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The University of Dublin, Trinity College



**Defining the role of the AP2/ERF transcription
factors Rap2.4 and Rap2.4b in stress responses in the
model plant *Arabidopsis thaliana*.**

Liina Rae

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

Doctor of Philosophy

**Department of Genetics
Trinity College
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Thesis 9347

DECLARATION

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SUMMARY

This study characterises the molecular properties and the function of two *Arabidopsis* AP2/ERF transcription factors, Rap2.4 and Rap2.4b. The two genes belong to the DREB subgroup, members of which have been demonstrated to function in plant responses to abiotic stresses. Identifying the transcription factors involved in mediating responses to abiotic stress is a topic of great interest in plant biology, as the genes controlling stress signalling cascades can offer significant potential for crop improvement.

Rap2.4 and Rap2.4b are highly similar genes that have originated from a common ancestor via duplication of a large chromosomal DNA segment. Expression profiling utilizing Northern blotting technique and the GUS reporter gene showed only partially overlapping expression patterns for the two genes in different plant tissues and under various abiotic stress conditions.

Investigations of the molecular properties of the encoded proteins revealed further similarities. The amino acid region that functions as a nuclear localisation signal for Rap2.4 is fully conserved in Rap2.4b, and the DNA-binding preferences, determined by gel-shift analyses, were very similar for both transcription factors. In addition, both genes acted as transcriptional activators in an *in vivo* transactivation assay.

Single knockout lines of either Rap2.4 or Rap2.4b, or a double knockout line had no discernible phenotype when grown under normal conditions or when exposed to abiotic stresses. However, global gene expression profiling of mRNAs from dehydrated tissues of the double knockout line revealed an interesting molecular phenotype. Simultaneous elimination of Rap2.4 and Rap2.4b resulted in down-regulation of a number of genes normally associated with drought responses. Most strikingly, expression of several genes coding for water channel proteins (aquaporins) was reduced as was expression of a dehydrin, the proline transporter AtProT3 and the ABC (ATP-Binding Cassette) family transporter AtWBC23, and two genes encoding DREB transcription factors.

Overexpression of Rap2.4 or Rap2.4b resulted in ABA hypersensitivity at germination. ABA hypersensitivity has been associated with increased tolerance to abiotic stresses in the published literature. However, although enhanced drought resistance was previously reported for Rap2.4b overexpression lines, our mutants failed to display such a trait. Global gene expression profiling of the Rap2.4 overexpression line revealed increased expression of several aquaporin genes, dehydrins, and genes associated with lipid and cell-wall metabolism. In addition, the two DREBs that showed reduced expression in the double knockout line were significantly upregulated in the overexpression line.

ABBREVIATIONS

° C	degrees celsius
2,4-D	2,4-dichlorophenoxyacetic acid
A	adenine
ABA	abscisic acid
ABA1/2/3	ABA-deficient 1/2/3
ABI1/2/3/4/5	ABA-insensitive 1/2/3/4/5
ABRE	ABA response element
ACC	aminocyclopropane-1-carboxylic acid
<i>Agrobacterium</i>	<i>Agrobacterium tumefaciens</i>
AP2 domain	AP2 DNA binding domain
APB	alkaline phosphatase buffer
<i>Arabidopsis</i>	<i>Arabidopsis thaliana</i>
AREB/ABF	ABA response element binding factor
ARF-Aux/IAA	Auxin response factor
arg/R	arginine
BAP	benzylaminopurine
BCIP	5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt
BLAST	basic local alignment search tool
bp	base pair
C	control/cytosine
CaMV 35S	Cauliflower mosaic virus 35S promoter
CBF	C-repeat binding factor
cDNA	complementary DNA
CDPK	Ca ²⁺ dependent protein kinases
CHX	cycloheximide
<i>cis</i> -element	<i>cis</i> -acting element
CO ₂	carbon dioxide
COR genes	cold responsive genes
cORF	composite open reading frame
d	days
DAG	diacylglycerole
DEPC	diethylpyrocarbonate
dH ₂ O	deionized water
DIG	digoxigenin
DKO	double knockout
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DRE/CRT	drought responsive/C-repeat element
DREB	DRE-element binding protein
DTT	dithiotreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EAR-domain	ERF-associated amphiphilic repression domain
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
ERF	ethylene response factor
EST	expressed sequence tag
EtBr	ethidium bromide
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FGCZ	Functional Genomics Center Zurich
g	gravitational acceleration/gram
G	guanine
GA	gibberellic acid
GEO	Gene Expression Omnibus
GFP	green fluorescent protein

GM	genetically modified
GUS	β -glucuronidase
h	hour
H ₂ O ₂	hydrogen peroxide
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
His ₆ tag	histidine tag
IP ₃	1,4,5-trisphosphate
IPTG	isopropyl β -D-thiogalactoside
JA	jasmonic acid
K ₂ HPO ₄	potassium phosphate, dibasic
kb	kilobase
KCl	potassium chloride
kDA	kilodalton
KH ₂ PO ₄	potassium phosphate, monobasic
LB	Luria Bertrani
LEA	Late Embryogenesis Abundant
LiCl	lithium chloride
lys/K	lysine
M	molar
MAPK	kinase mitogen associated protein kinase
Mbp	mega base pair
MES	2-(N-morpholino)ethanesulfonic acid
mg, ml, mM	milligram, millilitre, millimolar
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
MIP	membrane intrinsic proteins
mRNA	messenger RNA
MS	Murashige and Skoog medium
MUG	4-methylumbelliferyl β -D-glucuronide
<i>N. benthamiana</i>	<i>Nicotiana benthamiana</i>
Na ₂ CO ₃	sodium carbonate
Na ₂ HPO ₄	sodium phosphate, dibasic
NaCl	sodium chloride
NaH ₂ PO ₄	sodium phosphate, dibasic
NAN	cytoplasmic sialidase
NAOH	sodium hydroxide
NASC	European Arabidopsis Stock Centre
NBT	nitro-blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
ng, nm, nM	nanogram, nanometer, nanomolar
NLS	nuclear localisation signal
NOS	nopaline synthetase
OE	overexpression
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
<i>Pfu</i>	<i>Pyrococcus furiosus</i>
PIP	plasma-membrane intrinsic protein
PMSF	phenylmethanesulphonylfluoride
PP2C	protein phosphatase 2C
primer	oligonucleotide primer
RNA	ribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II
RNAse	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute

RT-PCR	reverse-transcriptase PCR
SA	salicylic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser/S	serine
SnRK2	SNF-related protein kinase 2
SSC	sodium chloride and sodium citrate buffer
SUMO	small ubiquitin-like modifier
T	thymine
TAIR	The Arabidopsis Information Resource
TBE	tris-boric acid-EDTA buffer
TCD	Trinity College Dublin
T-DNA	transferred DNA of the tumor-inducing (Ti) plasmid
TE	tris-EDTA buffer
TEMED	tetramethylethylenediamine
TF	transcription factor
thr	threonine
TIP	tonoplast intrinsic protein
TPP	trehalose-6-phosphate phosphatase
Tris-HCl	tris buffer, pH calibrated with HCl
UN	United Nations
uORF	upstream open reading frame
UTR	un-translated region
UV	ultraviolet
V	volts
w/v	weight/volume
WT	wild type
X-GLUC	5-bromo-4-chloro-3-indolyl- β -D-glucuronic
YM	yeast extract media
μ g, μ l, μ M	microgram, microlitre, micromolar

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Plant biology

Mankind is ultimately dependent on plants. Conversion of light energy into chemical energy through photosynthesis is the primary source of all food for humans and animals. The human population has quadrupled during the past 100 years and is expected to grow from 6.7 billion in 2006 to 9.2 billion by 2050 (UN, 2007). At this growth rate, an increase of 50 % in food production by 2050 will be required to sustain the resulting population. The availability of sufficient quantities of food is already an acute problem as demonstrated by rocketing prices for basic cereals such as wheat, corn and rice despite record crops in 2008, which led to riots in several countries. Food production increases in the past were achieved by expansion of cultivated land and through increases in yield due to the use of fertilizers and irrigation systems. However, yields have stabilized for cereals in the past decade and food production is unlikely to achieve the levels required by 2050 due to water scarcity, land degradation, urban build-up and the use of arable land for production of non-food crops, such as biofuels (Nellemann and Svihus, February 2009).

Water scarcity in particular is expected to have a substantial negative effect. Crop yields on irrigated lands are significantly higher and over 40 % of the food in the world is currently produced on irrigated lands. The availability of irrigation water which today largely originates from non-renewable sources (Rost et al., 2008), will possibly decline due to effects of climate change (Alcamo et al., 2003) and groundwater depletion through anthropogenic use in some areas (Rodell et al., 2009). In addition to water scarcity, increasingly higher salinity of the soil also poses problems. Unsustainably irrigated land is especially prone to salinization and nutrient depletion. Currently, saline soils are estimated to cover up to 10 % of the world's arable land and high salinity accounts for considerable losses in crop production every year. In irrigated areas where approximately 20 % of the land is salt-affected, the crop losses due to high salinity are even more marked (Nellemann and Svihus, February 2009; Szabolcs, 1994). It has been estimated that every minute at least 3 ha of arable land is lost due to excessive soil salinity (FAO, 2009).

Considering the projected increase in demand and the expected problems in meeting that demand, a great deal of effort has been invested in generating improved crop varieties that are able to withstand deteriorating environmental conditions. Despite the slow progress in breeding for tolerance to drought and high salinity using traditional methods, a few varieties of common cereals such as rice and wheat showing increased tolerance of these stresses have been produced on a commercial scale (reviewed in Witcombe et al., 2008). However, finding the appropriate sources for traits conferring abiotic stress tolerance is problematic as the availability of genetic variation within the germplasm collections of cultivated species is rather limited. Desired traits can be crossed into crop species from land races or wild relatives, but obtaining stress tolerant varieties

that also maintain high yields is time-consuming. In contrast, genetic engineering via plant transformation techniques can exploit novel sources of genetic variation and introduce genes from unrelated species, with the potential for more rapid progress.

Although genetic engineering technologies have been developed and employed commercially, the release of genetically modified (GM) crops has been met with concern and opposition in the affluent Northern hemisphere, particularly in Europe. From 1998 to 2004 a *de facto* moratorium suspended the approval of new genetically modified organisms for cultivation in the European Union (EU). More recently, because the ban has been replaced with a very strict regulatory regime, few EU countries are currently growing GM crops, although field trials are underway in many. Encouragingly, public support for GM foods is on the rise in Europe. In 2008 the Institute of Grocery Distribution in the UK found that nearly half of respondents regarded GM technology as potentially useful for dealing with global food shortages. Moreover, as demonstrated in a European Commission funded research project in five EU countries in 2007, the actual choices of European consumers are only about 20 % anti-GM.

GM crops enjoy more support in countries where environmental problems perhaps more immediately affect production of main food staples. For example, in Australia where the public awareness of potential GM crops with enhanced drought and salt tolerance, both acute problems there, has increased in the past years, over 70 % of the public holds a positive view towards GM technology (OECD, 2009; Rigaud, 2008). The biggest producer of GM crops is the USA where also the widest variety of GM crops are grown including soya beans, maize, rapeseed, cotton, squash, papaya, alfalfa and sugar beet (sourced from www.gmo-compass.org).

The GM crops in commercial use today are based on simple monogenic traits (resistance to herbicides, insects or viruses) that utilize genetic material from exogenous non-plant sources. Engineering for next generation crops, featuring improvements in more complex traits such as photosynthetic ability, nutrient up-take efficiency and stress tolerance, is likely to be based on modifying endogenous molecular pathways in the host plant. Deregulation of such crops is expected to be more straightforward than for first generation biotechnology crops (Century et al., 2008).

However, in order to fully harness the possibilities for increased food production offered by genetic engineering of crop species, greater knowledge of plant biology, especially in regard to plant responses and adaptation mechanisms to stresses, is essential. Understanding how some plant species are able to acquire tolerance to adverse environmental conditions through various biochemical and physiological processes will enable a more complete understanding of these complex traits and hopefully will help to identify specific molecular pathways that can be exploited in the generation of improved crop varieties.

1.2 The model plant *Arabidopsis thaliana*

Arabidopsis thaliana is a small dicotyledonous plant which belongs to the Brassicaceae family (Planta; Magnoliophyta; Magnoliopsida; Brassicales; Brassicaceae; genus *Arabidopsis*). Despite not being a crop species, it is one of the most widely used model organisms in plant

biology. *Arabidopsis* as a model plant possesses several desirable traits. It is easy to cultivate where space is restricted, has a short life cycle and produces large numbers of progeny. The genome of *Arabidopsis* is relatively small and can be manipulated through genetic engineering more easily and rapidly than any other plant genome. Different *Arabidopsis* accessions present researches with large variation in physiological traits and large numbers of mutant lines are available from international stock centres. Most importantly, a fully sequenced genome and the vast amount of knowledge obtained from years of studies contribute to the popularity of *Arabidopsis* as a model plant (The Arabidopsis Genome Initiative, 2000; Somerville and Koornneef, 2002; Somerville and Meyerowitz, 1994).

The main food crops are however monocotyledonous cereals, so the relevance of a dicotyledonous plant as a adequate model should be considered. Monocot and dicot plants last shared a common ancestor approximately 200 million years ago (Wolfe et al., 1989) and have diverged considerably since. Comparisons between the *Arabidopsis* and rice genomes and a collection of 240,000 sugarcane ESTs representing about 30,000 genes, suggest that roughly 14 % of sugarcane genes might confer monocot-specific traits while about 70 % of genes are similar in all three species (Vincentz et al., 2004). As expected, with over two thirds of genes presenting sequence similarity, the functions of many important genes are conserved between dicots and monocots.

The relevance of the *Arabidopsis* model is demonstrated by a proven track record in the identification of genes which can be used to improve stress tolerance in crop species. For example, a large-scale functional genomics program examining the effects of continuous expression of >1,500 *Arabidopsis* putative transcription factor genes, was recently used by Mendel Biotechnology Inc in order to identify molecular regulators of drought stress adaptation. The study resulted in the identification of approximately 40 transcription factors that were confirmed to act as regulators of drought tolerance enhancement. The *Arabidopsis* findings were applied in commercial crop improvement through a collaboration with the Monsanto Company. The maize homolog (ZmNF-YB2) of the *Arabidopsis* drought tolerance-conferring gene AtNF-YB1, significantly enhanced the yield of maize grown under drought conditions (Nelson et al., 2007).

1.3 Abiotic stress response pathways in *Arabidopsis*

Abiotic stresses such as high salinity and dehydration affect many aspects of plant physiology and metabolism. Exposure to abiotic stresses activates signal transduction pathways which lead to significant changes in gene expression. The adaptation to stress is thought to be mediated by both pre-existing and induced defenses (Hasegawa et al., 2000; Zhu, 2002). Different abiotic stresses involve complex stimuli that possess related yet different attributes. For example high salinity and dehydration both have an element of osmotic stress but excessive salt concentrations also encompass an ionic aspect. Freezing temperatures similarly limit the amount of water available to the cell but additionally result in mechanical constraints and altered activities of macromolecules. The appropriate adaptation mechanisms differ depending on the type of stress, but generally stress signalling can be divided into three functional categories based on the aspect

of plant physiology affected: ionic and osmotic stress signalling which acts to re-establish an appropriate homeostatic environment in cells under stress conditions; detoxification mechanisms controlling and repairing the cellular damage caused by stress; and lastly, signalling that acts to control (mainly limit) growth processes. The three signalling pathways interact – homeostasis signalling suppresses the detoxification response, while homeostasis and detoxification signals negatively regulate the growth inhibition response (Xiong et al., 2002b; Xiong and Zhu, 2002; Zhu, 2002).

Homeostasis at the macromolecular level

Compatible solutes/osmolytes play an important role during re-establishment of homeostasis under hyperosmotic stress conditions. Compatible solutes are highly soluble organic compounds such as sugars, sugar alcohols, amino acids and their derivatives that do not interfere with cellular metabolism even at very high concentrations. Osmolytes were originally thought to function in osmotic adjustment – a process that maintains an appropriate water potential gradient in plant roots upon changing soil conditions (Bray et al., 2002). Accumulating evidence, however, suggests that their main role lies in maintaining the natural conformation of macromolecules, possibly due to their ability to scavenge reactive oxygen species (Shen et al., 1997; Smirnov and Cumbes, 1989; Zhu, 2001). The amino acid proline is a potent scavenger of reactive oxygen species (Chen and Dickman, 2005) and increased levels of free proline have been observed in many transgenic abiotic stress-resistant plants. Transgenic plants overexpressing the heat shock protein HSP26 or the transcription factors GmDREB3, OsDREB1A and DREB1A, displayed elevated levels of free proline and soluble sugars, and enhanced tolerance to abiotic stresses (Chen et al., 2009; Ito et al., 2006; Xue et al., 2009). Negative regulators of stress responses, for example, ESKIMO1 and the maize protein phosphatase ZmPP2C also regulate proline levels. In *eskimo1* mutants, free proline levels were increased and plants showed enhanced freezing tolerance (Xin and Browse, 1998). Overexpression of ZmPP2C resulted in lowered proline levels and decreased tolerance to salt and drought (Liu et al., 2009).

Manipulating individual osmolyte biosynthetic pathways in order to achieve stress tolerance in crop species has had limited success. Nevertheless, engineering rice, wheat, sugar beet and tomato to accumulate sugars and polyols such as fructan, trehalose or mannitol, has been reported to confer enhanced salt or drought tolerance (Abebe et al., 2003; Cortina and Culia n-ez-Macia, 2005; Garg et al., 2002; Pilon-Smits et al., 1999). Enhanced accumulation of proline, glycine betaine or polyamines led to improved stress tolerance in rice and maize (Capell et al., 2004; Quan et al., 2004; Roy and Wu, 2002; Sakamoto et al., 1998).

Another class of protective molecules are the LEA (Late Embryogenesis Abundant) proteins which accumulate during the last stage of embryo maturation and in vegetative tissues in response to abiotic stress across the whole plant kingdom. Even though the function of LEA proteins is not clear, they are thought to act as molecular chaperones (Battaglia et al., 2008; Xiong and Zhu, 2002).

Cellular homeostasis depends not only on transcription and translation but also on the regulation of protein stability (or half-life), particularly in the case of regulatory proteins, and on the elimination of damaged proteins (Xiong and Zhu, 2002). This occurs via the ubiquitin-26S proteasome pathway which recognizes specific proteins and targets them, via ubiquitination, for destruction in the 26S proteasome. The critical importance of this pathway in *Arabidopsis* has been revealed by mutations in ubiquitination/proteasome components that block development, circadian rhythms, photomorphogenesis, hormone responses, senescence, and response to pathogen invasion (Craig et al., 2009). Ubiquitination-related proteins and proteases are often found among genes that show enhanced expression under stress conditions (reviewed by Ingram and Bartels, 1996). Interestingly, the *Arabidopsis* genome contains a very large number of ubiquitination-associated proteins. There are two ubiquitin-activating E1 enzymes, 37 ubiquitin-conjugating E2 proteins and 1415 ubiquitin E3 ligases (reviewed in Craig et al., 2009). In comparison to over 700 F-box E3 ligases in the *Arabidopsis* genome, only 14 and 78 are found, respectively, in fission yeast and humans (Semple, 2003). The E3 ligases are thought to confer the specificity to the ubiquitination process and the expansion of this specific subgroup in the plant lineage supports the idea that plants have greatly expanded the use of protein turnover as a means of actively and precisely modulating the complex interactions directing plant responses to internal and external signals (reviewed by Yee and Goring, 2009). Recent studies have demonstrated the importance of several ubiquitin E3 ligases in responses to abiotic stresses. Double knockout mutants lacking the DREB2A-INTERACTING PROTEIN1 (DRIP1) and DRIP2 which function as E3 ubiquitin ligases showed enhanced tolerance to dehydration (Qin et al., 2008). Overexpression of the RING-finger SDIR1, the F-box DOR or the U-box proteins PUB22 or PUB23 resulted in hypersensitivity to stress, illustrating the importance of E3 ligases in stress response pathways (Cho et al., 2008; Zhang et al., 2008b; Zhang et al., 2007c).

Sumoylation is a process controlling protein stability similar to ubiquitination and has been functionally linked to growth and flowering, defense and stress, and to ABA and phosphate starvation signalling (reviewed in Miura et al., 2007a). Addition of SUMO (Small ubiquitin-like modifier) tags can have different consequences for proteins – altered localization, altered activity or enhanced stability (reviewed by Geiss-Friedlander and Melchior, 2007). The main SUMO E3 ligase in plants is SIZ1. Knocking out SIZ1 causes sensitivity to ABA and freezing due to lack of sumoylation of the important regulators of ABA and cold stress response pathways ABI5 and ICE1, respectively (Miura et al., 2007b; Miura et al., 2009).

Ion homeostasis

Maintaining ion homeostasis is an important step in achieving salt stress tolerance. High salinity environments are most commonly due to high concentrations of NaCl, but result in altered cellular levels of K^+ and Ca^{2+} as well as Na^+ and Cl^- . High Na^+ concentration has a negative impact on K^+ uptake and causes Ca^{2+} accumulation in cells. Ion homeostasis is dependent on ion transport proteins, Ca^{2+} -ATPases, secondary active transporters and water channel proteins (reviewed in Hasegawa et al., 2000; Xiong and Zhu, 2002).

In particular, Na⁺-removing Na⁺/H⁺ antiporters are being studied as potential targets for improving crop plant salt tolerance. Up-regulation of AtNHX1 (Apse et al., 1999) and SOS1 (Shi et al., 2002) confers salt tolerance to transgenic *Arabidopsis* plants. The effectiveness of overexpressing a vacuolar Na⁺/H⁺ antiporter gene in improving salt tolerance has also been shown in tomato (Zhang and Blumwald, 2001), oilseed rape (Zhang et al., 2001a), rice (Ohta et al., 2002), perennial ryegrass (Wang et al., 2005b), wheat (Zhe et al., 2004) and common buckwheat (Chen et al., 2008).

Detoxification

Reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radicals and superoxide anions are produced by normal cellular activities such as photorespiration and β -oxidation of fatty acids, but their levels increase when plants are exposed to stress conditions. ROS play a dual role in abiotic stress responses: low levels of ROS function as parts of signalling cascades, but high concentrations of ROS have a deleterious effect. The capacity to control ROS levels and prevent ROS-mediated damage to macromolecules such as proteins and DNA is an important stress tolerance trait. ROS elimination is achieved by antioxidant compounds such as ascorbic acid, glutathione, thioredoxin and carotenoids and by ROS-detoxifying enzymes such as superoxide dismutase, glutathione peroxidase and catalase (reviewed by Hasegawa et al., 2000; Xiong and Zhu, 2002; Zhu, 2001). For example, the ascorbate peroxidase 1 (APX1) functions in regulating H₂O₂ levels and H₂O₂ signalling in plant cells and plays a key role in the acclimation of plants to a combination of drought and heat stress (Koussevitzky et al., 2008).

However, the capacity to scavenge ROS is limited, and prolonged abiotic stresses cause ROS production to overwhelm scavenging activities, resulting in extensive cellular damage and death. A number of transgenic plants, including the crop species potato and rice, which display increased abiotic stress tolerance have been obtained through enhancement of detoxification pathways (reviewed in Carvalho, 2008).

Growth regulation

Upon encountering stressful conditions, plants usually arrest the progression of cell division. The ability to recover and sustain growth is a determinant of stress tolerance. Mediators of stress responses such as the plant hormones ABA and jasmonic acid (JA) are known to affect the cell-cycle machinery (Swiatek et al., 2002). The participation of MAPK kinases in stress-signalling pathways suggests that stress signalling and cell-cycle regulation might have common aspects (Kovtun et al., 2000; Krysan et al., 2002). Cyclin-dependent kinases have also been implicated in mediating the effects of stress on the cell cycle. In wheat, reduced mitotic activity due to water stress was associated with deactivation of the cyclin-dependent protein kinase Cdc2 (Schuppler et al., 1998). In maize, inhibition of cell division by water deficit was also associated with reduction in expression of the mitosis-regulating cyclin dependent kinase ZmCdc2 (Setter and Flannigan, 2001). Furthermore, a stress-inducible cyclin-dependent kinase inhibitor (EL2) from rice was recently shown to link cell-cycle regulation to abiotic and biotic stress responses (Peres et al., 2007).

