perception and transduction in plant defense responses. *Genes Dev* **11**, 1621-1639.
- Ye, G.N., Stone, D., Pang, S.Z., Creely, W., Gonzalez, K., and Hinchee, M.** (1999). Arabidopsis ovule is the target for Agrobacterium in planta vacuum infiltration transformation. *Plant J* **19**, 249-257.
- Yu, D., Chen, C., and Chen, Z.** (2001). Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* **13**, 1527-1540.
- Zhang, J.Z.** (2003). Overexpression analysis of plant transcription factors. *Curr Opin Plant Biol* **6**, 430-440.
- Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X.** (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc Natl Acad Sci U S A* **96**, 6523-6528.
- Zhou, J., Tang, X., and Martin, G.B.** (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *Embo J* **16**, 3207-3218.
- Zhou, J.M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D.F.** (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol Plant Microbe Interact* **13**, 191-202.
- Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J.** (1998). PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. *Plant Cell* **10**, 1021-1030.
- Zhu, J.K.** (2001). Cell signaling under salt, water and cold stresses. *Curr Opin Plant Biol* **4**, 401-406.
- Zhu, J.K.** (2002). Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol* **53**, 247-273.
- Zhu, Y., Cai, X.L., Wang, Z.Y., and Hong, M.M.** (2003). An interaction between a MYC protein and an EREBP protein is involved in transcriptional regulation of the rice Wx gene. *J Biol Chem* **278**, 47803-47811.