Components of stress signalling pathways

Overall, *Arabidopsis* stress signalling pathways begin with signal perception, followed by the generation of secondary signalling molecules. Second messengers mediate the activation or induction of transcription factors that in turn regulate stress response genes. The stress response genes encode proteins with protective roles as well as regulatory functions. Stress responsive genes may participate in the generation of regulatory molecules, which can initiate a second round of signalling. The regulatory molecules participating in the stress-response pathways will be discussed at length below starting with essential stress hormone ABA and secondary messengers.

1.4 Regulatory molecules and genes in stress-signalling

Abscisic acid in stress signalling

The phytohormone abscisic acid (ABA) is produced in plants under abiotic and biotic stress conditions. Stress responses also involve other plant hormones like salicylic acid, ethylene and jasmonic acid but ABA remains the essential phytohormone for stress signalling.

Salt and drought stress and to a small extent cold stress cause increased biosynthesis of ABA and following stress relief ABA levels return to pre-stress levels (Stewart and Voetberg, 1985; Zeevaart, 1980). The ABA biosynthesis pathway is well-studied in *Arabidopsis*. Zeaxanthin epoxidase ZEP (also known as ABA1, LOS6) regulates the first step in ABA biosynthesis by catalyzing the epoxidation of zeaxanthin and antheraxanthin into violaxanthin (Schwartz et al., 1997b). The 9-cis-epoxycarotenoid dioxygenase (NCED) generates xanthoxin which is converted to ABA-aldehyde by the short-chain alcohol dehydrogenase encoded by ABA2 (Gonzalez-Guzman et al., 2002; Leon-Kloosterziel et al., 1996; Schwartz et al., 1997a). ABA-aldehyde is direct precursor of ABA and the last step in the biosynthetic pathway is controlled by ABA aldehyde oxidase AAO (Seo et al., 2000) and the molybdenum cofactor sulfurase MSCU encoded by ABA3/LOS5 (Bittner et al., 2001). The genes encoding ABA biosynthetic enzymes respond to salt and drought stress with the exception of the NCEDs, and are also up-regulated by ABA (Iuchi et al., 2001; Xiong et al., 2001b; Xiong et al., 2002a). The feedback loop in biosynthesis is necessary for the proper regulation of the ABA response pathway under stress conditions. Mutations in the RNA metabolism-associated SAD1 impairs the ABA regulation of AAO and MSCU causing enhanced drought sensitivity (Xiong et al., 2001a).

Overexpression of ABA biosynthetic genes has been shown to result in transgenic plants with improved drought and salt tolerance (Iuchi et al., 2001; Zhang et al., 2009b). ABA catabolism also plays a significant role in abiotic stress response pathways. CYP707A3 oxygenase is the major enzyme in ABA-depleting oxidative pathways whereas the β -glucosidase AtBG1 plays an important role in releasing ABA from a preformed pool of its inactive sugar-conjugated form (Lee et al., 2006; Umezawa et al., 2006).

The activation of ABA response pathways upon application of exogenous ABA suggests that plant cells are able to sense ABA levels outside the cell walls. In recent years, a number of candidates for ABA receptors have been proposed in the literature. The first report indicating the flowering time regulator FCA acts as an ABA receptor was retracted shortly after publication;

another report implicating an enzyme coding for magnesium chetalse as an ABA receptor was challenged by other research groups. Similarly, suggestions that G-protein coupled receptors act as ABA receptors have been met with caution (reviewed by Pennisi, 2009). Most recently, two independent studies published in the journal *Science* reported that members of the cyclase subfamily of the START domain superfamily bind ABA and regulate ABA-responses through protein phosphatase 2C (PP2C) proteins (Ma et al., 2009; Park et al., 2009). However, as with earlier putative ABA receptors, the latest findings have also been met with skepticism.

The down-stream portion of the ABA signalling cascade is often regulated by phosphorylation events. Protein kinases and phosphatases have both been implicated in mediating ABA responses. For example, three *Arabidopsis* SnRK2 protein kinases, SRK2D, SRK2E and SRK2I are essential for the ABA-mediated control of seed development and dormancy (Fujii and Zhu, 2009; Nakashima et al., 2009). Mutant *Arabidopsis* plants lacking SnRK2E show a reduced ability to close stomata under dehydration stress and have a wilted phenotype (Yoshida et al., 2006). PP2C proteins act immediately down-stream of putative ABA receptors and have been demonstrated to function as essential regulators of ABA responses. For example, a triple mutant lacking the genes *ABI1*, *ABI2* and *HAB1* coding for PP2Cs showed an extreme response to exogenous ABA which included impaired growth, and a partial constitutive response to endogenous ABA (Rubio et al., 2009).

Phosphorylation-regulated transcription factors carry out the last steps in the ABA-signalling cascade. Many ABA-responsive genes have specific ABA-responsive *cis*-elements named ABRE (ABRE-ABA response element; C/TACGTGGC) in their promoters. However, a single copy of ABRE is not sufficient for ABA-responsive expression and either additional copies of ABRE-elements or coupling elements are required. In *Arabidopsis*, ABRE-binding transcription factors (ABFs) belong to the family of bZIP transcription factors (Yamaguchi-Shinozaki and Shinozaki, 2006). Several ABF transcription factors contain a recognition element for the SnRK2 kinases (Shukla and Mattoo, 2008). ABF2, ABF3 and ABF4 which are up-regulated by ABA, dehydration and high salinity act as transcriptional activators (Uno et al., 2000). Overexpression of ABF3 and ABF4 leads to ABA hypersensitivity and enhanced drought tolerance indicating a positive role for ABFs in ABA response signalling (Kang et al., 2002). The target genes of ABFs include LEA genes *RD29A* and *RAB18*, the PP2C genes *ABI1* and *ABI2*, the cell cycle regulator cyclin-dependent kinase inhibitor *ICK1*, and potassium channels *KAT1* and *KAT2* (reviewed in Kim, 2006).

Second messengers in stress signalling

Second messenger molecules produced/released within plant cells form an important part of ABA-dependent and ABA-independent stress signalling cascades; Ca^{2+} , phospholipids and reactive oxygen species such as hydrogen peroxide all have roles in plant stress responses.

All abiotic stresses have been shown to increase the concentration of cytoplasmic Ca^{2+} , indicating that Ca^{2+} channels might act as stress sensors (Tuteja and Sopory, 2008; Xiong et al., 2002b). The increase in Ca^{2+} may also result from the activation of phospholipid signalling which

releases Ca^{2+} from intracellular Ca^{2+} stores (Zhu, 2002). An important feature of Ca^{2+} signalling is repetitiveness - early Ca^{2+} signalling can induce production of secondary messengers such as ABA which in turn may generate a second wave of Ca^{2+} signalling (reviewed in Xiong et al., 2002b). The fluctuating Ca^{2+} levels are perceived in cells by various Ca^{2+} binding proteins. Ca^{2+} dependent protein kinases (CDPKs) contain catalytic Ser/Thr kinase domains which can directly relay the signal to phosphorylation cascades. Ca^{2+} binding calmodulin-like (CML) and calcineurinB-like (CBL) proteins lack enzymatic activity and interact with other proteins in order to transmit the signal (Kolukisaoglu et al., 2004, and references therein). Examples of each class of Ca^{2+} binding protein have been implicated in stress responses, and Ca^{2+} signalling has been shown to play an important part in ABA-mediated signal transduction. Ca^{2+} accumulates in plant cells upon ABA application (reviewed in Hirayama and Shinozaki, 2007) and ABREs have been demonstrated to function as Ca^{2+} responsive *cis*-elements (Kaplan et al., 2006). *Arabidopsis* CML9 knockouts are hypersensitive to ABA and display enhanced tolerance to salt stress and water deficit (Magnan et al., 2008), whereas the loss of the CDPKs CPK4 and CPK11 caused ABA insensitivity and reduced salt tolerance (Zhu et al., 2007). The central negative regulators of ABA responses, ABI1 and ABI2 interact with protein kinase 3 (PKS3) which in turn interacts with Ca^{2+} binding ScaBP5 (SOS3-like calcium binding protein 5) further supporting the links between ABA and Ca^{2+} signalling (Guo et al., 2002).

Phospholipid messengers generated from membrane phospholipids also play an important role in stress signalling. In particular, high levels of IP_3 have been reported to accumulate in response to stress in various studies (DeWald et al., 2001; Drobak and Watkins, 2000; Takahashi et al., 2001). In plants, phospholipase C (PLC) which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to 1,4,5-trisphosphate (IP_3) and diacylglycerole (DAG) is induced by abiotic stresses (Hirayama et al., 1995) as is the PIP_5K gene encoding a phosphatidylinositol 4-phosphate 5-kinase – an enzyme responsible for the synthesis of the IP_3 precursor PIP_2 (Mikami et al., 1998). IP_3 releases Ca^{2+} from intercellular stores triggering further stress signalling cascades while DAG activates protein kinase C. In the *Arabidopsis fry1* mutant elevated IP_3 levels were correlated with superinduction of stress-responsive gene transcription (Xiong et al., 2001c). *FRY1* encodes an inositol polyphosphate-1-phosphatase that is involved in breakdown of IP_3 . Interestingly, despite enhanced stress gene expression, *fry1* mutants are more susceptible to damage by salt, drought and cold stress. It has been hypothesized that the elevated IP_3 levels may interrupt cellular homeostasis and the enhanced expression of stress-responsive genes is a measure to limit the stress injury (Zhu, 2002).

Phospholipase D (PLD) and AtPLC1 have also been implicated in plant stress signalling. PLD which generates phosphatidic acid (PA) from phospholipids mediates ABA-induced stomatal closure (Jacob et al., 1999) and AtPLC1 is required for full induction of the ABA-responsive genes RD29A, KIN2 and RD22 (Sanchez and Chua, 2001).

As mentioned earlier reactive oxygen species (ROS) may also function in the stress signalling cascade. In several studies heat shock transcription factors (Hsfs) have been described as sensors for ROS under high light and high temperature stress conditions (reviewed in Miller and Mittler, 2006). In humans, *Drosophila* and yeast binding of ROS to Hsfs has been shown to induce a conformational change that leads to increased DNA binding and transcription activation activity (Ahn and Thiele, 2003; Lee et al., 2000; Zhong et al., 1998). Hsfs control heat shock proteins (HSPs) which primarily function as molecular chaperones to ensure the correct function of many cellular proteins under conditions of elevated temperature. Hsfs and HSPs accumulate in plants in response to different abiotic stresses and overexpression of HSP has been shown to enhance tolerance to salt and water stress as well as high temperature (Alvim et al., 2001; Oono et al., 2006; Seki et al., 2001; Sugino et al., 1999; Sung and Guy, 2003).

The ROS are thought to be involved in mediating ABA signalling and controlling ABA biosynthesis. Hydrogen peroxide regulates ABA-induced stomatal closure in broad beans (*Vicia faba*) and *Arabidopsis* (Pei et al., 2000; Zhang et al., 2001b). In maize, ABA regulation of Catalase1 is mediated by H₂O₂ and general thermotolerance is associated with the antioxidant system (Gong et al., 1998; Guan et al., 2000).

ABA-independent stress signalling

Several stress-responsive genes are induced in mutant lines insensitive to ABA or deficient in ABA biosynthetic enzymes indicating the existence of ABA-independent stress response pathways in addition to ABA-dependent ones. Research has shown that stress-responsive gene induction can be ABA-dependent, ABA-independent or partially ABA-dependent (reviewed by Zhu, 2002). ABA-independent stress signalling is mediated through above-mentioned second messengers, phosphorylation cascades and transcription factors belonging to the AP2/ERF, NAC and zinc finger homeodomain families (reviewed in Yamaguchi-Shinozaki and Shinozaki, 2006).

Partially ABA-dependent genes require both ABA-mediated and ABA-independent signalling for maximal activation of stress conditions. For example, the expression of RD29A is controlled by both an ABRE-element recognised by ABA-dependent ABFs and a DRE-element to which ABA-independent AP2/ERF transcription factors bind. In transgenic lines overexpressing either ABF2 or DREB1/2-type transcription factors, RD29A expression is increased under non-stress conditions (Kim, 2006; Sakuma et al., 2006a). Under drought conditions RD29A can be partially induced in an ABA-independent manner in plants through the DRE *cis*-element (Yamaguchi-Shinozaki and Shinozaki, 1993; Yamaguchi-Shinozaki and Shinozaki, 1994). However, the importance of cooperation between the different *cis*-elements and the respective transcription factors depends on the nature of the stress. While the lack of drought-inducible DREB2 genes does not impair RD29A induction in drought (Sakuma et al., 2006a), RNAi knockdown lines with reduced levels of the cold-responsive DREB1A and DREB1B accumulate significantly less RD29A mRNA than wild-type upon exposure to low temperatures (Novillo et al., 2007).

Transcription factor families associated with stress responses in *Arabidopsis thaliana*

Identifying the transcription factors that regulate transcriptional cascades in response to biotic and abiotic stresses is a major research goal in plant biology. Indeed transcription factors are increasingly considered to be key targets for improving stress tolerance in crop species (Century et al., 2008).

The 125 Mbp genome *Arabidopsis* encodes over 27,000 protein coding genes (TAIR7 genome annotation), out of which approximately 1500 (5 %) are transcription factors (Riechmann et al., 2000). Most *Arabidopsis* transcription factors can be grouped into gene families based on the DNA-binding domain they contain (Riechmann et al., 2000; Riechmann and Ratcliffe, 2000). Several families of transcription factors have expanded considerably in plants or are plant lineage-specific. Families such as MYB, bZIP, NAC and MADS are not especially numerous in fungi or animals but have been significantly amplified in the plant lineage (for a recent review see Libault et al., 2009). The largest transcription factor families not present in animals or yeast include the AP2/ERF, AUX-IAA-ARF, Dof, GRAS, TCP and WRKY families. An outline of transcription factors associated with abiotic stress response pathways is given in Figure 1.1.

NAC (NAM, ATAF1,2 and CUC2) genes play a role in flower development, reproduction and senescence as well as in responses to hormones, stress and light (Olsen et al., 2005). The NAC transcription factor ATAF1 negatively regulates the expression of stress responsive genes under drought stress in *Arabidopsis* (Lu et al., 2007), while overexpression of the dehydration-induced NAC TF RD26 induced a whole array of stress-responsive genes and led to ABA hypersensitivity (Fujita et al., 2004), and overexpression of ANAC019, ANAC055, or ANAC072 significantly increased drought tolerance (Tran et al., 2004).

WRKY transcription factors participate in protective and defensive reactions and have also been implicated in senescence-related processes (Eulgem et al., 2000). WRKY proteins are of particular interest regarding engineering crop plants for increased disease resistance (Gurr and Rushton, 2005b) but several WRKY TFs also confer enhanced abiotic stress tolerance when overexpressed. For example, seeds of transgenic *Arabidopsis* plants overexpressing WRKY25 exhibit enhanced thermotolerance (Li et al., 2009), and OsWRKY11 overexpression in rice leads to increased heat and drought resistance (Wu et al., 2009).

ARF-Aux/IAA transcription factors are responsible for mediating responses to the plant hormone auxin and are involved in regulating development, growth and floral meristem patterning (Guilfoyle et al., 1998). Dof (DNA binding with one finger) transcription factors have functions in seed germination, responses to auxin and gibberellin and defense responses (Yanagisawa, 2002). Dof proteins have been associated with stress responses through their interaction with the OBF (*ocs*-element binding factors) class of bZIP transcription factors that are thought to play a role in oxidative stress responses (Chen et al., 1996; Zhang et al., 1995).

Members of the plant-specific AP2/ERF family are involved in key developmental steps, such as flower organogenesis and seed development, and in many stress responses (Riechmann and Meyerowitz, 1998). The AP2/ERF family is discussed in greater detail below.

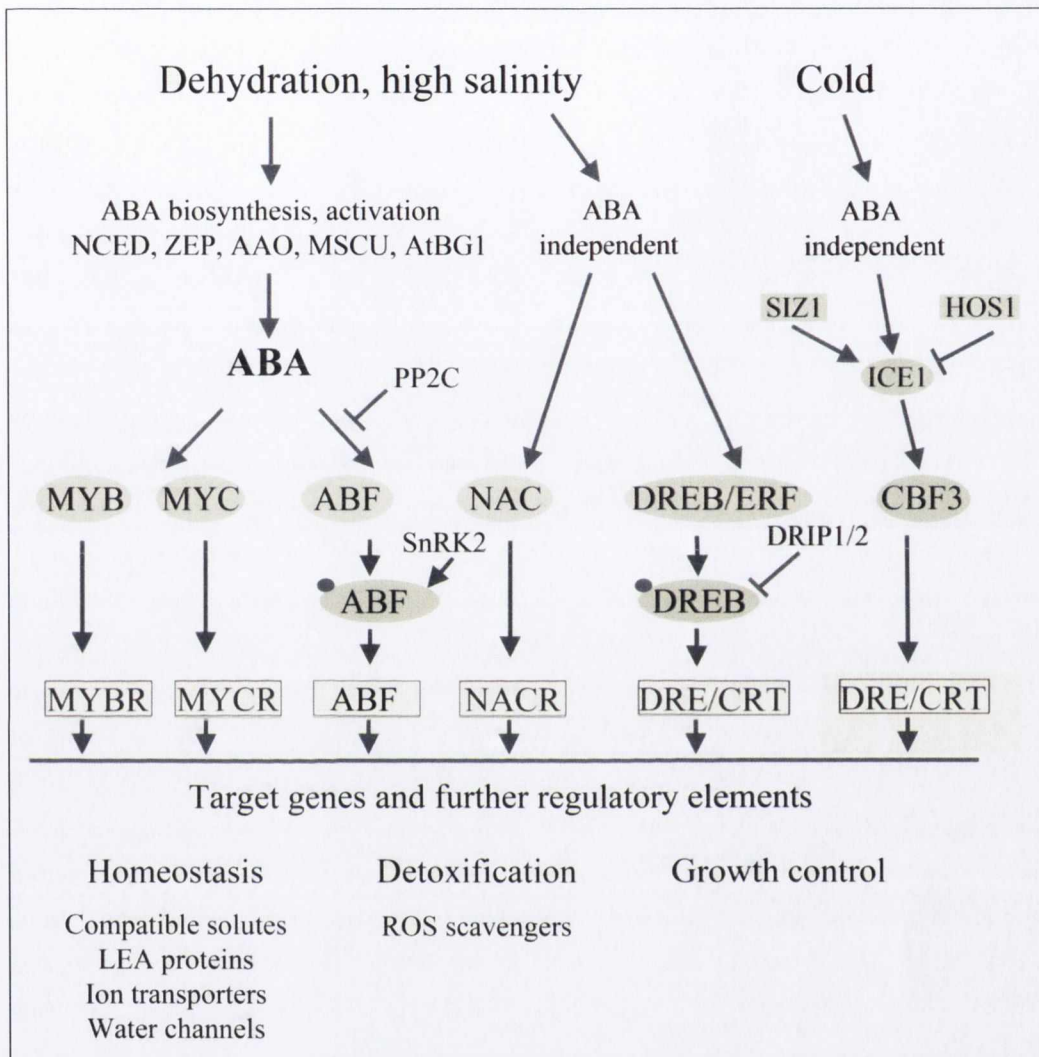


Figure 1.1 Schematic representation of transcription factors associated with abiotic stress signalling pathways in *Arabidopsis*. Transcription factors are shown as grey ovals, DNA *cis*-elements as white boxes and determinants of ICE1 stability as grey boxes. • marks putative post-translational modifications. PP2C – protein phosphatase 2C.

1.5 AP2/ERF transcription factors

The AP2/ERF transcription factors share a conserved DNA binding domain (AP2) of approximately 60 amino acids. The AP2 domain was first identified as a repeated motif in the APETALA2 gene which regulates several processes during flower development in *Arabidopsis* (Jofuku et al., 1994). In tobacco, four AP2 domain containing transcription factors were identified as proteins binding to the ethylene-responsive GCC-box *cis*-elements present in pathogenesis-related genes (Ohme-Takagi and Shinshi, 1995). The AP2 domain of ethylene-responsive element binding proteins (EREBPs or ethylene response factors (ERFs) according to Suzuki et al. (1998)) was found to be sufficient for sequence-specific DNA binding activity (Ohme-Takagi and Shinshi, 1995). The first AP2/ERF gene to have an established role in abiotic stress responses was CBF1 (also called DREB1B) which was demonstrated to recognize the drought-responsive element (DRE) in the promoter of a COR gene upon cold exposure (Stockinger et al., 1997).

The first identified AP2/ERF proteins illustrate the diversity of functions carried out by different members of this transcription factor family. More extensive characterization confirmed their involvement in the transcriptional regulation of a variety of biological processes related to growth, development, and responses to abiotic and biotic stresses (reviewed in Agarwal et al., 2006; Gutterson and Reuber, 2004; Kizis et al., 2001; Riechmann and Meyerowitz, 1998; Xu et al., 2008a).

AP2/ERF proteins are found in species across the entire plant kingdom. In addition to AP2/ERF genes identified in numerous angiosperm species all four major lineages of gymnosperms – conifers, ginkgo, cycads, gnetum - contain AP2/ERF genes (Nilsson et al., 2007; Shigyo et al., 2006; Vahala et al., 2001). An AP2 domain containing EST was also among stress-responsive genes identified in the seedless vascular resurrection plant *Selaginella lepidophylla* (Iturriaga et al., 2006) and several AP2/ERF genes were identified in the highly abiotic stress-tolerant moss *Physcomitrella patens* (Frank et al., 2005). Furthermore, the green alga *Chlamydomonas reinhardtii* EST collection has several entries coding for AP2 domain containing genes (Magnani et al., 2004; Nakano et al., 2006; Shigyo et al., 2006).

Interestingly, the functions of AP/ERF genes also seem to be conserved across wide evolutionary distances. The PpDBF1 gene from *Physcomitrella patens* is induced by salt, cold and drought stresses and when overexpressed in transgenic tobacco confers enhanced tolerance to all of these abiotic stresses (Liu et al., 2007). The APETALA2-like gene PaAP2L2 from the conifer *Picea abies* was able to promote petal-identity in an *ap2-1* background when expressed in *Arabidopsis* despite the lack of petal-like structures in gymnosperms (Nilsson et al., 2007).

The AP2/ERF family is no longer considered a strictly plant-specific group since proteins containing homologs of the AP2 domain have been found in the cyanobacterium *Trichodesmium erythraeum*, the ciliate *Tetrahymena thermophila*, and the viruses *Enterobacteria phage RB49* and *Bacteriophage Felix 01* (Magnani et al., 2004). However, these newly identified proteins bearing an AP2/ERF domain are predicted HNH endonucleases (Magnani et al., 2004).

The AP2 domain

The crystal structure of the DNA-binding AP2 domain has been resolved for ERF1 (Allen et al., 1998). The AP2 domain forms a three-stranded anti-parallel β -sheet and an α -helix packed approximately parallel to the β -sheet. The AP2 domain interacts with DNA through the β -sheet.

AP2/ERF transcription factors are structurally divergent outside the AP2 domain (Okamuro et al., 1997) but still share two features that are characteristic of transcription factors in general: region(s) of biased amino acid composition typical of transcription activation or repressor domains and putative nuclear localization signals (Riechmann and Meyerowitz, 1998). Sequence analysis has demonstrated significant variability among different AP2/ERF proteins in the putative transcription activation domains. For example, APETALA2 (AP2) contains an acidic and serine rich sequence; in AINTEGUMENTA (ANT), an extended region rich in serine and threonine is present, while CBF1 contains an acidic domain that acts as a transcriptional activation domain (Riechmann and Meyerowitz, 1998). The putative nuclear localization signals usually consist of short stretches of basic amino acids in proximity to the N-terminus of the AP2 domain (Riechmann and Meyerowitz, 1998).

Recent research has shown, unexpectedly, that the AP2 domain can play a role in protein dimerization in addition to its DNA binding function. The *DRN* (DORNRÖSCHEN) and DRNL (DORNRÖSCHEN-LIKE) AP2 domain alone is sufficient for mediating heterodimerization with PHV (PHAVOLUTA) (Chandler et al., 2007). Dimerization could affect the regulation of target genes if it interfered with the DNA-binding affinity or specificity of the AP2 domain (Chandler et al., 2007). The AP2 domain of CBF1 interacts with transcriptional co-activators (ADA2 proteins) which in turn interact with the histone acetyltransferase GCN5 (Mao et al., 2006). GCN5 is a major regulator of transcription - mutation of the gene induces pleiotropic effects on plant development, and affects the expression of a large number of genes (Servet et al., 2008).

Post-translational modifications such as phosphorylation have been reported to significantly affect AP2/ERF protein function. Phosphorylation, for example, enhanced the binding of tomato Pti4 protein to its target sequence (Gu et al., 2000), but in the case of PgDREB2A, abolished binding to its recognition element (Agarwal et al., 2007). The evidence from investigations of dimerization and post-translational modifications may indicate further complexity in AP2/ERF transcription factor functions.

The AP2/ERF family in *Arabidopsis thaliana*

Riechmann et al. (2000) proposed the existence of 144 AP2/ERF genes in *Arabidopsis*; Sakuma et al. (2002) added two genes and Nakano et al. (2006) identified one more previously unrecognised AP2/ERF gene. The 146 members of the AP2/ERF transcription factor family were divided into three classes based on the number of DNA binding domains they contain (Sakuma et al., 2002). One gene (At4g13040) contains an AP2 domain but its homology in comparison with the other AP2/ERF genes is too low to warrant placing it in any of the recognized subgroups (Nakano et al., 2006). An overview of the AP2 family is given in Figure 1.2. With 147 members the AP2/ERF gene family is one of the largest families of transcription factors in *Arabidopsis*. The

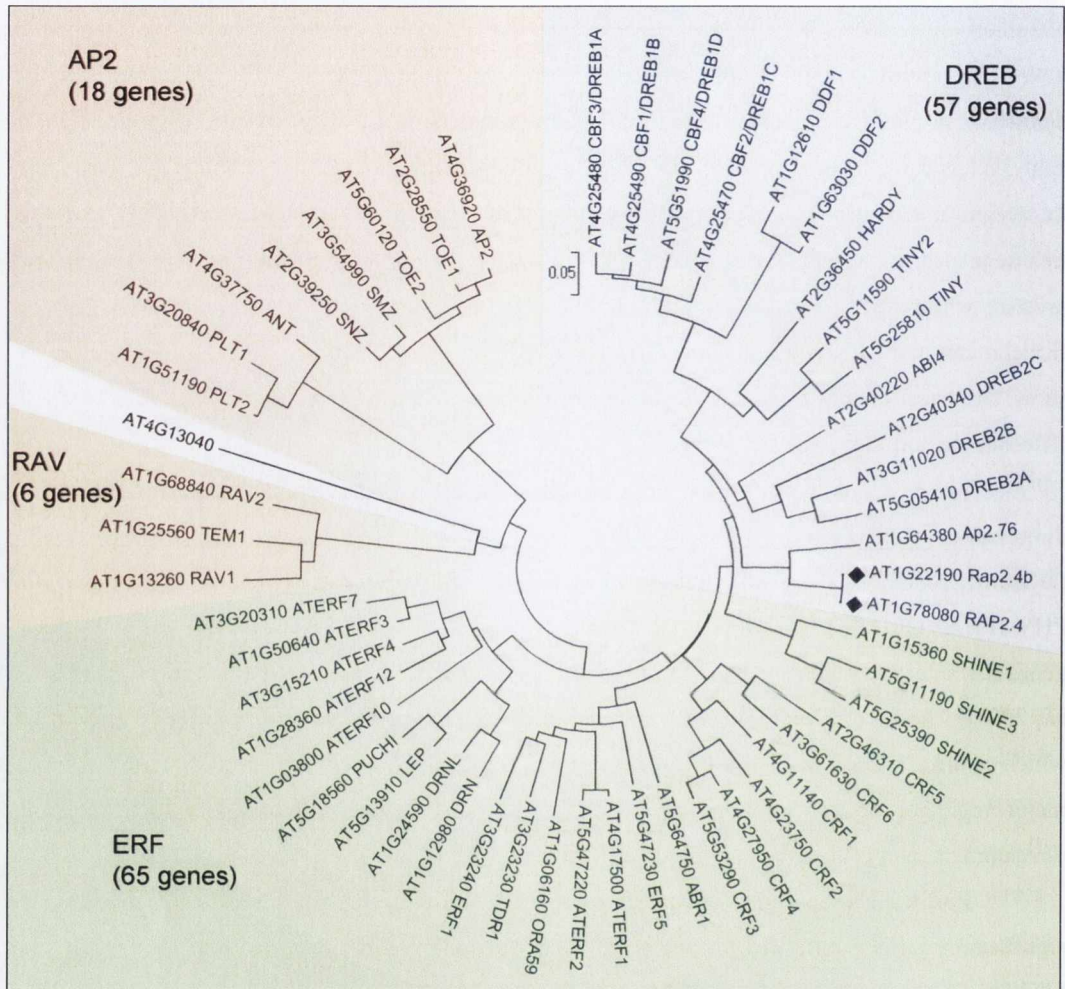


Figure 1.2. Evolutionary relationships in the AP2/ERF family. The four subfamilies are outlined on different backgrounds. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

expansion of AP2/ERF family is thought to have been driven by ancient transposition and homing events, in addition to the duplication of single loci and larger genomic segments (Magnani et al., 2004; Riechmann and Meyerowitz, 1998).

The AP2 subfamily

The AP2 subfamily comprises 4 proteins that have a single AP2 DNA-binding domain and 14 proteins that have two AP2 DNA-binding domains (referred to as R1 and R2) (Sakuma et al., 2002). A given R1 domain is more closely-related to the R1 domains of other AP2 TFs than to the R2 domain to which it is physically linked and *vice versa*, suggesting possibly distinct functional constraints for R1 and R2 domains (McGrath et al., 2005; Riechmann and Meyerowitz, 1998). The sequence of the region between R1 and R2, called the “linker” is also highly conserved, indicating functional restrictions. Mutation in the linker sequence of ANT generates a strong loss-of-function phenotype further demonstrating the importance of the region (Klucher et al., 1996). The AP2 domains of the 4 AP2 subfamily members containing only one DNA binding sequence resemble the R1 domain of the other 14 AP2 subfamily transcription factors (McGrath et al., 2005).

Several members of the AP2 subfamily are key regulators of various developmental processes including (i) specification of the floral meristem (Irish and Sussex, 1990; Shannon and Meeks-Wagner, 1993); (ii) establishing sepal and petal identity (Bowman et al., 1989; Bowman et al., 1991; Kunst et al., 1989); and (iii) ovule and seed development (Jofuku et al., 1994; Modrusan et al., 1994). The ANT gene plays a role in floral organ growth and is required for ovule development (Elliott et al., 1996; Klucher et al., 1996). TOE1 (TARGET OF EAT1-1) and TOE2 function in the control of flowering time as floral repressors (Aukerman and Sakai, 2003).

PLETHORA1 (PLT1) and PLT2 genes are essential for stem cell specification and activity in *Arabidopsis* roots (Aida et al., 2004). SCHLAFMÜTZE (SMZ) and SCHNARCHZAPFEN (SNZ) contain only one AP2 domain and act as repressors of flowering in *Arabidopsis* (Schmid et al., 2003).

The RAV subfamily

The second subfamily (the RAV – related to ABI3/VPI subfamily) includes 6 genes containing two unrelated DNA-binding domains, one AP2 domain and one B3 domain each of which recognise different DNA motifs (Kagaya et al., 1999). The B3 domain is conserved in the VPI/ABI3 (Viviparous1/abscisic-acid-insensitive3) genes (Suzuki et al., 1997). RAV1 (At1g13260) and RAV2 (At1g68840) are regulated by touch-related stimuli in *Arabidopsis* (Kagaya and Hattori, 2009).

Interestingly, the RAV genes TEMPRANILLO1 (TEM1, At1g25560) and TEMPRANILLO2/RAV2 (TEM2) have recently been shown to act as repressors of flowering. In *Arabidopsis*, flowering is accelerated by exposure to long day (LD) via signalling mediated by CONSTANS (CO) and FT. Castillejo and Pelaz (2008) showed that the quantitative balance between the activator CO and the repressor TEM determines FT levels which in turn control the start of flowering.

RAV genes in other plant species have been associated with stress responses. The RAV gene *CARAVI* from sweet pepper (*Capsicum annuum*) was found to be involved in defence reactions following bacterial infection and to enhance resistance to pathogens and osmotic stresses such as high salinity and dehydration when ectopically expressed in transgenic *Arabidopsis* plants (Sohn et al., 2006). It is possible that the *Arabidopsis* RAV genes also play a role in defence responses, based on their ethylene-inducibility and on the ethylene-insensitivity of loss-of-function alleles for four out of the six RAVs (Alonso et al., (2003).

The ERF/DREB subfamily

The third class contains 122 transcription factors containing one AP2 domain and is referred to as the ERF/DREB subfamily (Sakuma et al., 2002). The ERF/DREB transcription factors are mainly involved in defence and abiotic stress responses. The transcription factors of this subfamily can be further divided into two subclasses based on the amino acid sequence similarity in the AP2 domain and the specific DNA element to which they bind (Sakuma et al., 2002).

The proteins encoded by the first subclass (65 in total) specifically bind to the GCC-box sequence containing the core GCCGCC element, which is present in the promoter regions of a large number of ethylene-inducible genes encoding pathogenesis-related proteins, and are referred to as the ERF proteins. The GCC-box is necessary and sufficient for transcriptional regulation by ethylene in tobacco (Ohme-Takagi and Shinshi, 1995).

The proteins in the second class specifically bind to the DRE/CRT (drought responsive *cis*-element/C-repeat motif) sequence containing the core sequence A/GCCGAC and are referred to as DREB proteins (57 genes). The DRE/CRT *cis*-element was first identified in the promoter of RD29A and is shared by many dehydration, high-salinity induced and cold-responsive genes (Yamaguchi-Shinozaki and Shinozaki, 1994; reviewed in Yamaguchi-Shinozaki and Shinozaki, 2005; 2006).

Two conserved amino acids in the AP2 domains differ between ERFs and DREBs creating a potential basis for different DNA binding specificity (Liu et al., 1998). The amino acids valine in the 14th and glutamic acid in the 19th position of the AP2 domain are conserved among DREB proteins. The position 14 residue valine is 100 % conserved among DREBs and is critically important for the specificity of DNA binding (Sakuma et al., 2002). ERF proteins mostly contain alanine in the 14th position and aspartic acid in the 19th position. Positions 14 and 19 are located within the part of the AP2 domain that is responsible for the protein-DNA interaction (Allen et al., 1998).

Generally ERFs and DREBs are thought to function in biotic or abiotic stress pathways respectively, due to binding to different *cis*-elements, and subsequently regulate the expression of different target genes. However, a certain amount of cross-talk has been demonstrated for ERF and DREB proteins. Some respond to abiotic and biotic stresses and some bind to both DRE and GCC

cis-elements suggesting that they could regulate either type of stress response. For example, the *Arabidopsis* DREB proteins CBF1 and TINY bind to both DRE and GCC elements with similar affinity (Hao et al., 1998; Sun et al., 2008) and TINY2 expression is induced by abscisic acid, cold, drought, wounding, salinity (Wei et al., 2005). DREB2A also binds to both DRE- and GCC-boxes (Sakuma et al., 2002) but functions only in abiotic stress responses (Sakuma et al., 2006a; Sakuma et al., 2006b). It is likely that many AP2/ERF transcription factors play multiple roles in the regulation of stress-responsive genes and consequently the signalling networks involving these factors are complex.

The ERF transcription factors in *Arabidopsis*

The ERF transcription factors were first identified as mediators of ethylene signalling pathways in pathogenesis-related response cascades via binding to ethylene-responsive *cis*-elements known as GCC-boxes (Buttner and Singh, 1997; Ohme-Takagi and Shinshi, 1995). Manipulation of ERF gene expression can provide tools for altering resistance to pathogens. For example, overexpression of ERF ORA59 increased resistance to pathogens (Pre et al., 2008) and loss-of-function mutants of AtERF14 displayed increased susceptibility to pathogen infection (Onate-Sanchez et al., 2007). Interestingly, the overexpression of certain ERF genes has led to enhanced resistance to both abiotic and biotic stresses making this family particularly attractive as potential targets in improving crop yields.

The ERF genes respond at the transcriptional level to biotic stresses such as pathogen infections; various abiotic stresses such as drought, cold and high salinity; and to treatments with the phytohormones jasmonic acid, ABA and ethylene. The response-pathways to different stresses and treatments involve different subsets of ERF genes which in turn control different subsets of GCC-box containing target genes. Interestingly, several ERF genes contain GCC-box elements in their own promoters suggesting the existence of regulatory loops where ERF genes are either autoregulated or controlled by other ERF proteins (reviewed by Shinshi, 2008).

One of the best characterized ERF transcription factors in *Arabidopsis* is ERF1. ERF1 (At3g23240) ethylene-inducibility is mediated by EIN3 and EIN3-like (EIL) DNA-binding factors which in turn are controlled by the central regulator of ethylene signalling EIN2 (Alonso et al., 1999; Solano et al., 1998). In addition to ethylene, ERF1 responds to jasmonic acid, a key phytohormone in controlling plant defence responses against pathogens and environmental stresses such as wounding, water deficit and ozone exposure (for a recent review see Balbi and Devoto, 2007). Furthermore, ERF1 induction is impaired in mutants blocking either hormone signalling pathway and ERF1 overexpression induces expression of both ethylene- and jasmonic acid-responsive genes (Lorenzo et al., 2003). Overall, methyl-jasmonate induces the expression of 9 ERF genes, including AtERF1/2/4 (At4g17500, At5g47220, At3g15210), ORA59 (At1g06160) and TDR1 (At3g23230) (McGrath et al., 2005).

Although most ERF transcription factors have been demonstrated to act as transcriptional activators some function as repressors. AtERF1, AtERF2, AtERF5 were demonstrated to activate the transcription of reporter genes via the GCC-box element in transient expression experiments,

whereas AtERF3 and AtERF4 are active repressors that can down-regulate the expression of reporter genes and impair the activity of other transcriptional activators (Fujimoto et al., 2000). The repression activity of certain ERF proteins is conferred by a motif named the EAR-domain (ERF-associated amphiphilic repression domain) with the conserved amino acid sequence (L/F)DLN(L/F)xP (Ohta et al., 2001). The EAR motif is found in eight ERF repressors, namely AtERF3, AtERF4, and AtERF7 to AtERF12. The transcription factors AtERF4, AtERF7, and AtERF10 to AtERF12 have also been experimentally proven to act as repressors (Yang et al., 2005). In addition to defence-related roles AtERF4 and AtERF7 are involved in ABA-mediated abiotic stress signalling. AtERF4 overexpression reduces the expression levels of ABA-responsive genes and renders transgenic plants hypersensitive to salt stress (Yang et al., 2005). AtERF7 has been demonstrated to interact with PKS3, an important regulator of ABA-responses, and overexpression of AtERF7 leads to increased transpirational water loss (Song et al., 2005).

Coordinated expression of activators and repressors under stress conditions could be part of a fine-tuning mechanisms to elicit the most appropriate response and thereby avoid self-inflicted damage (Kazan, 2006). Interestingly, the transcriptional activators AtERF1, AtERF2 and AtERF5 are more sensitive to mutations in the GCC-box recognition element than the transcriptional repressors AtERF3 and AtERF4 (Fujimoto et al., 2000). The differences in binding preferences could also be important in achieving the correct balance between activation and repression depending on the prevailing conditions.

Another ERF gene implicated in ABA responses is ABR1 (ABA repressor 1). ABR1 is strongly induced by exogenous ABA and abiotic stresses such as cold, drought and high salinity. The *abr1* mutant is hypersensitive to ABA and osmotic stress and enhances the ABA-responsive expression of a number of abiotic stress marker genes including RD29A, RD29B, RD22, RAB18, COR47 and COR15A (Pandey et al., 2005).

The genes belonging to the SHINE clade of ERFs have a unique function in plant defence and stress responses. Overexpression of the three SHINE genes increases cuticular wax levels, alters leaf structure and enhances drought tolerance (Aharoni et al., 2004).

Several ERF genes have established roles in development. The DRN (also known as ENHANCER OF SHOOT REGENERATION1; ESR1) and DRN-LIKE (also known as ESR2, BOLITA and SOB2) control embryo patterning. The DRN gene is important in meristem organisation and cytokinin-independent root regeneration (Chandler et al., 2007, and references therein) while DRN-LIKE is required for stamen emergence and affects cell expansion, proliferation, and differentiation pathways (Marsch-Martinez et al., 2006; Nag et al., 2007). The DRN-LIKE homolog LEAFY PETIOLE (LEP) is a positive regulator of gibberellic acid-induced germination (Ward et al., 2006). Finally, the auxin-regulated PUCHI contributes to lateral root morphogenesis by affecting cell division patterns (Hirota et al., 2007).

CRFs (CYTOKININ RESPONSE FACTORS) are the final group of characterized ERF genes in *Arabidopsis*. Exogenous cytokinin application affects CRFs in two ways – the accumulation of CRF mRNAs is altered and the CRF proteins relocate to the nucleus. The loss of

CRF proteins affects cell expansion, with phenotypes growing more radical as more CRF genes are inactivated by mutation (Rashotte et al., 2006).

In summary, the *Arabidopsis* ERF proteins are involved in numerous processes including biotic and abiotic stress responses, hormone signalling transduction, growth and development.

In other plant species ERFs have mainly been characterized as genes involved in both abiotic and biotic stress response pathways. For example, overexpression of tobacco Tsi1 enhanced salt tolerance and resistance to *Pseudomonas syringae* pv *tabaci* (Park et al., 2001). Tsi1 overexpression in hot pepper resulted in increased resistance to multiple pathogens (Shin et al., 2002). Similarly, the OPBP1 (OSMOTIN PROMOTER BINDING PROTEIN 1) from *Nicotiana tabacum* enhanced resistance to multiple pathogens and salt stresses when overexpressed in transgenic tobacco and rice (Chen and Guo, 2008; Guo et al., 2004). Overexpression of the tomato TERF1 or TSRF1 enhanced abiotic stress resistance in transgenic tobacco (Gao et al., 2008; Zhang et al., 2007a). Transgenic *Arabidopsis* plants expressing the hot pepper CaPF1 were more tolerant to freezing stress and bacterial pathogen attack (Yi et al., 2004). Finally, overexpression of TaERF1 from wheat or GmERF3 from soya bean increased multiple stress tolerance in transgenic tobacco (Xu et al., 2007; Zhang et al., 2009a).

The DREB proteins in *Arabidopsis*

The DREB TFs are the second subgroup of genes of AP2/ERF family that contain one AP2 DNA-binding domain. The DREB proteins bind to the DRE/CRT element present in promoters of many abiotic stress-inducible genes and regulate gene expression in response to drought, cold and high salinity. Interestingly, one DREB gene, ABI4, has a particularly well-established role in development. ABI4 (abscisic acid insensitive 4) controls the seed development and seed-specific ABA response but is not induced by stress treatments (Söderman et al., 2000). Recently the transcription factor was shown to be involved in three separate chloroplast-to-nucleus signalling pathways. ABI4 is activated downstream of *GUNI* (genomes uncoupled1) – a central mediator of retrograde signalling in plants - and binds to specific *cis*-elements in DNA thereby repressing the expression of target genes (Koussevitzky et al., 2007).

In *Arabidopsis* the DREB subgroup contains two extensively studied clusters of genes: the DREB2 and the DREB1/CBF genes. Also the DREB genes TINY and TINY-like, DDF1/2 and HARDY have been characterized in detail. Recent work in this lab has characterized another member of the DREB subclass – *AP2.76* (At1g64380) (PhD thesis, Brennan, 2004, TCD).

The DREB1 gene cluster

The DREB1 cluster contains 4 genes DREB1A (CBF3), DREB1B (CBF1), DREB1C (CBF2) which are generally associated with cold responses and DREB1D (CBF4) which acts both in cold and drought response pathways. The first member of the DREB1 family identified as a regulator of abiotic stress was DREB1B (CBF1). CBF1 was found to contain the conserved AP2 domain, bind to the DRE/CRT element and act as a transcriptional activator in dehydration and

cold stress (Stockinger et al., 1997). CBF1 overexpression was found to strongly induce expression of the cold-regulated (COR) genes COR6.6 (also known as KIN2), COR15A, COR47 and COR78 (also known as RD29A) at normal growth temperatures and enhance freezing tolerance of non-cold-acclimated *Arabidopsis* plants (Jaglo-Ottosen et al., 1998). Gilmour et al. (1998) found CBF1 to be one of three related genes (CBF1/2/3) all situated in close proximity on chromosome four. DREB1A(CBF3) was also shown to strongly activate the expression of *RD29A* via the DRE-element and enhance plant tolerance to drought, salt loading and freezing (Liu et al., 1998) whereas the CBF2 gene acted as a negative regulator of CBF1 and CBF3 expression (Novillo et al., 2004). Despite being very similar CBF1 and CBF3 have both overlapping and distinct functions during the response to low temperatures regulating expression of the same set of genes. Proper cold acclimatisation nevertheless requires simultaneous expression of both genes (Novillo et al., 2007).

CBF4 (DREB1D) on the other hand lies on the fifth chromosome and is thought to have originated from the common ancestor of CBF1/2/3 before it underwent duplication which gave rise to the three CBFs on chromosome 4. While the three other CBF genes are more associated with cold-stress, CBF4 is up-regulated during drought exposure, and overexpression of CBF4 resulted in increased tolerance to both dehydration and freezing (Haake et al., 2002).

In addition to enhancing stress tolerance in *Arabidopsis*, DREB1 genes have also been successfully utilized to enhance abiotic stress tolerance in crop species such as wheat, tomato, tobacco (*Nicotiana tabacum*), strawberry (*Fragaria* spp), rice, oilseed rape (*Brassica napus*) and potato (reviewed in Century et al., 2008). However, overexpression of DREB1 genes can result in growth retardation. The dwarf phenotype of transgenic lines overexpressing CBF1 was due to enhanced expression of gibberellic-acid (GA)-inactivating GA 2-oxidase genes which resulted in accumulation of growth-repressing DELLA proteins (Achard et al., 2008). Further research showed that it is possible to avoid the negative effects on growth by using appropriate inducible promoter systems. Kasuga et al. (1999) utilized the stress-inducible RD29A promoter to drive the expression of CBF3 and achieved significant improvement in stress tolerance without any unwanted side-effects on growth.

In addition to stress treatments, CBF genes are also induced by inhibition of protein synthesis and are regulated by the circadian clock (Fowler et al., 2005; Zarka et al., 2003). Moreover, the induction of CBF genes and CBF target genes such as COR15A is impaired in the dark (Kim et al., 2002).

CBF3 and its downstream targets are controlled by the gene ICE1 (Inducer of CBF expression 1). ICE1 is a constitutively expressed MYC-like bHLH transcription factor that enhances the expression of the CBF3 regulon when overexpressed (Chinnusamy et al., 2003). Recently, an ICE1 homolog ICE2 has been identified as enhancer of CBF1 (Fursova et al., 2009). Expression of the ICE1 homolog from rice OrbHLH2 in *Arabidopsis* also up-regulated the CBF3 regulon (Zhou et al., 2009).

In *Arabidopsis* ICE1 is regulated by HOS1 and SIZ1. HOS1 is a RING finger E3 ubiquitin ligase that physically interacts with ICE1 and mediates the ubiquitination and

degradation of ICE1 thereby attenuating cold responses via the ubiquitination/26S proteasome pathway. *hos1* mutants show enhanced cold-responsive gene expression whereas HOS1 overexpression represses the expression of CBFs and their downstream genes and confers increased sensitivity to freezing stress (Dong et al., 2006). On the other hand, a SUMO E3 ligase SIZ1 is a positive regulator of ICE1. SIZ1-dependent sumoylation that may act to stabilise or activate the ICE1 protein is induced by cold (Miura et al., 2007b). In addition to its involvement in cold responses, SIZ1 is also a central regulator of growth and drought signalling (Catala et al., 2007).

TINY, TINY2 and DDF1/2 are more closely related to DREB1 genes than DREB2 genes. TINY (At1g77200) was first identified via a mutant obtained through a transposon-tagging system designed to recover gain-of function mutants. Enhanced expression of the AP2 domain-containing TINY was found to result in a partial constitutive triple response i.e. significant reductions in plant height, hypocotyl length and fertility implying a function mediating an ethylene response (Wilson et al., 1996). TINY was shown to bind to the DRE-element and the GCC-box with similar affinity and to activate the expression of reporter genes via these interactions. TINY was induced by high salinity, ethylene and jasmonate indicating involvement in both abiotic and biotic stress response pathways. Consistently, TINY overexpression resulted in elevated levels of abiotic stress-responsive genes such as COR6.6, COR15A and RD29A as well as increased accumulation of transcripts of the defence-related PDF1.2 (Sun et al., 2008).

TINY2 (At5g11590) is a TINY-like gene that acts as a transcriptional activator and is strongly induced by cold, drought, wounding, salt stress treatments and exogenous ABA application. TINY2 was shown to bind to the DRE/CRT *cis*-element suggesting that it is probably involved in the activation of downstream genes in response to environmental stresses. Differently from TINY, TINY2 did not bind to the GCC-box and is therefore probably not associated with biotic stress signalling (Wei et al., 2005).

DDF1 and DDF2 (dwarf and delayed-flowering 1 and 2; At1g63030 and At1g12610) are DREB proteins strongly induced by high-salinity. Overexpression lines of DDF1 are dwarfs with increased tolerance towards salt stress. DDF2, encoding another DREB closely related to DDF1, also conferred the *ddf-1* like phenotype (Magome et al., 2004). Similarly to CBF1 overexpressor lines, the growth retardation of DDF1 overexpressors is due to defective gibberellic acid biosynthesis (Magome et al., 2004).

The DREB2 clade

The first DREB2-type gene characterized, DREB2A, was identified in the same study as DREB1A (CBF3). When DREB1A and its two homologs were induced by low temperatures, DREB2A and its single homolog responded to dehydration treatment suggesting that discrete sets of DREBs are specifically activated by the particular type of abiotic stress encountered.

DREB2A was shown to bind to the DRE-element and to transactivate transcription of a reporter construct. As observed for DREB1A, DREB2A overexpression caused growth retardation, but interestingly whereas DREB1A strongly induced the expression of stress-

responsive genes under non-stress conditions, the increases were not so marked in DREB2A overexpression lines (Liu et al., 1998). The DREB2A homolog DREB2B was induced by high salinity in root tissues and by dehydration in roots and stems although at much lower levels suggesting that DREB2A could be more important in stress signalling (Nakashima et al., 2000). DREB2C is closely related to other DREB2 genes and has been shown bind to and activate transcription through the DRE-element, and confer enhanced thermotolerance when overexpressed in *Arabidopsis* (Lim et al., 2007).

Sakuma et al. (2006a) showed that the weak induction of stress-responsive genes in the DREB2A overexpression lines was due to the presence of a negative regulatory domain within the DREB2A coding sequence. Overexpression of a constitutively active version, in which amino acids 135-166 had been deleted, resulted in high activation of stress-responsive genes and enhanced survival under drought and heat stress (Sakuma et al., 2006a; Sakuma et al., 2006b). The dwarf phenotypes that were observed in transgenic lines constitutively overexpressing DREB2A were avoided by using the stress-inducible RD29A promoter.

Evidence suggests that the native form of DREB2A is unstable and that a posttranslational modification might be required for its activation (Sakuma et al., 2006a). Qin et al. (2008) showed recently that DREB2A activity is regulated by two C3HC4 RING domain-containing proteins that function as E3 ubiquitin ligases. DREB2A-INTERACTING PROTEIN1 (DRIP1) and DRIP2 interact with the DREB2A protein in the nucleus and mediate its ubiquitination. Overexpression of DRIP1 in *Arabidopsis* delayed the expression of DREB2A-regulated drought-responsive genes whereas a *drip1drip2* double mutant displays significantly enhanced expression of drought-inducible genes. Overall, DRIP1 and DRIP2 are thought to act as novel negative regulators in drought-responsive gene expression by targeting DREB2A for proteolysis in the 26S proteasome.

DREB2A target genes have been determined by microarray analysis (Sakuma et al., 2006a). Most of its target genes belong to the LEA protein class which are thought to protect macromolecules from dehydration. DREB2A overexpression also up-regulated the expression of another DREB gene (At1g22985), suggesting the existence of further regulation of gene expression downstream of DREB2A.

DREB2-type genes have been identified in other plant species including wheat (*Triticum aestivum* and *Triticum durum*), rice (*Oryza sativa*), maize (*Zea mays* L.), oat (*Avena sativa*), perennial ryegrass (*Lolium perenne* L.), brassica species (oilseed rape *Brassica napus* and India mustard *Brassica juncea*), soya bean (*Glycine max*), aloe (*Aloe vera* L.), chrysanthemum (*Chrysanthemum*), perennial fescue grass (*Festuca arundinacea*), pearl millet (*Pennisetum glaucum*), cotton (*Gossypium hirsutum*), poplar (*Populus Trichocarpa*) and the moss *Physcomitrella patens* (recently reviewed in Garg et al., 2008).

DREB2 homologs from some of these species have been successfully used to enhance tolerance to abiotic stresses. For example, expression of the abiotic stress-inducible soya bean gene GmDREB2 (Chen et al., 2007) or the wheat gene TaAIDFa (Xu et al., 2008b) conferred drought and high-salt tolerance in transgenic *Arabidopsis*. Similarly, the *Arabidopsis* HARDY

gene (At2g36450) improved water use efficiency in rice (Karaba et al., 2007). Interestingly, transgenic tobacco plants overexpressing the DRE-binding transcription factor from *Physcomitrella patens* gained higher tolerance to salt, drought and cold illustrating the conservation of DRE-mediated stress responses across wide evolutionary distances (Liu et al., 2007).

1.6 The objectives of the study

Plants have evolved complicated mechanisms to detect pathogens and activate a battery of local and systemic defence responses, though they lack an immune system. Also, in unfavourable environmental conditions, a number of biochemical and physiological processes are induced in order to acquire stress tolerance. Perception of a pathogen or exposure to various stresses triggers a signal transduction pathway which leads to transcriptional activation and/or up-regulation of numerous genes. Identifying the transcription factors that regulate the responses to biotic and abiotic stresses is a major research goal in plant biology, made possible by the completed genome sequence of *Arabidopsis thaliana*.

Recent work in the host laboratory (as part of the EU-funded REGIA project) characterized two members of the AP2/ERF family, *AP2.72* (At5g13330) and *AP2.76* (At1g64380). These studies established that *AP2.76*, belonging to the DREB subclass, is involved in abiotic stress pathways and wounding. *AP2.72* belongs to the ERF subclass and was found to function in the Systemic Acquired Resistance (SAR) response which confers broad-spectrum resistance to many pathogens.

The aim of the present study was to investigate the expression and role of two closely-related members of the DREB clade i.e. *Related to apetala (Rap)2.4* (At1g78080) and *Rap2.4b* (At1g22190) during the life-cycle of *Arabidopsis*, and to define the functional characteristics of the two proteins in terms of promoter *cis*-element binding and transactivation properties. In addition, ectopic overexpression and knockout lines were used to identify the regulatory pathways in which *Rap2.4* and *Rap2.4b* are involved.

In Chapter 2, *Rap2.4* and *Rap2.4b* were characterized using a bioinformatics approach to assign putative functions. The expression patterns at different stages of the *Arabidopsis* life-cycle, in response to exposure to various potentially inducing stimuli were studied for both genes utilizing Northern blotting techniques and reporter gene fusions with the promoters of *Rap2.4* and *Rap2.4b*.

Chapter 3 presents the results of a detailed investigation into the molecular properties of the *Rap2.4* and *Rap2.4b* proteins which includes their subcellular localization, DNA-binding preferences and transcriptional activation activity.

Chapter 4 describes the generation and analysis of knockout and overexpression mutants for both genes. Also double knockouts lacking the expression of both *Rap2.4* and *Rap2.4b* were generated and analysed. Microarray experiments which compared global gene expression profiles of (i) wild-type vs. double knockout under drought conditions and (ii) wild-type vs. a *Rap2.4* overexpression line were conducted in order to identify the target genes of *Rap2.4* and *Rap2.4b*.

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CHAPTER 2

RAP2.4 AND RAP2.4B GENE STRUCTURE AND EXPRESSION

2.1 Introduction

The fully sequenced genome of *Arabidopsis thaliana* has presented scientists with the challenge of assigning functions to all of the nearly 27,000 annotated genes. To address this challenge many software tools have been designed in order to predict various characteristics of the given protein based on the DNA sequence of the gene of interest. Bioinformatic approaches combining several prediction tools can help to shed light on the function of the gene under investigation. Usually, such functions are assigned by sequence similarity of the coding sequences to known genes, or by identification of transcriptional *cis*-regulatory elements that are known to be involved in regulation of a specific pathway.

We used several public databases and tools in order to examine the promoters, coding sequences, protein characteristics and expression patterns of Rap2.4 and Rap2.4b. The data obtained through bioinformatics analysis are probabilistic and although they can be a useful indicator of gene function, assigning definite and precise function to any gene requires experimental evidence to support the hypothetical function.

Phylogenetic origins of the DREB subfamily

Much of the 125 Mbp of the *Arabidopsis* genome is internally duplicated. Different mechanisms can lead to duplication of whole genomes, long chromosomal regions or individual genes (The Arabidopsis Genome Initiative, 2000; Bowers et al., 2003). Vision et al. (2000) identified 103 duplicated blocks in the *Arabidopsis* genome containing seven or more matching composite open reading frames (cORFs) and found that 81 % of the 18,596 cORFs fell within the bounds of at least one block. However, they found only 28 % of these cORFs to be actually present in duplicate. Nearly 25 % of all duplicated cORFs were found in two or more duplicated blocks providing evidence of multiple duplication events. Bowers et al. (2003) circumscribed 34 non-overlapping chromosomal segment pairs that encoded 89 % of *Arabidopsis* proteins.

Transcription factors often comprise families of related proteins that are defined by a shared DNA-binding domain. The genes encoding the ancestral DNA-binding proteins have been amplified during evolution by duplication and re-arrangement events, resulting in large gene families (Riechmann and Meyerowitz, 1998). Classical models predict that gene loss is the most likely outcome following gene duplication, however genes involved in signal transduction or transcription have been preferentially retained in duplicate in *Arabidopsis* (Blanc and Wolfe, 2004).

The AP2/ERF is one of the largest gene families in *Arabidopsis* consisting of 147 proteins, each containing one or two AP2 DNA-binding domains. As described in Chapter 1, the DREB/ERF subfamily of 122 proteins can be further divided on the basis of subtle differences in the amino acid sequences of the AP2 domains into 57 DREB (DRE-element binding proteins) and

65 ERF (ethylene response element binding) proteins. Sakuma et al. (2002) divided the DREB genes into 6 small subgroups (A1 to A6) based on the similarities in the DNA binding domain (Figure 2.5).

The DREB transcription factors are generally thought to function in abiotic stress signalling where they regulate the transcription of target genes through the DRE *cis*-element. Given the large number of similar proteins in the DREB subgroup, it is expected that many of the members must have arisen through the genome duplication events. Thanks to the fully sequenced *Arabidopsis* genome it is possible to trace duplication events within the DREB subgroup and investigate how the duplicated genes have diverged.

Analysis of publicly available expression data.

High-throughput gene expression analysis utilising microarray techniques have created a wealth of publicly available expression data for *Arabidopsis*. As the large datasets from microarray experiments become an increasingly important resource for biological investigation, tools for surveying and summarizing the results have been developed that are accessible to non-bioinformatics specialists.

Through a number of user-friendly web-based data mining tools one can retrieve expression patterns for individual *Arabidopsis thaliana* genes or groups of genes at specific developmental stages or in response to environmental stimuli. Gene expression profiles can also be followed in a large number of mutant lines under normal and/or stress conditions.

For example the Genevestigator database and web-browser data mining interface developed by the ETH Centre, Switzerland, covers the results of over 4000 Affymetrix GeneChip microarray experiments from several databases (FGCZ, NASC, GEO, ArrayExpress, AtGenExpress and TAIR). Genevestigator offers facilities for examining the expression patterns of over 22,000 *Arabidopsis* genes in different organs, at various developmental stages or under specific environmental conditions. Alternatively one can search for genes specifically expressed in certain organs or in response to stresses (Zimmermann et al., 2004).

The eFP (Electronic Fluorescent Pictograph) browser available through the TAIR website or on <http://www.bar.utoronto.ca>, offers graphic depiction of gene expression data from the AtGenExpress Consortium. The eFP browser presents the user with idealized images of *Arabidopsis* tissues which are coloured according to the expression level of the query gene in a particular tissue under particular treatment (Winter et al., 2007).

The purpose of both software applications is to aid gene function discovery and offer direction in designing new experiments by providing plant biologists with contextual information on the expression of genes. However, it is important to remember that the expression data from microarray experiments only refers to the abundance of mRNAs at the time of extraction. No predictions regarding the protein abundance or stability can be made and therefore any functional hypothesis made based on the microarray data needs substantial experimental support.

Cis-elements databases and organisation

Cis-motifs are important regulatory elements that control the expression of genes through regulating transcription. *Cis*-elements consisting of 5 up to 20 nucleotides are generally found near the 5' end of the genes i.e. in the promoter region. Promoter regions can be divided into proximal and distal parts, the former controlling basal level of expression through RNA polymerase II (RNAPII) specific elements (Nikolov and Burley, 1997; Roeder, 1991), and the latter containing elements that control the spatial-temporal aspects of gene expression via interactions with transcription factors (Fessele et al., 2002; Tjian and Maniatis, 1994).

Molecular and genomic analyses have identified many sets of *cis*-acting elements that are involved in abiotic stress responses. In unfavourable conditions stress-responsive transcription factors bind to their specific *cis*-elements in the promoters of stress-inducible genes and by activating or repressing transcription, generate networks of signalling and direct physiological responses (reviewed in Yamaguchi-Shinozaki and Shinozaki, 2005).

There are several publicly available resources enabling exploration of plant promoters and visualization of plant specific *cis*-motifs. For example, the Athamap (<http://www.athamap.de/index.php>) provides depictions of putative transcription factor binding sites mapped onto genomic regions; PlantCare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>), AtcisDB (<http://Arabidopsis.med.ohio-state.edu/AtcisDB/>) and PLACE (<http://www.dna.affrc.go.jp/PLACE/>) are databases of *cis*-acting regulatory elements with tools for scanning DNA sequences. The PLACE database contains 469 entries covering sequences of *cis*-elements with brief descriptions of the motifs and GenBank nucleotide accession numbers from vascular plants and *Chlamydomonas reinhardtii* (Higo et al., 1999).

The GUS reporter system

The *E. coli* hydrolase β -glucuronidase (GUS) is encoded by the *uidA* locus and catalyses the cleavage of a wide variety of β -glucuronides (Jefferson et al., 1986). GUS is a stable enzyme with several attractive properties that have made it a widely used tool for studying protein localization and exploring temporal and spatial patterns of gene expression under the control of specific promoter sequences in plants.

The main advantages of the GUS reporter system are lack of intrinsic glucuronidase activity in plants, stability of the GUS enzymatic activity when fused with other proteins, availability of inexpensive substrates for spectrophotometric, fluorometric or histochemical assays and the high sensitivity of assays (Jefferson et al., 1987). Histochemical assays are based on the use of colourless substrate 1mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid which gives a clear blue coloured product upon cleavage by the GUS enzyme. Fluorometric assays are based on MUG substrate (1mM 4-methylumbelliferyl β -D-glucuronide) which in presence of GUS enzymatic activity yields a fluorescent product that can be measured with excitation at 365 nm and emission at 455 nm (Jefferson et al., 1987).

GUS has been the reporter of choice for studying gene regulation and function in many genetics applications. When studying a specific gene, generation of transgenic plants where GUS is driven by the respective promoter, is considered a standard approach. Libraries of insertional mutant lines carrying the GUS reporter gene within the transferred T-DNA are available from *Arabidopsis* seeds stock centres. Provided the insertion is located in a regulatory region of the gene of interest, quantitative and qualitative measurement of GUS activity can provide valuable information concerning its expression during the plant life-cycle.

Chapter Aims

The aim of this chapter was to collect and review the publicly available bioinformatic and expression data for Rap2.4 and Rap2.4b and to experimentally explore expression patterns in different organs of mature *Arabidopsis* plants, and in response to various stress treatments. The availability of the complete *Arabidopsis* genome sequence enabled us to generate transgenic plants in which the Rap2.4 and Rap2.4b promoters were used to drive GUS expression. To support the observed GUS expression patterns, Rap2.4 and Rap2.4b mRNA levels were also assessed by Northern blotting. Rap2.4 and Rap2.4b expression patterns were studied using mature greenhouse-grown plants, while mRNA levels following exposure to various abiotic stresses and treatments with plant hormones were characterized using seedlings grown under sterile conditions.

2.2 Materials and methods

2.2.1 DNA and protein sequence analysis

Database analyses were carried out using BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>). Vector NTI version 6 (InforMax Inc., 1999) was used for DNA restriction analysis and primer design. Conserved protein domains were identified using RPSBLAST: a database of conserved domains (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Multiple alignments of protein sequences were done with Clustal W program (<http://www.ch.embnet.org/software/ClustalW.html>, (Thompson et al., 1994).

The PSORT program (<http://wolfsort.seq.cbrc.jp/>; (Horton et al., 2006)) was used in order to predict protein subcellular localization signals. Putative phosphorylation sites were identified using the NetPhos 2.0 Prediction Server (<http://www.cbs.dtu.dk/services/NetPhos/>, (Blom et al., 1999)). The PEST sequence was identified using the PESTFIND tool (<http://bioweb.pasteur.fr/seqanal/interfaces/Pestfind-simple.html>; (Rogers et al., 1986)).

A search for conserved *cis*-elements in the promoter regions of Rap2.4 and Rap2.4b was performed using PLACE: A Database of plant *cis*-acting regulatory DNA Elements (<http://www.dna.affrc.go.jp/PLACE/>, (Higo et al., 1999)).

2.2.2 Phylogenetic analysis

Protein sequences of DREB transcription factors were downloaded from the TAIR website. Sequences were aligned using the Windows version of Clustal X version 1.81 (Thompson et al., 1997), edited by the GeneDoc program (Nicholas et al., 1997). Neighbour-joining trees were generated and the bootstrap values of 1000 pseudoreplicates were calculated using Clustal X. Phylogenetic trees were visualised using the NJ Plot tool (Perrière and Gouy, 1996).

2.2.3 Promoter-GUS study

2.2.3.1 Generation of transgenic plants

Plasmids used:

p2CaMV-NG – a binary vector made in this lab based on pFGC5941. The plasmid contains duplicated CaMV 35S promoter with translational enhancer from the Tobacco Etch Virus (TEV), translational fusion of NAN and GUS reporter genes followed by polyadenylation signal from octopine synthase gene in the T-DNA region. The vector contains BAR resistance marker for selection of transformed plant tissue and kanamycin marker for selection process in bacterial cultures.

pFGC5941 is a 11405 bp binary vector (Figure 2.15) constructed by Kerschen et al. (2004). The GenBank accession number of pFGC5941 is AY310901. The vector contains the left and right border sequences necessary for T-DNA transfer into plant cells. The T-DNA contains a BAR gene conferring resistance to the herbicide phosphinotricine (BASTA) that functions as a selectable marker for transformed plant cells. Next to the BAR marker gene, a CaMV 35S promoter is located, followed by a synthetic polylinker for inserting sequences of interest to be expressed in transformed plants. In the original vector, a chalcone synthase intron from *Petunia hybrida* is located in the multiple-cloning site. The polylinker is followed by the polyadenylation signal from the octopine synthase gene and the right border sequence. Outside the T-DNA region a kanamycin marker is situated to facilitate selection in bacterial cultures.

Oligonucleotide primers

Construct	Primer	Oligonucleotide sequence 5' to 3'
Rap2.4prom	R24p2F	CGGAATTCGGTCTGTGAACTGTGAAGTGTGAACAC
::GUS	R24p2R	CGGGATCCGTCCATGAGTTCACCACCAGAGT
Rap2.4prom	R24Pdel1F	CGGAATTCGAAACGGAAAATATCGTTGG
Δ1::GUS	R24p2R	As above
Rap2.4prom	R24Pdel2F	CGGAATTCTAGACGAAATGAAACAATTCTGG
Δ2::GUS	R24p2R	As above
Rap2.4bprom	R24bpromF	CGGAATTCATTTCCCCTGTGGACACGCTCGG
::GUS	R24bpromR	CGGGATCCACCACCGAATGGATCAGATTGTTG

The 23rd codon of the Rap2.4 open reading frame is shown in bold letters in the R24p2R oligonucleotide sequence.

The 21st codon of the Rap2.4b open reading frame is shown in bold letters in the R24bpromRBam oligonucleotide sequence

Underlining indicates sequences added in order to create restriction sites.

The following primer was used to sequence the Rap2.4 promoter::GUS fusion.

GUSRN 5'-TTGCCCCGGCTTTCTTGTAACGCGCTTTCCCA-3'

2.2.3.2 Analysis of GUS expression in Rap2.4 promoter::GUS and Rap2.4b promoter::GUS transgenic *Arabidopsis*

Identification of GUS positive transgenic plants

Dry seeds were collected from transformed plants and sowed on soil. The seeds were stratified on wet soil at 4° C for 2-3 days and transferred to the greenhouse (20-24° C, 16 hours light, 8 hours dark). Germinated two-day old seedlings were sprayed with BASTA (phosphinotricin, 100 mg/L) twice with an interval of 48 hours.

Quick GUS assay

The presence of the GUS transgene in putative transgenic plants was tested by a quick GUS assay. A piece of leaf was ground in GUS buffer (50 mM Na₂HPO₄ pH 7.0, 10 mM β-mercaptoethanol, 1mM 4-methylumbelliferyl β-D-glucuronide (MUG). Blue fluorescence marking the presence of transgene in the plant was visualised by UV-transillumination.

GUS histochemical staining

Histochemical GUS assays were performed as described by Kirby and Kavanagh (2002). Plant tissues such as flowers, stems, siliques and leaves were collected from mature transgenic plants grown in the greenhouse. Seedlings were grown on MS medium with added vitamins and antifungal reagent amphotericin (2.5 µg/ml). Plant tissues were immersed in GUS staining solution (50 mM Na₂HPO₄ pH 6.8, 1 mM EDTA, 0.06 % TritonX-100, 0.1 mM potassium ferricyanide, 1mM potassium ferrocyanide, 1 mM 5-bromo-4-chloro-3 — indolyl-β-D-glucuronic acid) and vacuum infiltrated for 15 minutes. After incubation at 37°C (overnight for organs of mature plants, 10 minutes to 1 hour for seedlings), chlorophyll was removed with 70 % ethanol. Stained samples were stored in 20 % ethanol at 4°C.

Photography

GUS staining in plant organs was analysed under Olympus BX40 light and Olympus SZX9 stereo microscopes. Photos were taken with an Olympus digital camera. Images were manipulated with Adobe Photoshop software.

2.2.4 Expression profiling by Northern blotting

Arabidopsis mutant lines

aba1-5 – mutant line obtained from NASC (seed stock ID N155). The ABA1 gene (AGI At5g67030) encodes a zeaxanthin epoxidase which catalyses the first step in ABA biosynthesis – the conversion of zeaxanthin and antheraxanthin to violaxanthin. The *aba1-5* mutant has significantly lower levels of endogenous ABA in both normal and stress conditions, is less sensitive to salt at germination and displays reduced dormancy. For the *aba1-5* mutants, growth is less vigorous, fresh weight is reduced and plants wilt quicker than wild-type (Leon-Kloosterziel et al., 1996).

aba2-4 - mutant line obtained from NASC (seed stock ID N3835). The ABA2 gene (AGI at1g52340) encodes a cytosolic short-chain dehydrogenase/reductase involved in the conversion of xanthoxin to ABA-aldehyde (Cheng et al., 2002; Schwartz et al., 1997a). The *aba2-4* mutant was characterized by Laby et al. (2000) as a plant line insensitive to inhibitory effects of sugars on early seedling development. The *aba2-4* plants display reduced growth, reduced plant size and vigour, increased rate of water loss leading to wilting especially under low relative humidity and in water stress.

aba3-1 - mutant line obtained from NASC (seed stock ID N157). The ABA3 gene (AGI At1g16540) encodes a molybdenum cofactor sulfurase that is required for the last step of ABA biosynthesis – the conversion of ABA-aldehyde to ABA (Bittner et al., 2001). The mutant line was identified by Leon-Kloosterziel et al. (1996) and displays similar phenotype to *aba1-5*. *aba3-1* mutant phenotype is less severe than that of *aba1-5* due to slightly higher endogenous ABA levels.

hos1 - mutant line obtained from NASC (seed stock ID N157). Generated by EMS mutagenesis in the C24 background, the affected locus is At2g39810. Mutant plants are less cold hardy than the wild type and show enhanced expression of cold-responsive genes in response to low-temperature conditions. *hos1* plants are smaller and less vigorous than wild-type, with fewer leaves and shorter siliques; they flower early and are constitutively vernalized (Lee et al., 2001).

ice1 – mutant line obtained from the laboratory of J.-K. Zhu (Department of Plant Sciences, University of Arizona, Tucson, Arizona). An *Arabidopsis ice1* mutant was isolated in a screen for mutations that impair cold-induced transcription of a CBF3 promoter-luciferase reporter gene. The *ice1* mutation confers reduced chilling and freezing tolerance due to a block in the expression of CBF3 which decreases the expression of many genes downstream of CBFs (Chinnusamy et al., 2003). The control line for the *ice1* mutant is Columbia carrying the CBF3 promoter::luciferase construct.

fiery1 - mutant line obtained from NASC (seed stock ID N24933). The plant line carries a recessive mutation in the At5g63980 locus. *fiery1* plants have increased sensitivity to abiotic stresses and enhanced expression of ABA- and stress-responsive genes when treated with ABA, low temperature, drought, or salt stress. Seed germination and postembryonic development of *fiery1* mutants is more sensitive to ABA or stress inhibition (Xiong et al., 2001c).

abi3-1 - mutant line obtained from NASC (seed stock ID N24). The *abi3-1* mutation is in the Landsberg genetic background, affecting the ABI3 gene in the locus At3g24650. The mutant plants are ABA resistant and show reduced dormancy (Nambara et al., 1992).

fus3-8 - mutant line obtained from NASC (seed stock ID N157). Generated by gamma rays in the Columbia ecotype and backcrossed to the Landsberg background. The affected locus is At3g26790. Mutants are defective in late embryo development and germination, intolerant to desiccation, have reduced seed dormancy and seed storage protein levels, and germinate precociously (Raz et al., 2001).

Stress treatments

Arabidopsis thaliana ecotype Columbia plants were grown for 2-3 weeks on MS plates with 0.8 % agar and exposed to various abiotic stresses. For drought stress plants were dehydrated on Whatman paper under normal growth conditions. Cold stress was carried out by placing the plants on MS plates at 4°C, heat stress by incubating the MS plates at 38°C for defined time periods. Salt and osmotic stress treatment were carried out by spraying plants with 200 mM NaCl, 10 mM LiCl, 100 mM KCl or 300 mM mannitol.

Hormone treatments were carried out by spraying plants with sterile distilled water containing 100 µM abscisic acid (ABA), 5 µM aminocyclopropane-1-carboxylic acid (ACC), 2 µM salicylic acid (SA), 5 µM jasmonic (JA) acid, 1 mM synthetic auxin (2,4-D), 100 µM gibberellic acid (GA) or 5 µM synthetic cytokinin benzylaminopurine (BAP1).

The protein synthesis inhibitor cycloheximide (CHX) was applied by spraying plants with a 10 µM CHX solution.

RNA extraction and Northern blotting

The relevant experimental procedures are described in Chapter 5.

Primers used for generating Rap2.4 and Rap2.4b specific probes

Rap2.4-specific 244 bp DIG-labelled probe was generated using following primers:

Rap24P2F: 5'-ATCTCACATCGGAGGCGATTTCCGG-3'

Rap24P2R: 5'-TCCAGCGGTGGACTCTTCAAACCTCCGT-3'

Rap2.4b-specific 285 bp DIG-labelled probe was generated using following primers:

Ftdna591: 5'-TGAGACGACGCAGAAACAGG-3'

R24orfRBamHI: 5'-CGGGATCCCTAATTTACAAGACTCGAACA-3'

2.3 Results

2.3.1 Bioinformatic studies of Rap2.4 and Rap2.4b

Rap2.4 gene and protein structure

The open reading frame of Rap2.4 (*related-to-Apetala2-4*, *Arabidopsis* Genome Initiative Identifier At1g78080) consists of 1005 bp and encodes a putative transcription factor of 334 amino acids. The ORF is flanked by a 542 bp 5'UTR and a 278 bp 3' UTR (Figure 2.1). The predicted molecular weight of the protein is 36.61 kDa, isoelectric point 6.59, and a charge at pH 7.0 of -1.36. Rap2.4 contains a conserved AP2 DNA-binding domain which consists of 58 amino acids according to the NCBI conserved domain search. Directly at the N-terminus of the AP2 domain lies a putative nuclear localization signal. The Rap2.4 protein contains two regions rich in the amino acid serine that may act as transcriptional activation domains and several putative phosphorylation sites which may indicate that the activity of this transcription factor might be modified by post-translational phosphorylation (Figure 2.2). Rap2.4 also contains, in the C-terminus, a region of 49 amino acids rich in proline, glutamic acid, serine and threonine known as the PEST region. The presence of PEST regions has been shown to result in rapid turnover of proteins containing them (Rogers et al., 1986). The PEST region of Rap2.4 has a score of 11.12. According to Rogers et al. scores above 5 should be regarded as "interesting" as it could indicate fast turnover of the protein.

The 5' UTR of the Rap2.4 mRNA contains a short open-reading frame potentially encoding a peptide of 27 amino acids.

Comparison of the cDNA of Rap2.4 and the chromosomal Rap2.4 gene revealed that the gene is not interrupted by introns.

Rap2.4b gene and protein structure

The open reading frame of *Rap2.4b* (*related-to-Apetala2-4 b*, AGI At1g22190) consists of 786 bp and encodes a putative transcription factor of 261 amino acids. The ORF is flanked by a 463 bp 5'UTR and a 258 bp 3' UTR (Figure 2.3). The predicted molecular weight of the protein is 29.14 kDa, isoelectric point 6.01, and a charge at pH 7.0 of -2.78. Rap2.4b contains a conserved AP2 DNA-binding domain which consists of 58 amino acids according to the NCBI conserved domain search.

The protein contains several putative phosphorylation sites which indicate that the activity of this transcription factor might be modified by post-translational phosphorylation (Figure 2.4). Rap2.4b also contains a PEST region of 52 amino acids in the C-terminus. The Rap2.4b PEST sequence has a slightly weaker score (9.70) than that of Rap2.4, but could still contribute considerably to fast protein turnover.

Comparison of the cDNA of Rap2.4b and the chromosomal Rap2.4b gene revealed no introns.

-541 atcgggtgagggttgagagtaattcactacacacacacaaaaaataaattgagtgccctcccc
 -481 caaaaacaaaatggtagataacgagcaattggtttttttcagatttgatcctgaatttt
 -421 tacatttttttttgcaatctccccctaatctgtggtttctcgtctctctctggttaa
 -361 tcactctgtctttcaaaagaaagaaa aaagaaaaa ttcgatttc tgggttgt ttttgtc
 -301 atacagaaaaaatcaagcttatgaaatgtgttt aatttttg ttttaatttgaaaggc
 -241 aggtttttttcagaacgagatcgttttttcaaatttcttctgattttacctctttttttct
 -181 tcttagatttttagtgaatcgagggtgaaatttttgattccctcttttcggatctacacag
 -121 aggtttgc ttatttcaaaccttttagatccattttttttaattttctcggaaaatccct
 -61 gtttctttactttttttataagctcaggttcaattttttcggatcaaaattttatttta
 M A A A M N L Y T C S R S F Q D S G G E
 -1 aATGGCAGCTGCTATGAATTTGTACACTTGTAGCAGATCGTTTC AAGACTCTGGTGGTGA
 L M D A L V P F I K S V S D S P S S S S
 61 ACTCATGGACGCGCTTGTACCTTTTATCAAAAGCGTTTCCGATTCTCCTTCTTCTTCTC
 A A S A S A F L H P S A F S L P P L P G
 121 TGCAGCGTCTGCGTCTGCGTTTCTTCCCTCTCGTCTTCTCCTCCTCCTCCTCCTCCTC
 Y Y P D S T F L T Q P F S Y G S D L Q Q
 181 TTATTACCGGATTCAAGTTCTTGA CCAACCGT TTTTCATACGGGTGGATCTTCAACA
 T G S L I G L N N L S S S Q I H Q I Q S
 241 AACCGGGTCATTAATCGACTCAACAACCTCTCTTCTTCTCAGATCCACCAGATCCAGTC
 Q I H H P L P P T H H N N N N S F S N L
 301 TCAGATCATCATCCTCTTCTCCTCGACGATCACAACAACAACAACCTCTTCTCGAATCT
 L S P K P L L M K Q S G V A G S C F A Y
 361 TTCCAGCCCAAAGCCGTTACTGATGAAGCAATCGGAGTCGCTGATCTGTTTCGCTTA
 G S G V P S K P T K L Y R G V R Q R H W
 421 CGGTTCAAGGTGTTCTTCGAAGCCGACGAAGCTTTACAGAGGTG TGAGGCAACGTCACTG
 G K W V A E I R L P R N R T R L W L G T
 481 GGGAAAA TGGGTGGCTGAGATCCGTTTGCCGAGAAATCGGACTCTGCTCTGGC TTGGGAC
 F D T A E E A A L A Y D K A A Y K L R G
 541 TTTTGACACGGCCGGAGGAAGCTGCGCTATGATAAGCCGGCGTACAAGCTGCGCGG
 D F A R L N F P N L R H N G S H I G G D
 601 CGATTTCCCGGCTTAACTTCCCTAACCTACGTCATAACGGATCTCACATCGGAGGCGA
 F G E Y K P L H S S V D A K L E A I C K
 661 TTTCCGGTGAATATAAACTTCTTACTCTCAGTCGACGCTAAGCTTGAAGCTATTTGTAA
 S M A E T Q K Q D K S T K S S K K R E K
 721 AAGCATGCGGAGACTCAGAAACAGGACAAATCGACGAAATCATCGAAGAAACGTGAGAA
 K V S S P D L S E K V K A E E N S V S I
 781 GAAGGTTTCGTCGCCAGATCTATCGGAGAAAGTGAAGGCGGAGGAGAATTCGGTTTCGAT
 G G S P P V T E F E E S T A G S S P L S
 841 CGGTGGA TCTCCACCGGTGACGGAGTTTGAAGAGTCCACCCTGATCTTCGC CGTTGTC
 D L T F A D P E E P P Q W N E T F S L E
 901 GGACTTGACGTTTCGCTGACCCGGAGGAGCCGCCAGTGGAAACGAGACGTTCTCGTTGGA
 K Y P S Y E I D W D S I L A .
 961 GAAGTATCCGTCGTACGAGATCGATTGGGATTCGATTCTAGCTTAGggggcaaaataggaa
 1021 attcagcgcgttgcaatggagttttgtgaaatgcatgactggcccagagt aat taat
 1081 taaat atggattagtgttaaatttcgtatgttaat atttgtattatgggttgt attagtc
 1141 tctctgtgctggtcagcttgcggtttttgtcaggtcgcacatgcccagct tttcatt
 1201 ttatgtaatctttttttctttttgtctatgtaatttgtagctc agt tttctc atctata
 1261 atgcaat tttattatgattatgtaa

Figure 2.1. *Arabidopsis* cDNA encoding the Rap2.4 protein. The protein coding sequence is shown in upper case, the 5' UTR and 3'UTR in lower case. The short open reading frame in the 5'UTR is marked in blue. The deduced amino acid sequence is shown above the nucleotide sequence in each row. The start and stop codons are highlighted in yellow. The amino acids of the AP2 domain are shown in red.

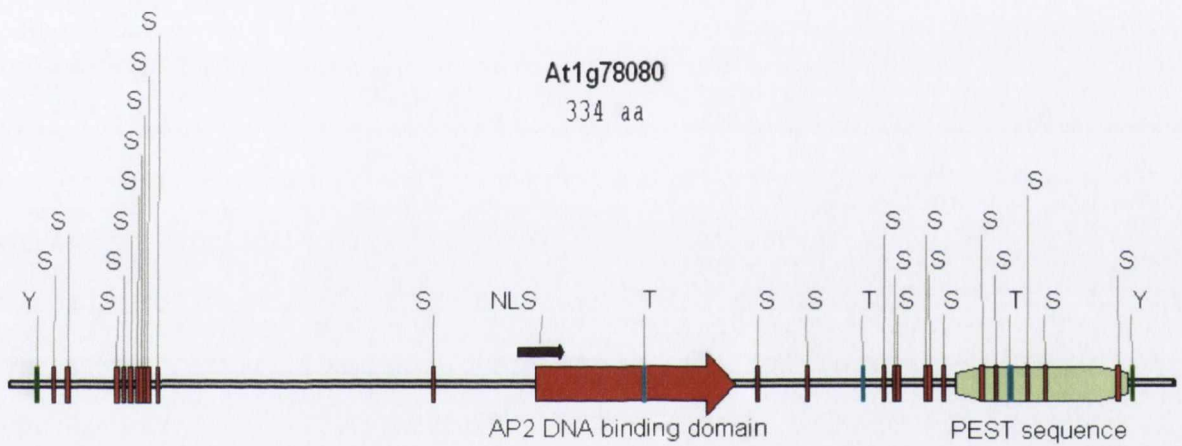


Figure 2.2. Schematic illustration of Rap2.4 protein. The AP2 domain is indicated with a red arrow. The PEST sequence is indicated with a green box. Nuclear localisation signal is indicated with a brown arrow. Putative serine, threonine and tyrosin phosphorylation sites are indicated with short vertical bars and marked with letters S, T and Y, respectively.

```

-463 aacaagagccaagc taatgagataagtgatcggtgaggctgagagttattcacttaca
-403 aaaaaaaaaaaaaaacttgagtgtaaccaaaaaaaaaaagttgatatactttctggtttctc
-343 cttaactttttatctttacaaatccatcccccttagatctgtttatttcccgtactttg
-283 attcattttctgttagtaatctgtctttcgtatagaagaaaactgatttcttggtttgtat
-223 tttcttaagagatcaatctttttttttttttgatctctcttgtgttttttttctttgta
-163 gaattaatcgtttgtgagggtatttttttaattccctcctctcagaaatctacacagagg
-103 ttttttattttataaacctctttttcgtattttcttgaaaacaaaaaatcctgtctcttac
                                     M T T S M D
-43  tttttttacaagaacaagggaaaaaaatctcttttttattagaaATGACAACTTCTATGGA
    F Y S N K T F Q Q S D P F G G E L M E A
18  TTTTACAGTAACAAAACGTTTCAACAATCTGATCCATTCCGGTGGTGAATTAATGGAAGC
    L L P F I K S P S N D S S A F A F S L P
78  GCTTTTACCTTTTATCAAAAAGCCCTTCCAACGATTTCATCCGGCTTTGCGTTCCTCTTACC
    A P I S Y G S D L H S F S H H L S P K P
138 CGCTCCAATTTTCATACGGGTCGGATCTCCACTCATTTCCTCACCATCTTAGTCCTAAACC
    V S M K Q T G T S A A K P T K L Y R G V
198 GGTCTCAATGAAAACAAAACCGGTACTTCCGCGGCTAAACCGACGAAGCTATACAGAGGAGT
    R Q R H W G K W V A E I R L P R N R T R
258 GAGACAACGTCACTGGGAAAATGGGTGGCTGAGATTGTTTTACCAGGAATCGAACTCG
    L W L G T F D T A E E A A L A Y D K A A
318 ACTTTGGCTCGGAACATTTCGACACGGCGGAGGAAGCTGCTTTAGCTTATGACAAGGCGGC
    Y K L R G D F A R L N F P D L R H N D E
378 GTATAAGCTCCGAGGAGATTTTTCGCGGCTTAATTTCCCTGATCTCCGTCATAACGACGA
    Y Q P L Q S S V D A K L E A I C Q N L A
438 GTATCAACCTCTTCAATCATCAGTCGACGCTAAGCTTGAAGCTATTTGTCAAAACTTAGC
    E T T Q K Q V R S T K K S S S R K R S S
498 TGAGACGACGCAGAAAACAGGTGAGATCAACGAAGAAGTCTTCTTCTCGGAAAACGTTTCATC
    T V A V K L P E E D Y S S A G S S P L L
558 AACCGTCGCAGTGAAACTACCGGAGGAGGACTACTCTAGCGCCGGATCTTCGCCGCTGTT
    T E S Y G S G G S S S P L S E L T F G D
618 AACGGAGAGTTATGGATCTGGTGATCTTCTTCCCGGTTGTGCGGAGCTGACGTTTGGTGA
    T E E E I Q P P W N E N A L E K Y P S Y
678 TACGGAGGAGGAGATTCAGCCGCCGTGGAACGAGAACGCGTTGGAGAAGTATCCGTCGTA
    E I D W D S I L Q C S S L V N *
738 CGAGATCGATTGGGATTCGATTCTTCAGTGTTTCGAGTCTTGTAATTAGatgttgccata
798 ggggtattttagggacttttagagctctctgcatggagtttttgggtcattgcagagattt
858 tattattattaaggggggtttggtatggttaatatcaataagtttatctactttgatggtta
918 attagtggttaatctctgcatgggtccaagctgttttttttggcatgcttcgaccgtgtg
978 agatttcttatgtaattttttagtattccttgattttcttagttcaagttaaatggcaca
1038 aaagaac

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Figure 2.3. *Arabidopsis* cDNA encoding the Rap2.4b protein. The protein coding sequence is shown in upper case, the 5' UTR and 3'UTR in lower case. The deduced amino acid sequence is shown above the nucleotide sequence in each row. The start and stop codons are highlighted in yellow. The amino acids of the AP2 domain are shown in red.

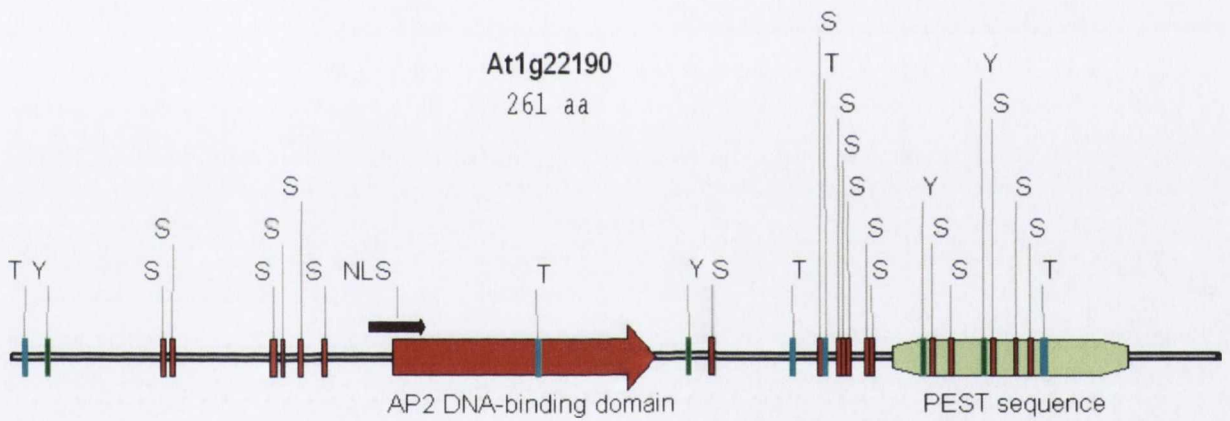


Figure 2.4. Schematic illustration of Rap2.4b protein. The AP2 domain is indicated with a red arrow. The PEST sequence is indicated with a green box. Nuclear localisation signal is indicated with a brown arrow. Putative serine, threonine and tyrosin phosphorylation sites are indicated with short vertical bars and marked with letters S, T and Y, respectively.

Phylogenetic analysis of Rap2.4 and Rap2.4b

Based on the TIGR listing of genomic duplications (http://www.tigr.org/tdb/e2k1/ath1/duplication_listing.html) 50 out of the 56 DREBs are found within large duplicated segments and only six reside outside annotated duplicated regions. Most of the DREBs have clearly arisen through large scale duplications as 34 of the DREBs are located within large duplicated regions forming 17 defined pairs. The 16 remaining DREBs that are found within large duplicated regions lack a copy that would be annotated as a DREB gene in the respective partner region.

As expected, the AP2 DNA-binding domains of paralogous DREBs are mostly more similar to one another than to the AP2 domains of other DREBs. The duplicated genes can usually be found in close proximity to each other on the phylogenetic tree of DREB proteins (Figure 2.5). The paralogs that do not lie on the same nodes in the phylogenetic tree constructed on the basis of the AP2 domain similarities, can be found on the same nodes when a phylogenetic tree is calculated based on the whole protein sequences. The chromosomal locations of the duplicated genes are outlined on Figure 2.6.

Rap2.4 and Rap2.4b are found within a 2 Mbp duplicated segment on chromosome 1 (chromosome 1, 5898710 bp-7890453 bp and 27224738-29455456). Rap2.4 and Rap2.4b contain identical DNA-binding domains and share a significant amount of amino acid sequence outside the conserved AP2 domain (Figure 2.7). Rap2.4 and Rap2.4b belong to the A-6 subgroup of DREBs. The AP2 domains of the proteins in the A-6 subgroup differ from those of Rap2.4 and Rap2.4b by 3-11 amino acids (Figure 2.8).

As Rap2.4 and Rap2.4b are paralogs which have arisen from duplication of a large segment, their chromosomal contexts are similar with respect to surrounding genes. Surprisingly we found that 4 other genes in the A-6 subgroup to which Rap2.4 and Rap2.4b belong to, also share elements of the genomic context of Rap2.4 and Rap2.4b. As expected a second pair of paralogs in the A-6 subgroup that have arisen by duplication of large regions, At2g22200 and Atg439780, share more genomic context with each other than with other DREBs in the clade (Figure 2.9). Most noticeably, 6 of the 10 DREBs in the A-6 subgroup are either immediately flanked by or at least in very close proximity to a gene coding for trehalose phosphate phosphatase (TPP).

Analysis of Rap2.4 and Rap2.4b publicly available expression data

The Genevestigator expression database offers facilities for examining the expression patterns of over 22 000 *Arabidopsis* genes by collating data from over 4000 individual microarray experiments. Prior to conducting expression profiling by Northern blotting we reviewed the data available for the transcription factors under study here, Rap2.4 and Rap2.4b.

During the life-cycle of *Arabidopsis*, Rap2.4 (262135_at probe on the Affymetrix array) was expressed at higher levels than Rap2.4b (255926_at probe on the Affymetrix array). The mRNA of Rap2.4 and Rap2.4b was more abundant in the young rosette stage compared to other stages of plant life-cycle (Figure 2.10, stage 3 on A and B). Rap2.4b was also strongly expressed

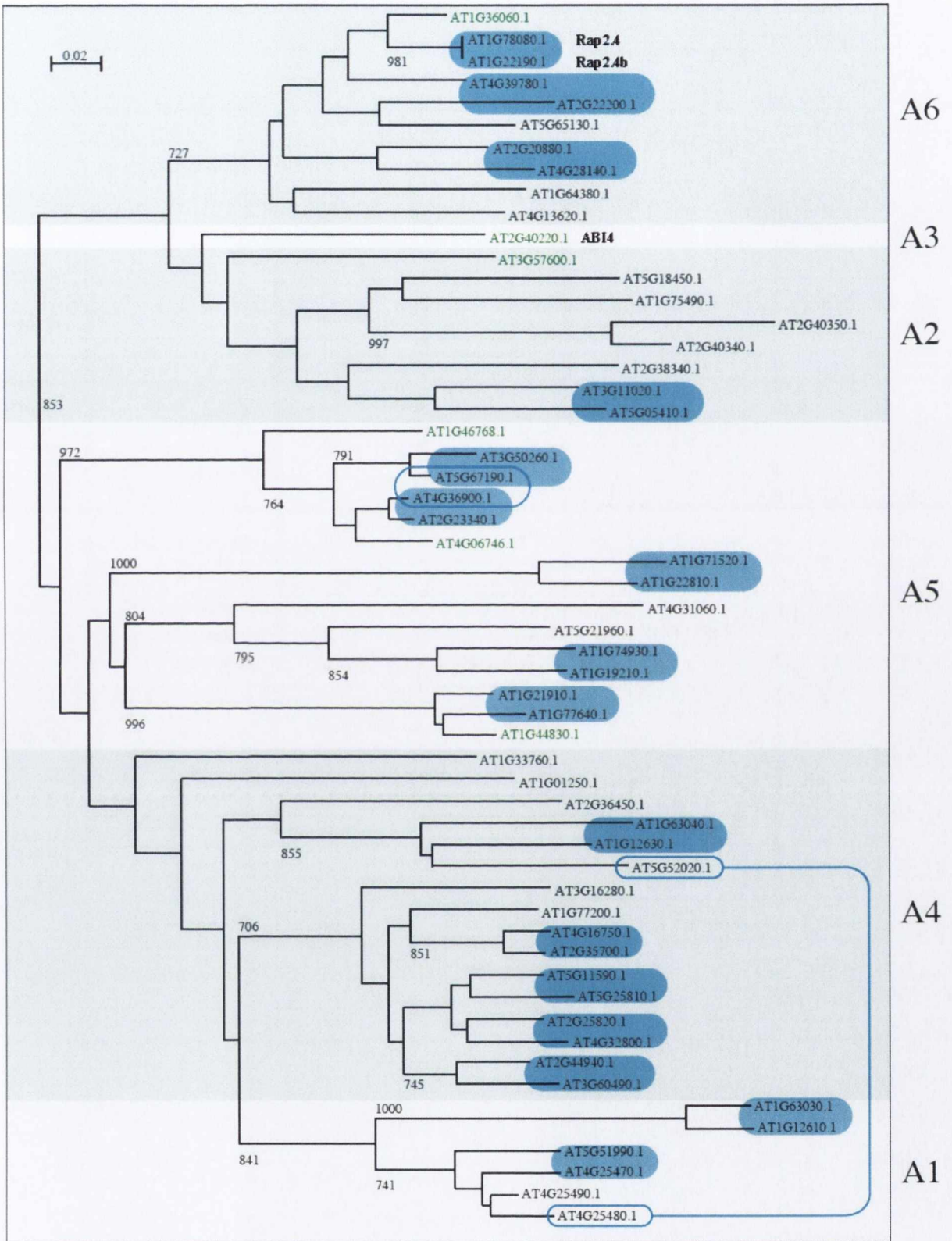


Figure 2.5. Phylogenetic tree of *Arabidopsis* DREB transcription factors. Subgroups A1 to A6 according to Sakuma et al (2002) are outlined as blocks of white and blue. Paralogous genes are indicated with blue rounded rectangles. DREBs that do not fall within large duplicated segments are in green lettering. Only bootstrap values of over 700 are displayed.

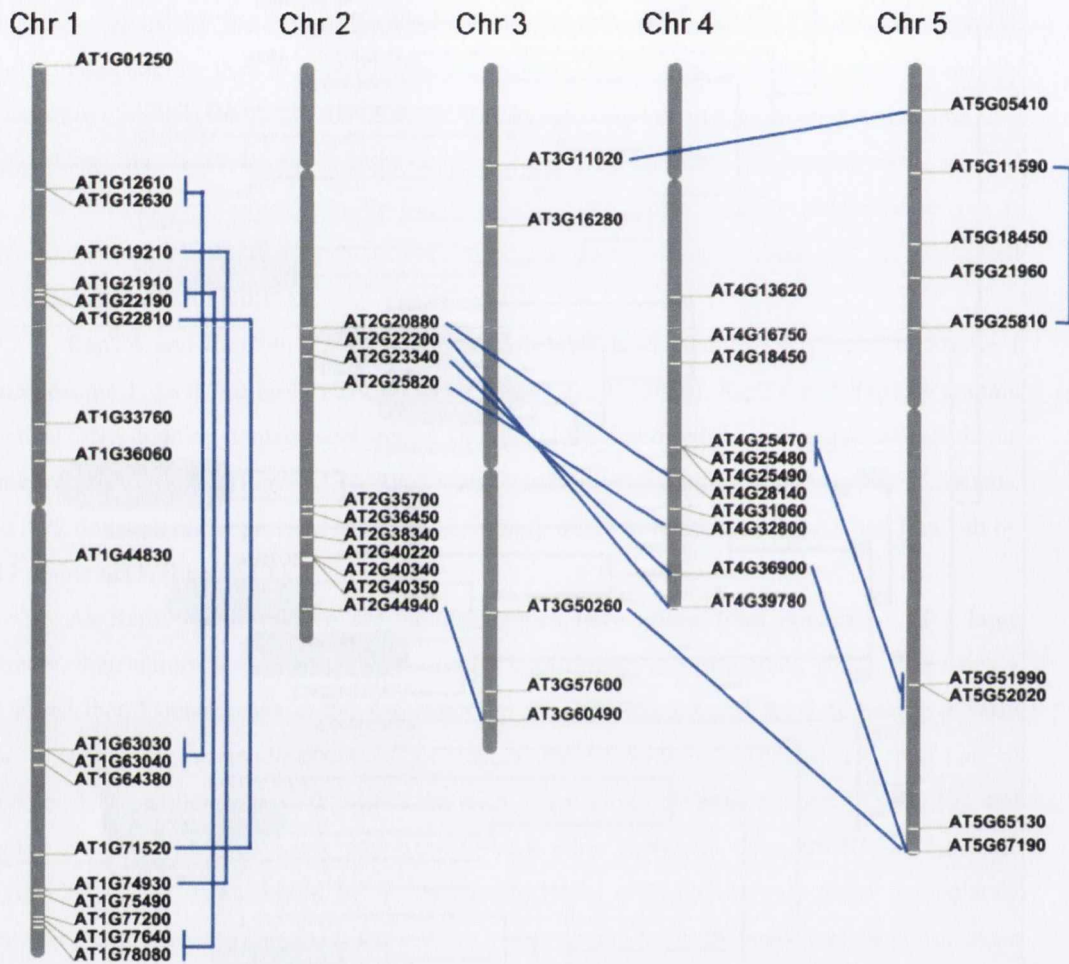


Figure 2.6. Duplicated DREB transcription factors outlined on the 5 chromosomes of *Arabidopsis*. The duplicated gene pairs are connected with blue lines.

```

Rap2.4 : MAAAMNLYTCSRFSQDS---GGELMDALVPIKSVSDSPS : 37
Rap2.4b : MTTSMDFYSN-KTFQSDPFGGELMEALLPIK----SPS : 35
          M M1 Y3 43FQ S GGELM AL6PIK SPS

Rap2.4 : SSSAASASAFHLHPSAFSLPPLPGYYPDSTFLTQPFPSYGS : 77
Rap2.4b : NDSSA-----FAFSLP-----APIPSYGS : 54
          S A AFSLP P SYGS

Rap2.4 : LQQTGSLIGLNNLSSSQIHQIQSQIHHPLPPTHNNNNSF : 117
Rap2.4b : LH-----SF : 58
          L SF

Rap2.4 : SNLLSPKPLLMKQSGVAGSCFAYGSGVPSKPTKLYRGVRQ : 157
Rap2.4b : SHHLSPKPVSMTQTS-----AAKPTKLYRGVRQ : 88
          S LSPKP6 MKQ3G KPTKLYRGVRQ

Rap2.4 : RHWGKWVAEIRLPRNRTRLWLGTFTAEAAALAYDKAAYK : 197
Rap2.4b : RHWGKWVAEIRLPRNRTRLWLGTFTAEAAALAYDKAAYK : 128
          RHWGKWVAEIRLPRNRTRLWLGTFTAEAAALAYDKAAYK

Rap2.4 : LRGDFARLNFPNLRHNGSHIGDFGEYKPLHSSVDAKLEA : 237
Rap2.4b : LRGDFARLNFPDLRHN-----DEYQPLQSSVDAKLEA : 160
          LRGDFARLNFP1LRHN EY PL SSVDAKLEA

Rap2.4 : ICKSMAET-QKQDKSTKSSKKREKQVSSPDLSEKVKAEEN : 276
Rap2.4b : ICQNLAETTQKQVRSTKSSSRKR---SSTVAVKLPEEDY : 197
          IC 6AET QKQ 4STK S R 4 S 6 K6 E

Rap2.4 : SVSICGSPPVTEFEESTAGSSPLSDLTFADPEEPPQ--WN : 314
Rap2.4b : S-SAGSSPLLTESYSGGSSSPLSELTFGDTEEEIQPPWN : 236
          S S G SP 6TE S SSPLS LTF D EE Q WN

Rap2.4 : ETFSLKYPSEYIDWDSILA----- : 334
Rap2.4b : EN-ALKYPSEYIDWDSILQCSSLVN : 261
          E LEKYPSEYIDWDSIL

```

Figure 2.7. Rap2.4 and Rap2.4b deduced amino acid sequence alignment. The AP2 domains are highlighted in yellow, conserved amino acids are shown on grey background. Protein sequences were aligned with ClustalW web-tool and visualised with GeneDoc program. Dashes indicate gaps introduced in order to align the sequences.


```

Rap2.4
AT1G78080: KLYRGVQRHWGKWVAEIRLPRNRTRLWLGTFTAEAAALAYDKAAYKLRGDFARLNF
Rap2.4b
AT1G22190: KLYRGVQRHWGKWVAEIRLPRNRTRLWLGTFTAEAAALAYDKAAYKLRGDFARLNF

AT4G28140: KLYRGVQRHWGKWVAEIRKPSRRLWLGTFTAEAAAMAYDRQAFKLRGHSATLNF
AT2G20880: KLYRGVQRHWGKWVAEIRKPRNRTRLWLGTFTAEAAAMAYDREAFKLRGETARLNF
AT1G36060: KLYRGVQRHWGKWVAEIRLPRNRTRLWLGTFTAEAAALAYDRAAFKLRGDSARLNF
AT2G22200: NLYRGVQRHWGKWVAEIRLPKNRTRLWLGTFTAEKAALAYDQAAFOLRGDIAKLNF
AT4G39780: KLYRGVQRHWGKWVAEIRLPKNRTRLWLGTFTAEAAAMAYDLAAYKLRGEFARLNF
AT5G65130: KLYRGVQRHWGKWVAEIRLPKNRTRLWLGTFTAEQAAALAYDQAAHKIRGDNARLNF
AT4G13620: KLYRGVQRHWGKWVAEIRLPRNRTRVWLGTFTAEQAAAMAYDTAAYILRGEFAHLNF
AT1G64380: KLYRGVQRHWGKWVAEIRLPQNRMRVWLGTVDTAEAAAYAYDRAAYKLRGEYARLNF

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Figure 2.8. Alignment of the AP2 domains of the DREB genes that belong to the A-6 subgroup. The amino acids differing from those present in Rap2.4 and Rap2.4b are highlighted with grey background.

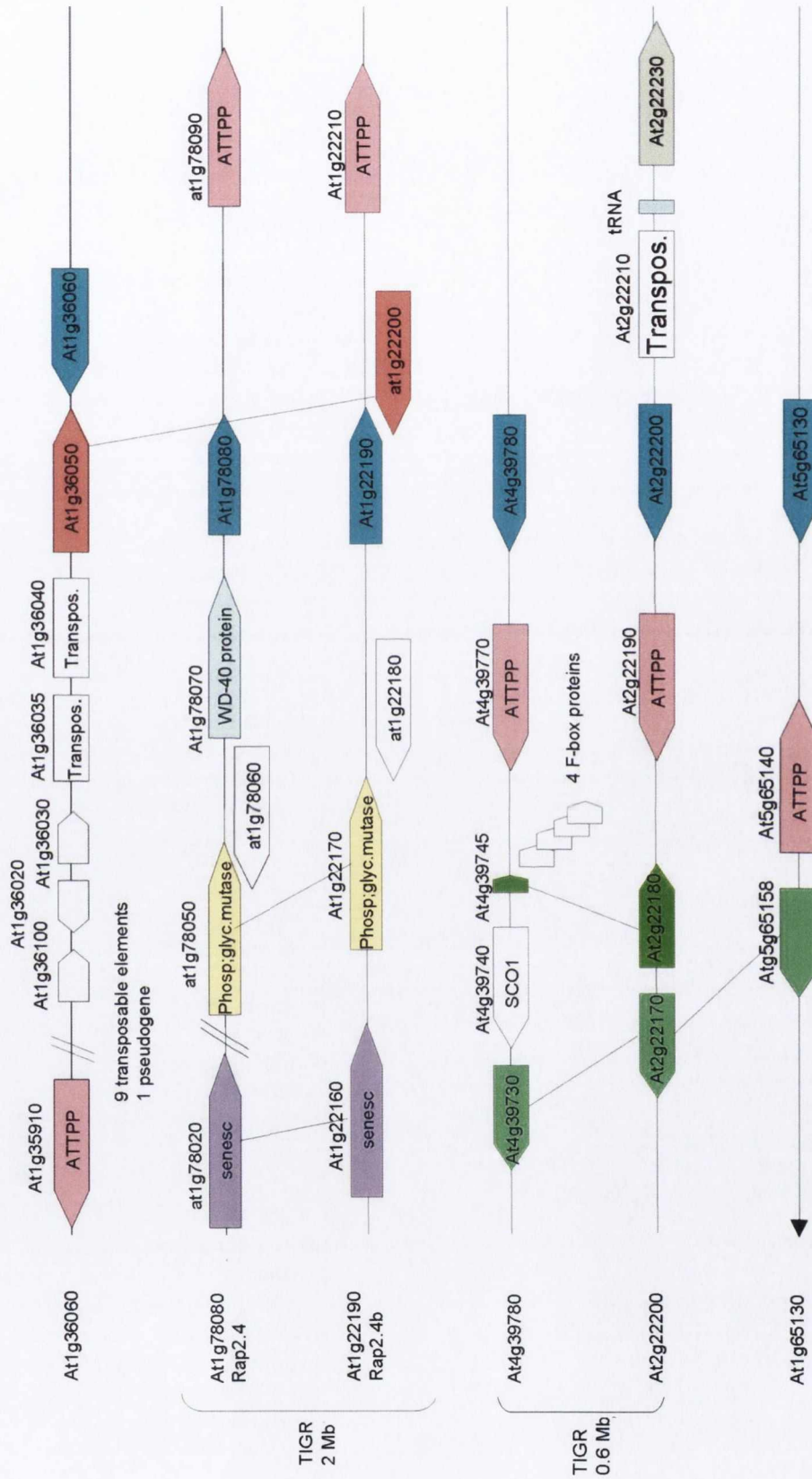


Figure 2.9. Genomic contexts of 6 DREBs belonging to the A-6 subgroup. DREBs are shown in blue block arrows, trehalose phosphatases in pink block arrows. Genes that are annotated as similar in TAIR database are marked by block arrows in the same respective colours.

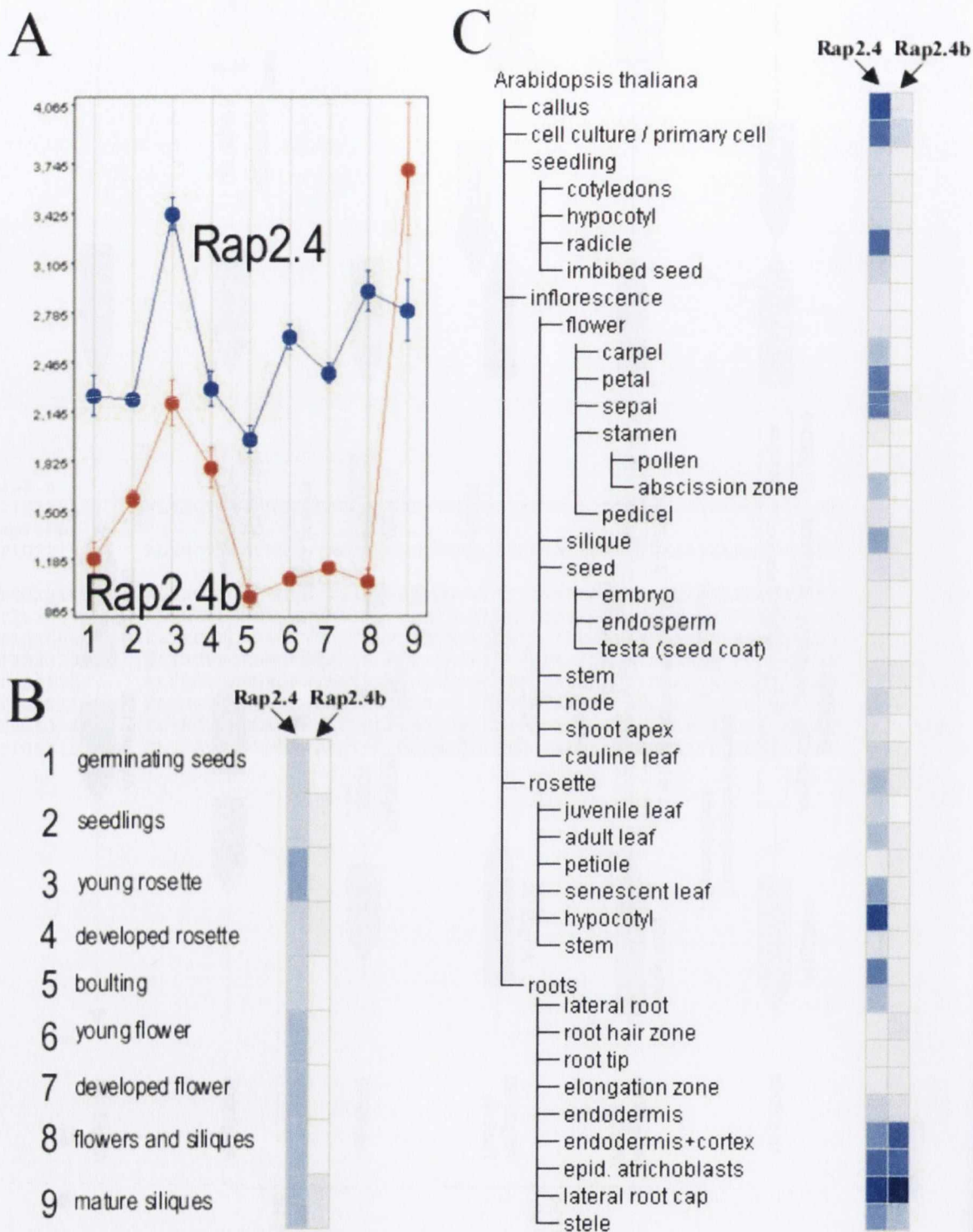


Figure 2.10. Rap2.4 and Rap2.4b expression according to Genevestigator expression viewer. A) Graph of Rap2.4 and Rap2.4b expression in different stages of *Arabidopsis* life-cycle. B) Heat map of Rap2.4 and Rap2.4b expression in different stages of *Arabidopsis* life-cycle. C) Heat map of Rap2.4 and Rap2.4b expression in different tissues of *Arabidopsis* seeds, seedlings and mature plants.

during the last stage of the *Arabidopsis* life-cycle defined by the presence of mature siliques (Figure 2.10, stage 9 on A and B).

Organ-specific expression patterns showed Rap2.4 to be expressed in almost all tissue types of *Arabidopsis* with strong levels observed in callus, cell culture, radicles of seedlings, petals and sepals of inflorescences, abscission zones, siliques and certain parts of roots. Rap2.4b was expressed at higher levels in callus, cell culture and some tissues in roots (Figure 2.10, C).

Reviewing the Genevestigator data concerning exposure to pathogens, stress treatments, chemical hormone applications, Rap2.4 and Rap2.4b were found to be induced by several abiotic stresses, to respond to changes in light and photoperiod conditions and to be influenced by levels of nutrients. Both transcription factors were induced by senescence and repressed by cold. In some aspects the expression patterns of Rap2.4 and Rap2.4b differed. For example, Rap2.4b was induced by application of protein synthesis inhibitor cycloheximide and low CO₂ levels, whereas Rap2.4 mRNA abundance was increased in low O₂ environment (Figure 2.11).

Rap2.4 promoter studies

Bioinformatic analysis of the Rap2.4 promoter using the PLACE database revealed that the 1.4 kb region upstream of Rap2.4 transcription start site contains a number of *cis*-acting elements which may provide clues about the expression and function of the gene.

A typical TATA-box was identified at -35 to -29 upstream of transcription start site. Several motifs associated with light responsiveness are present in the Rap2.4 promoter. BOX-1 elements are found in light responsive tobacco genes and I-BOX motifs are conserved in both monocots and dicots. Regarding abiotic stress responses, a number of binding sites for dehydration-associated MYB transcription factors were identified along with A-rich ANAERO1 consensus motifs. ANAERO1 consensus sequences are over-represented in promoters of genes involved in fermentative pathways. Several W-box elements associated with wounding stress and response to salicylic acid were found within the Rap2.4 promoter. The presence of the abovementioned *cis*-elements suggests a stress-response related role for Rap2.4.

Regarding spatial control of expression, 7 TAPOX1 (ROOTMOTIFTAPOX1) motifs are present in the Rap2.4 promoter. TAPOX1 elements are associated with high root-specific expression levels. A partial recognition site for the seed-maturation controlling transcription factors ABI3 and FUS3 (Monke et al., 2004), called the RY-repeat, was identified 1.2 kb upstream of the transcription start site. All promoter elements are depicted on the Rap2.4 promoter sequence in Figure 2.12 and a summary of the predicted *cis*-elements is given in Table 2.14.

Rap2.4b promoter studies

The Rap2.4b promoter was scanned for *cis*-elements using the SignalScan tool in the PLACE database. A typical TATA-box was identified at -60 to -53 bp upstream of transcription start site.

Like the Rap2.4 promoter, the Rap2.4b promoter contains several BOX-1 and I-BOX elements associated with light responsiveness. Also abiotic stress related MYB and ANAERO1 consensus motifs were identified in the Rap2.4b promoter. A cold-responsive LTRE-

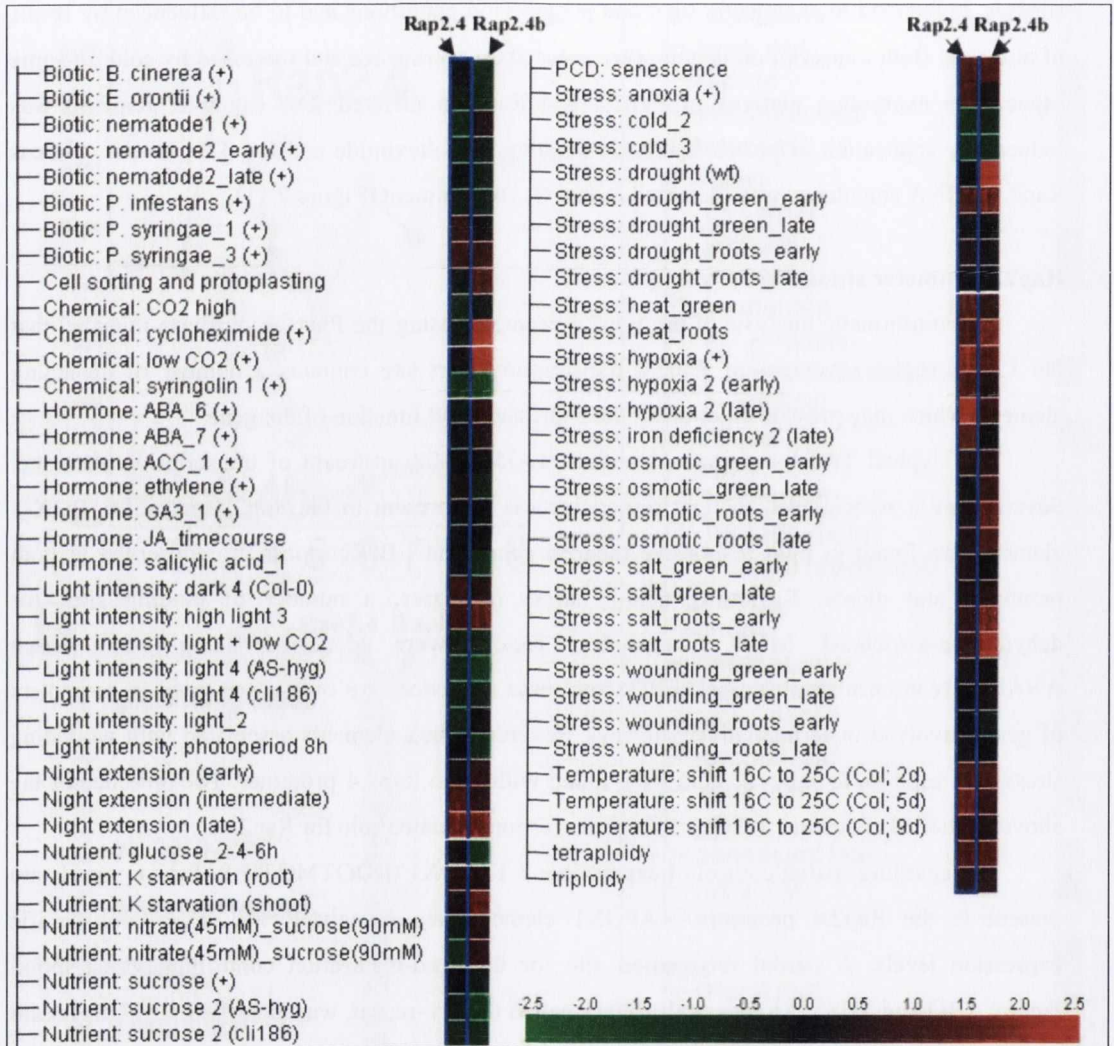


Figure 2.11. Rap2.4 and Rap2.4b expression in stress conditions according to Geneinvestigator expression viewer. The map depicts expression levels in response to various environmental stimuli, hormone applications and nutrient stresses.

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                                                    CCAATBOX
-1395      GAAACTAGAG CCTAAAAGTC AGCAAGTTTA TGTAGTCATG ATTATAACCA TTAACCAATA
           W-box barley      W-box barley MYB1AT1 MYB1AT1
           CCAATBOX
-1335      AGAGTATGAG ACCAATAAAA GAGAGATGAT TAAAACACAG AACCTAGCTA AGCAATAACA
           ANAERO1con
           MYB1AT
-1275      AACAAACAAA TAAGCAAAAT GGTCTGTGAA CTGTGAAGTG TGAACACGAA CCGGATCGGT
           W-BOX      W-box barley      ABRELATERD1
-1215      CAACAAAATC AGGTATGACT ATTTACTCCA CAAAGCACAA ATAATATGAA GACACGTGTT
           ASF-1      RY element
-1155      TTTGAATTAT GTATTCCTAT TTGTATAATA CACGCGTCAT TCACTCATTA GCATGATGTC
-1095      ATTTTCTCA TCCGCTCGCA GGTAGGAAAA ATACAGTGTA CCTCGCGCCG ATTATATGAA
           I-BOX      MYB1AT
-1035      TTTATCAGCC TCATCATAAT TCATAAATAA TAAATAGACG AAATGAAACA ATTCTGGAAT
           BOX-1      ANAERO1cons      MYB1AT
-975       AGAAATATGC TGATCTAAC AAAATGGATG ATTATAAAAA TATATAAACA ATTTTAAGAA
           MYB1AT
-915       AATTTGGTGG ATACTTTTCT TATTTTGAA AAATTCATG AAAAGAGAGA ATGTAAAAAT
           I-BOX      MYB1AT
-855       AGACTTGTTA TCTTACAATT GTTTTCTACA TTGCGCTACA TAAAAAATTA AGAGTACATA
           W-box barley
-795       AATATATTCG TTTGAACATT TATTCCTTTT ATAAAATTAT GTTAATGTAG TCATGTTTCT
           MYB barley
-735       AAATTAATAT AACAAAATGT TAATGTAGAT TAAATATCTT ACTTTTTTTT GAAATTTCAA
           BOX-1
-675       TTAGTTTCCA AAATATAAAT TTTTCTAAT ATAGTATAAA ATAATTCATA ATCAAATCCT
           MYB barley      W-BOX
-615       AATTGAAAAG AAGTAAAGAT TATTTTAAACA AAAAAGGGGT TTGACATTAG AGGGTAAAGA
           I-BOX      MYB26
-555       CGATAACTTT AACGACAAGT TTTAGGTGGA TTAAAAAAAG AAAAGTTAGG TTGTATCATT
-495       AATATATCCA TATGCATCAT TTTAAGAGGT GTTTAAAATT TAAACACAA ATTTAAAGAA
-435       AAACACGCTA TTTTATTTA ATTTACTATT TTATATAAAA CGCCAGAAAA TAAAATAAAT
           CCAATBOX
-375       ACAAGTATTC AACTAATAAA AACTATATAT AAAATATGAT TGGTCAGAAA AAAGTAAAGT
-315       GAAAGATCGC TAAAACATCA CTTAAAGTAA TTCTGAGGAA GTATTAGTAT TTATGGTAAT
           I-BOX
-255       CATTTATCAT TACTATATTT TTTTACTAAC ACAGTCTAAT TTTATTTTTT CTAACACTAC
           W-BOX      ANAERO1c
-195       TACAGTTTTA TTTACTGTCA AAAAGTTTTT ACCATCTTGT GTGTCATCAA ACAAACTAC
           MYB1AT
-135       TCGCTTAGGT ATTTTGAAAT ATAAAATTGC CTAAGAAACG GAAAATATCG TTGGAAAAAA
           MYB1AT
-75       CCCGGAAAAT ATGAAAATAG TGTGAGGAGA CTACACCCAC TATATAAACA AGAGCCAAGC
           MYB1LEPR
-15       AGGTGAGGTT AGTTCATCGG TGAGGTTGAG AGTAATTCAC TACACACACA CAAAAATAA
46       ATTGAGTGCC TCCCCAAAA ACAAAATTGG TAGATAACGA GCAATGTTT TTTTTCAGAT
106      TTGATCCTGA ATTTTACAT TTTTTTTTT GCAATCTCC CTAATCTGT TGTTCCTGC
166      TTCTTCTCT GTTAATCATC TGCTTTTCAA AAAGAAAGAA AAAAGAAAAA TTCGATTTCT
226      GGGTTTGTG TTGTCATACA GAAAAAATC AAGCTTATGA ATTTGTGTTT AATTTTTTGT
296      TTTAATTTGA AAGGCAGGTT TTTTCAGAAC GAGATCGTTT TTTCAAATTT CTTCGTGATT
346      TACCTCTTT TTTCTTCTA GATTTTAGTG AATCGAGGTT GAAATTTTTG ATTCCCTCTT
406      TTCGATCTA CACAGAGGTT GCTTATTTC AACCTTTAG ATCCATTTT TTTAATTTT
466      CTCGGAAAA TCCTGTTC TTTACTTTT TATAAGTCTC AGGTTCAATT TTTTCGGATT
526      CAAATTTT TTTTAAATGG CAGCTGCTAT GAATTTGTAC ACTTGTAGCA GATCGTTTCA
586      AGACTCTGGT GGTGAECTCA TGGAC

```

Figure 2.12. Promoter sequence of Rap2.4 covering 1395 bp upstream of the transcription start site, 542 bp of 5' UTR and 69 bp encoding the first 23 codons of the open reading frame. W-box elements are highlighted in pink, MYB-elements in blue, light-regulation related elements in yellow, anaerobic stress-related elements in red, abiotic stress related elements in green. TAPOX1 elements are highlighted with green and TATA-box is highlighted in yellow. The transcribed region is underlined; the coding region underlined and highlighted in red.

element was found approximately 700 bp upstream of the transcription start site suggesting that Rap2.4b might be regulated by other DREB transcription factors.

Other possible upstream regulators of Rap2.4b expression are the WRKY transcription factors as there are several binding sites (W-box) present within the promoter sequence.

The Rap2.4b promoter contains a number of ACGT sequences that are required for etiolation-induced expression of *early-response-to-dehydration1* (*erd1*) suggesting that the Rap2.4b gene might react to developmental stimuli.

Regarding tissue specific elements, 3 TAPOX1 elements were identified indicating that Rap2.4b might be expressed in the roots. A partial RY-repeat was identified in a similar position (1.2 kb upstream of the transcription start site) in the Rap2.4b promoter as in the Rap2.4 promoter. The recognition sequence is conserved better in the Rap2.4b promoter and is therefore more likely to confer expression in seeds.

All promoter elements are depicted on the Rap2.4b promoter sequence in Figure 2.13 and a summary of the predicted *cis*-elements is given in Table 2.14.

2.3.2 Expression profiling of Rap2.4 and Rap2.4b

2.3.2.1 Expression of the GUS reporter gene driven by the Rap2.4 and Rap2.4b promoters

Cloning the constructs

For constructing the Rap2.4 full-length promoter fusion with the GUS reporter gene a fragment containing a 1254 bp promoter region, 542 bp 5'UTR and the first 23 codons of the Rap2.4 open reading frame was amplified by PCR using oligonucleotide primers R24p2F and R24p2R and wildtype *Arabidopsis* DNA as template. The primers R24p2F and R24p2R were designed with incorporated EcoRI and BamHI sites, respectively. An Accuprime PCR kit (Invitrogen) and the accompanying protocol was used for PCR. The 1865 bp PCR fragment was digested with the abovementioned restriction enzymes and cloned to the pFGC5941- based (Figure 2.15) p2CaMV-NG binary vector where it replaced a fragment containing a duplicated CaMV (*Cauliflower Mosaic Virus*) 35S promoter, translational enhancer from TEV (*Tobacco Etch Virus*) and NAN reporter gene. The resulting plasmid was called pFGC-Rap2.4promoter::GUS (Figure 2.16). The plasmid was verified by sequencing with the GUSRN primer.

In order to construct the plant transformation vectors where GUS is controlled by two different truncated versions of the Rap2.4 promoter, the R24p2R primer was used in combination with R24Pdel1F or R24Pdel2F forward primers, resulting in amplified PCR fragments of 711 bp and 1611 bp, respectively. The primers R24Pdel1F and R24Pdel2F both incorporated an EcoRI site and the cloning was carried out as described before for pFGC-Rap2.4promoter::GUS. The resulting binary vectors were called pFGC-Rap2.4promDel1::GUS and pFGC-Rap2.4promDel2::GUS (Figure 2.16).

The promoter driving GUS expression in the vector pFGC-Rap2.4promDel1::GUS contains a 100 bp region upstream of the transcription start site, 542 bp of 5'UTR and the coding

-1532 CATTTCCCCT GTGGACACGC TCGGCTTACG GTTATGAAAT CATGCGTTGG AGTTTTTCTT
ANAERO1c
-1472 GCTTTTCCCCT TTTCTCATGC GTAGAGTTT TTTTCTTCT TTACAATTG TTTAACCATA
MYBIAT MYBIAT1
-1412 GTTTTATAGG TTTGTAGCTA TATGAGTGGT TAATTTATGG TCTAGCTAAT TGGTCTTTTT
W-BOX CCAATBOX
-1352 CTTATTCAGT TTTTGCATTA AATGTAGATA TCAACTACTGA AAGTTGACTT TTATCAAAAA
MYB barley I-BOX
-1292 TACATAACAA AGTATAAGAA AAATAACGCT TAGACAAGTT ATTACACATA TGCAATGTTC
RY element I-BOX I-BOX
-1232 ACCAAAGCAA GCAAGTACGA AATATACATG CATATGATAA TACATGTAAT CGAGATAAAT
-1172 CAGCATCGTA AAGATGAACT GGTAACACGG ACACGAGCCA TCCACATGGG TGGTACCTCC
-1112 TTATGCGCGT CTTCCTCAA AATATGATC ATTATAAAAT GATGACACCA TAGTCTAACC
W-BOX
-1052 TTGCAAATG ACGCCGTATT GATTGTAGTG ACATTTGTAC ACGATTACAC GAAGCAATAA
ASF-1
-992 ATTAAGTGAT GCAACTCTAT AAGATATCGT AAGATGATGA AATGTTAAT AAACATAA
W-BOX
-932 AATGGATGAT ATAATACGGA ATTGACATTT GAGGAGTTCT CAGCCATTCA TTCTAACCCCT
-872 CTAGACCCCT CTTTTTAAAT CTTGTATGAT TTATTATCTA AATAAAAGTC TAAACCCTC
I-BOX
-812 ATTAACATGA TATCATCATT ACATTGCGGAG CCAAAATTAT TGCTTAACAA TCATGAAGTC
ABRELATERD1 LTRE-barley
-752 TACGTGTGTC ACATTGGGAA CCTCCGAATA ATTATTTTTA TACTTTTTTCG GTTTGATATA
I-BOX
-692 CTTCAATATA TACTATTAAT TTGGATAATC TCATTCTTA TACTCTCTCA GTTCCATTTT
ANAERO1c
-632 ATTGAAAAT TTCAGAAGTT TTGTTTTTCG ATATATACAA AAGAATTCTA AATCTACTAG
CCAATBOX MYBIAT
-572 CTGGACTAGT GGCCAATTG TTGGACGCT CTACCAAATA AATCGTAAAA ATATGTTGAT
I-BOX
-512 TTCAATTTA TCAATTTGGC TGGAATCGAA GTGCTCGTGT ACATTAGTTT CAACCGCTTA
ACGTerd1
-452 GAAAGACGTC TAGCTAAATG AGTAGACAAC CATTTTTTTA ACTAATCTTT TAAAATTTAT
ACGTerd1 BOX-1 BOX-1 MYBIAT1
-392 GGAGTTGTGT ACGTCCACTC ACCTCTATTT GAATAGAAAA AAGATTAATA TGGTTATGGA
ACGTerd1
-332 AGAAACGTCC CATAAAGCCG GTGTGAATGT AATATAATGA AATGATTCTC CATCAAACATA
BOX-1
-272 TGATTGTAAA ATTAAAACGC TTCGATTATA GAATAAACTT GTAGAAACAT TTTAACTTGT
ANAERO1c MYBIAT
-212 AAAAAATTAAC CATTCTAAAA CAAAACCTGT TAAAACCTAA CTTACAACAA ATGTTTGATT
MYBIAT ACGTerd1 I-BOX
-152 GAAATTGAAT ATATATTATT TGGAGTACGT AAACATGGAT GATCTTATCT GAAATAGTAC
MYB barley
-92 ATAAAAATAC AAAAGAAAAA ATTAATTAAA CATATAAATT TGAGAAATAT GAGGAGGATG
W-box barley
-32 GAAAAAAGAA GTCACGCCAC GCCTCCTATA TAAACAAGAG CCAAGCTAAT GAGATAAGTG
29 TATCGGTGAG GCTGAGAGTT ATTCACCTTAC AAAAAAAAAA AAAAACTTGA GTGTAACCAA
89 AAAAAAAGT TGATATACTT TCTGGTTTTT TCCTTAACCTT TTATTCTTTA CAAATCCATC
149 CCCCTTAGAT CTGTTTATTT CCCGCTACTT TGATTCATTT CTGTTAGTAA TCTGTCTTTC
209 GTATAGAAGA AACTGATTT CTTGGTTTGT ATTTTCTTAA AGAGATCAAT CTTTTTTTAT
269 TTTTGATCTT CTTGTGTTTT TTTTCTTTG TAGAATTAAT CGTTTGTGAG GGTATTTTTT
329 TAATTCCTC CTCTCAGAAA TCTACACAGA GGTTTTTTAT TTTATAAAC TCTTTTTTCA
389 TTTTCTTGA AACAAAAAAT CCTGTTCTTT ACTTTTTTTA CAAGAACAAG GGAAAAAAT
449 TTCTTTTTAT TAGAAATGACAACCTTCTATG GATTTTTTACA GTAACAAAAA GTTTC

Figure 2.13. Promoter sequence of Rap2.4b covering 1532 bp upstream of the transcription start site, 463 bp of 5' UTR and 63 bp encoding the first 21 codons of the ORF. W-box elements are highlighted in pink, MYB-elements in blue, light-regulation related elements in yellow, anaerobic stress-related elements in red, abiotic stress related elements in green. TAPOX1 elements are highlighted with green and TATA-box is highlighted in yellow. The transcribed region is underlined; the coding region underlined and highlighted in red.

List of promoter elements found in Rap2.4 and Rap2.4b promoters

Motif	Sequence	Copies in R24	Copies in Rap2.4b	Function
ABRELATERD1	ACGTG	1	1	ABRE-like sequence, required for etiolation induced expression of <i>erd1</i> .
ACGTerd1	ACGT	0	4	required for etiolation induced expression of <i>erd1</i>
ASF-1	TGACG	1	1	Involved in transcriptional activation of several genes by auxin and/or salicylic acid. Could be relevant to light regulation
BOX-1 (BOXICHS)	ATAGAA	2	3	Light regulation
I-BOX	GATAA	4	7	Light regulation in both monocots and dicots
ANAERO1 consensus	AAACAAA	4	3	Found in promoters of genes involved in the fermentative pathway
CCAAT box	CCAAT	3	2	Co-operates with HSE to increase promoter activity in heat shock genes
LTRE barley	CCGAAA	0	1	Cold-responsive element
MYB1AT	A/TAACCA	8	5	Binding sites for various MYB transcription factors involved in drought response
MYB barley	TAACAAA	2	2	
MYB26	GTTAGGTT	1	0	
MYB1LEPR	GTTAGTT	1	0	
W-box barley	TGACT	4	1	Binding site for WRKY proteins
W-BOX	TTGAC	3	3	
TAPOX1	ATATT	7	3	Expression in roots
RY element	CATGCA(TG)	1	1	Interaction with ABI-3 and FUS3

Table 2.14. Cis-acting elements found in the promoters of Rap2.4 and Rap2.4b. Motif refers to the name of the cis-element in the PLACE database (<http://www.dna.affrc.go.jp/PLACE/>, (Higo et al., 1999)).

pFGC5941 original
11405 bp

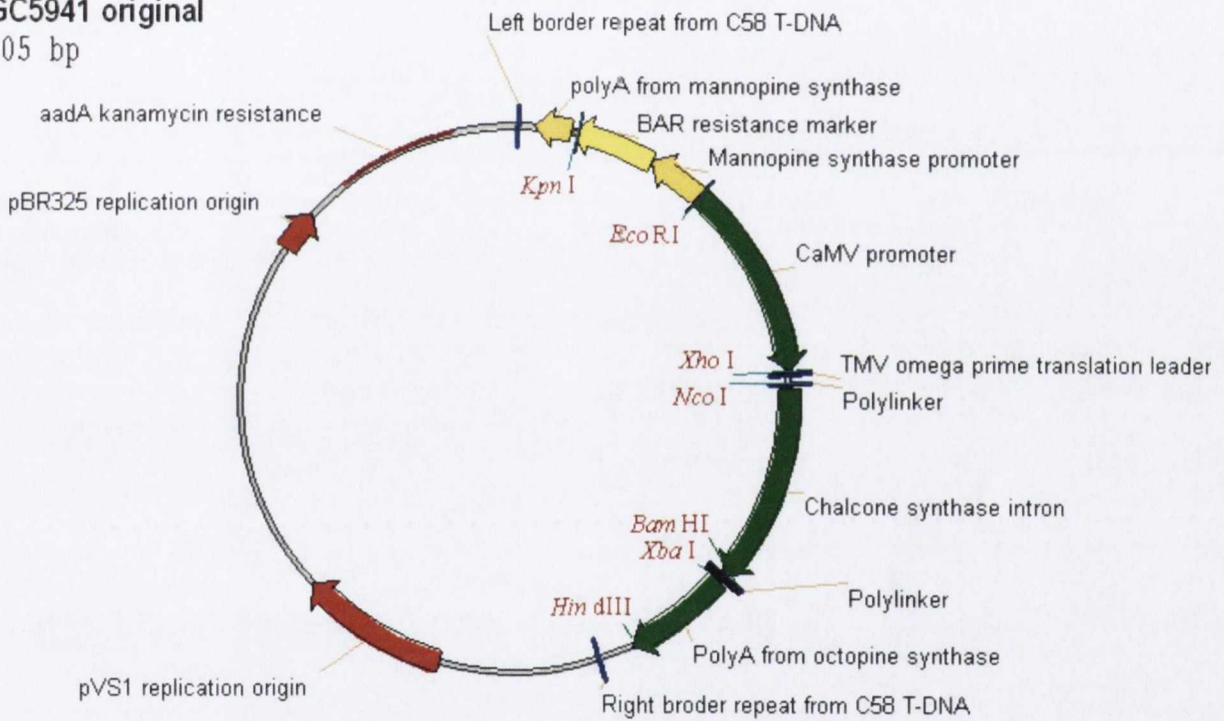


Figure 2.15. Schematic illustration of the pFGC5941 vector. Important features of the vector are marked with arrows, restriction sites are shown inside the vector diagram.

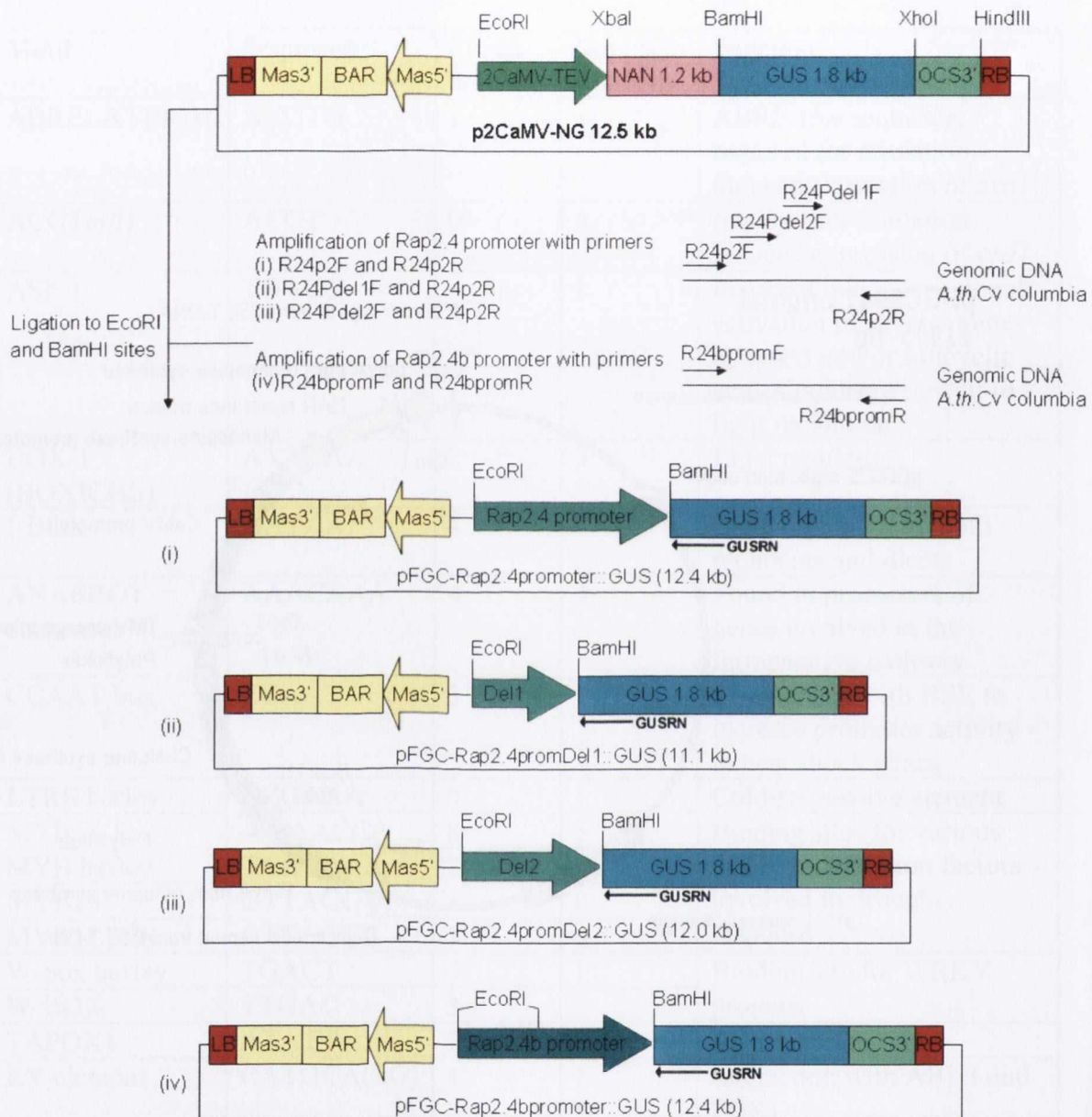


Figure 2.16. Schematic representation of the cloning procedure for construction of pFGC-Rap2.4promoter::GUS, pFGC-Rap2.4promDel1::GUS pFGC-Rap2.4promDel2::GUS and pFGC-Rap2.4b promoter::GUS binary vectors containing GUS reporter gene. The primers used for PCR amplifications and sequencing are indicated with black arrows.

sequence for the first 23 amino acids of the Rap2.4 protein. The promoter driving GUS expression in the pFGC-Rap2.4promDel2::GUS contains a 1000 bp promoter region, 542 bp of 5'UTR and the coding sequence for the first 23 amino acids of the Rap2.4 protein.

The Rap2.4b promoter was amplified using R24bpromF and R24bpromR primers and *Arabidopsis* genomic DNA as a template. The amplified fragment (2058 bp) contains a 1532 bp promoter region, 463 bp 5'UTR and 63 nucleotides encoding the first 21 amino acids of the Rap2.4b protein. The primers incorporated EcoRI and BamHI sites, respectively, that were employed for cloning the Rap2.4b promoter into the p2CaMV-NG as described above for Rap2.4 promoter constructs. The resulting plasmid was called pFGC-Rap2.4bpromoter::GUS.

The plasmids were verified by restriction analysis (Figure 2.17), transformed to *Agrobacterium tumefaciens* strain AGL-1 and used to transform *Arabidopsis thaliana* ecotype Columbia plants via "Floral dip" method (Clough and Bent, 1998).

Identification of GUS-positive transgenic plants at high density

BASTA-resistant seedlings were subjected to a quick GUS assay in order to verify the expression of transgene. For pFGC-Rap2.4promoter::GUS carrying transgenic plants 17 GUS expressing plants were identified. For pFGC-Rap2.4promDel1::GUS and pFGC-Rap2.4promDel2::GUS 18 and 14 independent lines were identified, respectively. For pFGC-Rap2.4bpromoter::GUS 20 independent lines were identified.

Histochemical analysis of Rap2.4promoter::GUS lines

Quick GUS assays verified the presence of GUS reporter gene in 17 individual plant lines transformed with pFGC-Rap2.4promoter::GUS.

In more detail, in the plant lines transformed with the full-length Rap2.4 promoter::GUS fusion, histochemical analysis showed GUS activity in the leaf vasculature and inflorescences (Figure 2.18). The GUS reporter gene was active in the styles and stigmas of carpels; anthers and filaments of stamens and in pollen grains (Figure 2.18, d to f). In siliques GUS was localized to abscission zones and stigmatic tissues (Figure 2.18, b).

Even though two of the pFGC-Rap2.4promoter::GUS transgenic lines displayed GUS staining in coats of a few seeds, no GUS activity was observed in embryos of any of the lines at either torpedo or mature stages (Figure 2.19, a and b). Upon radicle emergence, however, the GUS reporter gene was strongly induced and 3-4 days after sowing seedlings demonstrated high GUS activity levels in the roots (Figure 2.19, c to f).

These patterns of GUS activity suggest a strict spatial and temporal regulation of Rap2.4 expression in *Arabidopsis*. To further the investigation we constructed plasmids where the GUS reporter gene was placed under the control of truncated versions of the Rap2.4 promoter.

Histochemical analysis of Rap2.4b promoter::GUS lines

Histochemical analysis of the transgenic plants carrying the pFGC-Rap2.4bpromoter::GUS revealed expression patterns different from those observed in



Figure 2.17. Verification of the promoter::GUS fusion plasmids by restriction analysis. Plasmid DNA was isolated and digested with EcoRI and BamHI. Restriction digest gave a fragment of 1.86 kb for pFGC- Rap2.4promoter::GUS, 0.7 kb for the pFGC-Rap2.4promDel1::GUS and 1.6 kb for the pFGC-Rap2.4promDel2::GUS. For the pFGC-Rap2.4b promoter::GUS constructs two fragments of 0.95 and 1.1 kb, respectively, were observed. Molecular weight marker was loaded to lane marked with M.

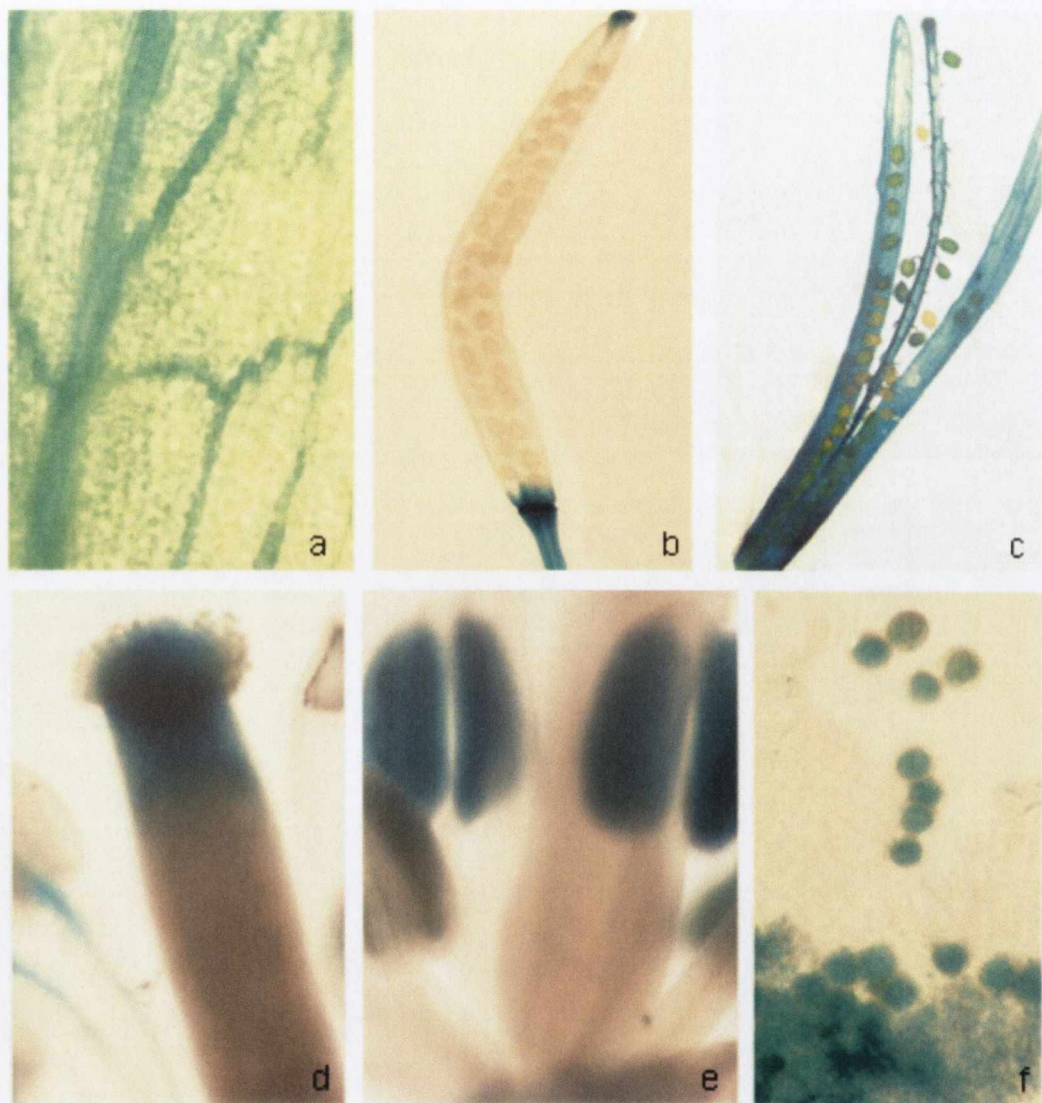


Figure 2.18. GUS expression patterns in mature transgenic plants containing the full-length Rap2.4 promoter::GUS construct.

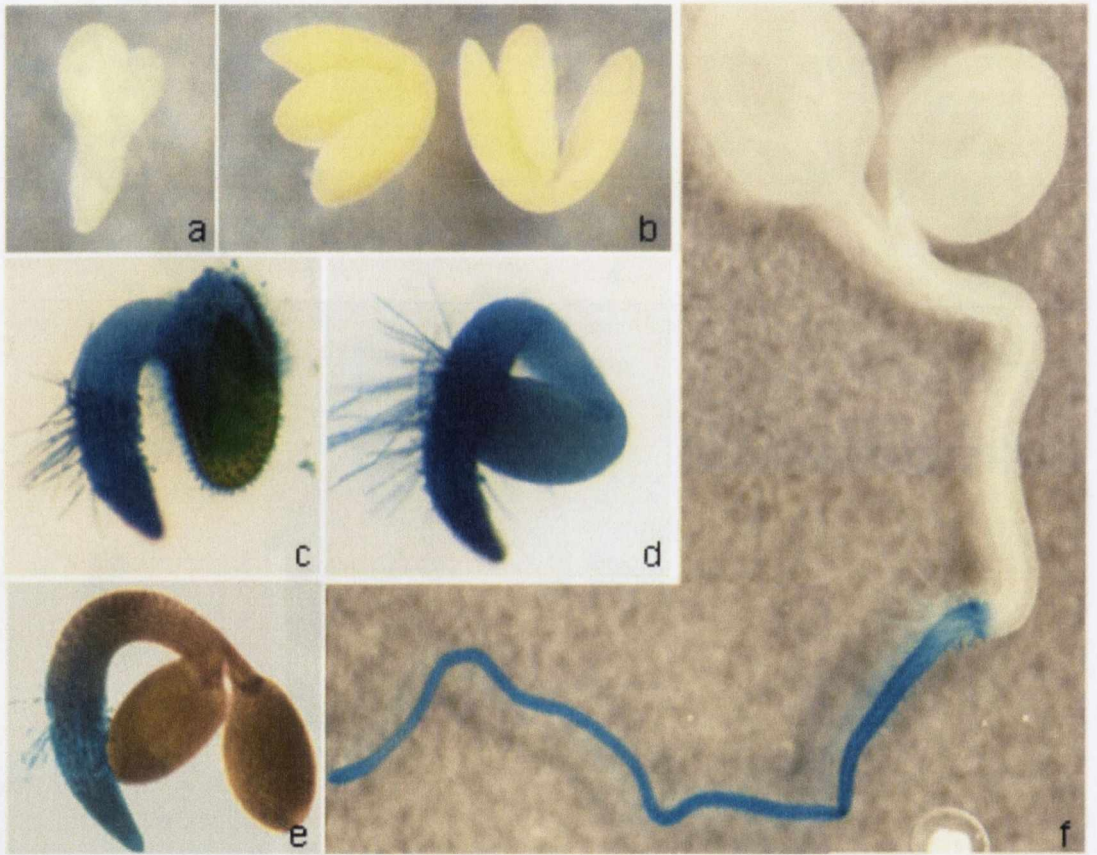


Figure 2.19. GUS expression patterns in seeds and seedlings of transgenic plants carrying the full-length Rap2.4 promoter::GUS construct.

Rap2.4bpromoter::GUS lines. The mature plants carrying Rap2.4bpromoter::GUS construct only showed GUS activity in the rosette leaves of 6 week-old plants (Figure 2.20).

Very strong GUS reporter gene expression was observed in dry seeds containing the Rap2.4bpromoter::GUS construct (Figure 2.21, a). When the staining period was reduced to 10 minutes, GUS expression was seen only at the micropylar end (Figure 2.21, b). However, in dry seeds the GUS staining was only observed in seed coats, the embryo itself did not stain in any of the Rap2.4bpromoter::GUS lines (Figure 2.21, c). During germination GUS activity disappeared from the seed coats. Upon rupture of the *testa*, GUS activity was observed in the emerging radicle (Figure 2.21, d to f). In young seedlings GUS reporter gene was active in the roots but not in any other parts of the seedlings (Figure 2.21, g to j).

Histochemical analysis of Rap2.4promDel1::GUS and Rap2.4promDel2::GUS lines

Quick GUS assays verified the expression of GUS reporter gene in 18 and 14 individual transformed plant lines identified for pFGC-Rap2.4promDel1::GUS and pFGC-Rap2.4promDel2::GUS, respectively.

Overall plants containing the pFGC-Rap2.4promDel1::GUS and pFGC-Rap2.4promDel2::GUS constructs displayed similar GUS patterns to transgenic plants containing Rap2.4 full-length promoter::GUS constructs. Truncating the promoter did not substantially affect the GUS reporter gene activity in the inflorescences, abscission zones of siliques or roots of mature plants; they stained strongly even when only 100 bp of the Rap2.4 promoter was driving reporter gene expression (Figure 2.22).

However the leaf veins did not stain in any of the lines carrying either of the truncated versions of Rap2.4 promoter. Seedlings carrying the truncated versions of Rap2.4 promoter displayed much less GUS activity – in only 40 % of lines carrying the pFGC-Rap2.4promDel2::GUS transgene was GUS staining evident.

The GUS activity in the progeny (T1) of primary transgenic plants (T0) showed GUS expression patterns consistent with those of the parent lines.

2.3.2.2 Rap2.4 and Rap2.4b expression profiling by Northern blotting

Rap2.4 and Rap2.4b expression in mature *Arabidopsis* organs, seeds and seedlings.

Organ-specific expression of Rap2.4 and Rap2.4b was studied in *Arabidopsis* ecotype Columbia. Total RNA for Northern analysis was extracted from flowers, green siliques, stems and leaves of mature plants grown in the greenhouse under normal conditions (20-24°C, 16 hours of light) and from roots of *in vitro* seedlings grown in the dark. Using Rap2.4 and Rap2.4b specific DIG-labelled probes, transcripts of 1.8 kb and 1.5 kb, corresponding to the wild type full-length mRNAs, respectively, were detected in almost all studied organs (Figure 2.23).

Rap2.4 mRNA was most abundant in root tissue. Rap2.4 expression of was also high in stems but very low in siliques, leaves and flowers (Figure 2.23, A). Rap2.4b on the other hand showed high expression in the stems of mature plants whereas only low levels of Rap2.4b mRNA

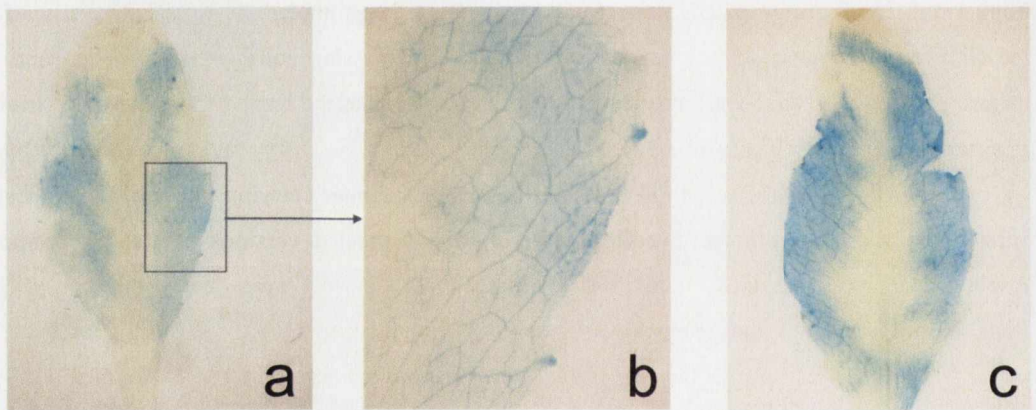


Figure 2.20. GUS expression patterns in mature transgenic plants containing the full-length Rap2.4 b promoter::GUS construct.

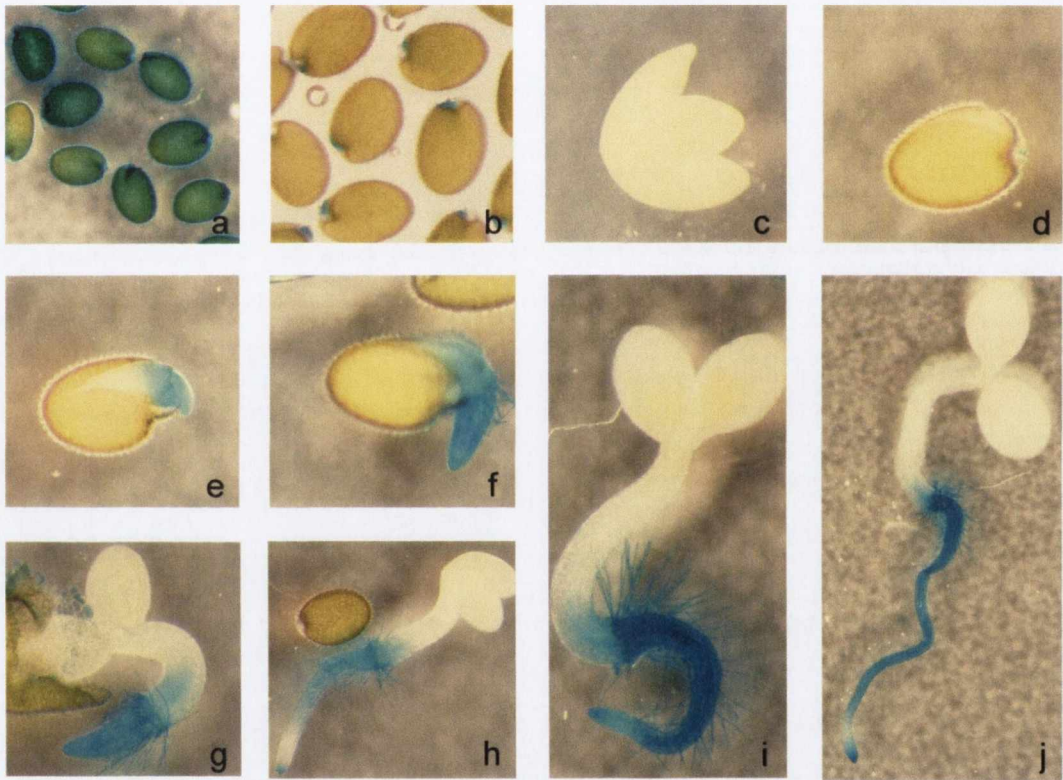
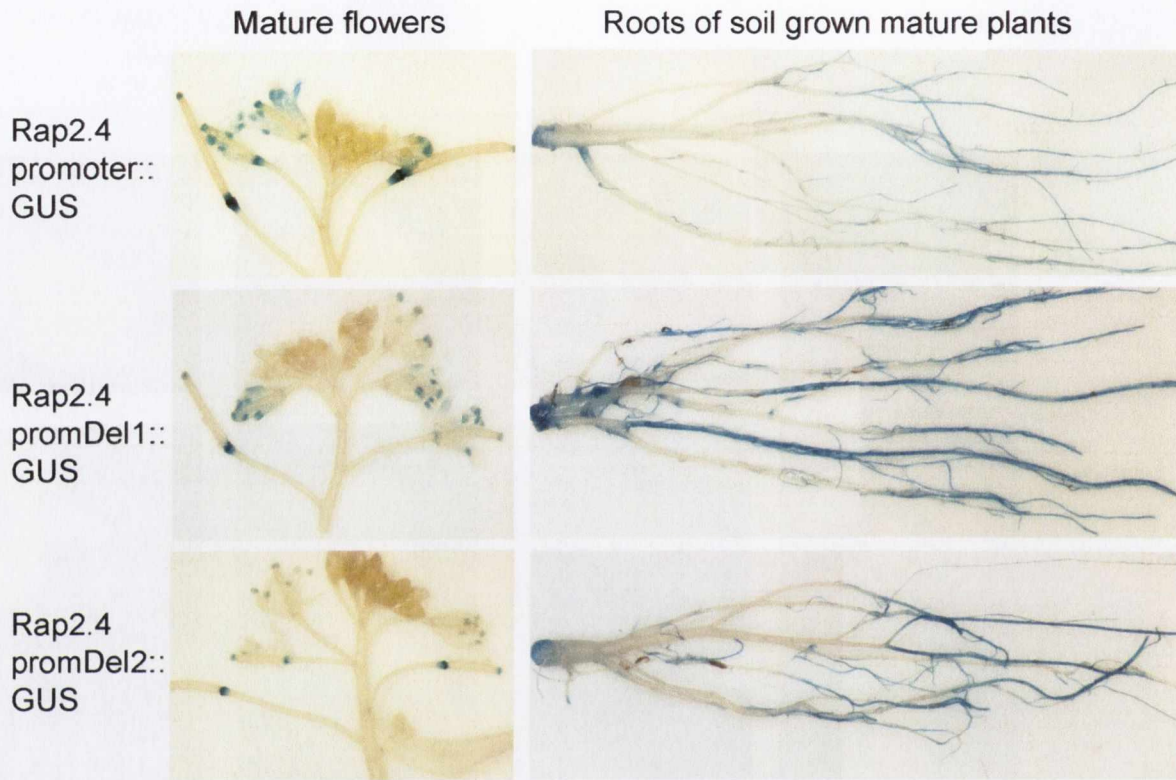


Figure 2.21. GUS expression patterns in seeds and seedlings of transgenic plants carrying the Rap2.4b promoter::GUS construct.

A



B

	Rap2.4 FL	Rap2.4 Del1	Rap2.4 Del2	Rap2.4b
Leaves	15	0	0	20
Stems	4	0	0	0
Inflorescences	17	12	10	2
Siliques	17	18	14	0
Roots	17	18	14	0
Seeds	2	0	0	20
Young seedlings	17	0	6	20
Total number of lines	17	18	14	20

Figure 2.22. Rap2.4 promoter deletions fused with GUS reporter gene. A) GUS expression patterns in inflorescences (left panel) and roots (panel on the right) of transgenic plants carrying pFGC-Rap2.4promoter::GUS (row 1), pFGC-Rap2.4promDel1::GUS (row 2), pFGC-Rap2.4promDel2::GUS and pFGC-Rap2.4b promoter::GUS (row 3) constructs. B) Overview of GUS expression patterns in the Rap2.4 and Rap2.4b promoter::GUS transgenic lines.

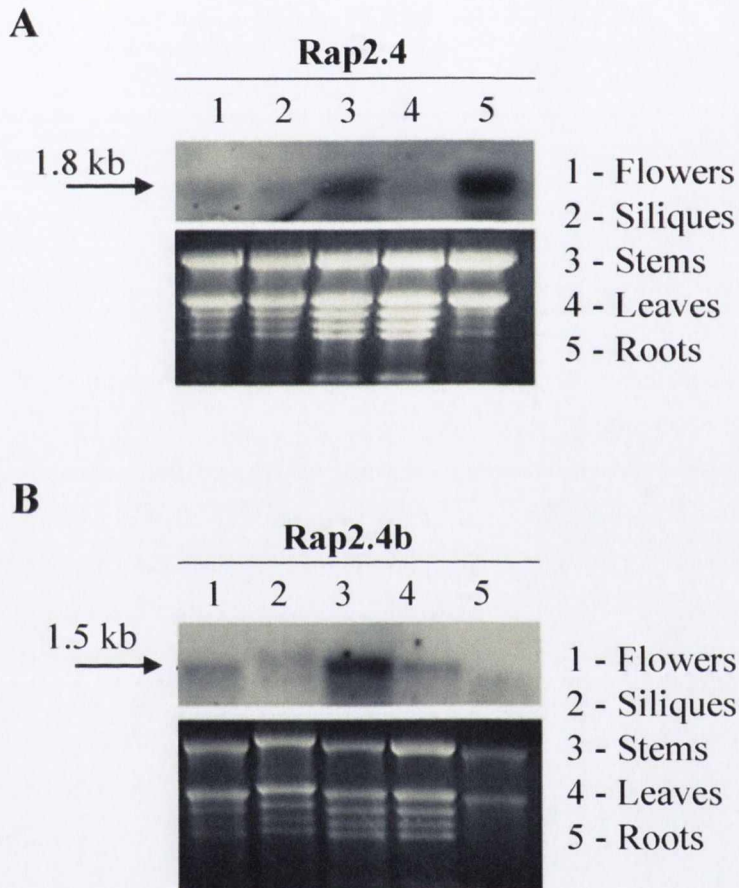


Figure 2.23. Organ-specific expression of Rap2.4 and Rap2.4b. Total RNA was isolated from various organs of mature *Arabidopsis* plants and analysed by RNA gel blot hybridization. RNA samples were loaded as follows: 1, flowers; 2, siliques; 3, stems; 4, leaves; 5, roots. A) Hybridization with Rap2.4 specific DIG-labelled probe detected a single band of 1.8 kb (top panel). Approximately 20 μ g of RNA was loaded into each lane. B) Hybridization with Rap2.4b specific DIG-labelled probe detected a single band of 1.5 kb (top panel), approximately 10 μ g of RNA was loaded into each lane. Bottom sections on both A and B show RNA loading by ethidium bromide staining.

were found in other organs such as flowers, siliques or leaves. No Rap2.4b-specific band was detected in root tissue but this could be due to unequal loading issues (Figure 2.23, B).

To examine the expression of Rap2.4 and Rap2.4b in early stages of development, RNA was extracted from dry *Arabidopsis* seeds, germinating seeds following 46 h of imbibition in water, and young *in vitro* grown seedlings.

Rap2.4 showed no expression in dry and imbibed seeds, but was induced upon germination reaching its highest levels 3 weeks after germination (Figure 2.24, A). Surprisingly Rap2.4b was expressed at extremely high levels in dry seeds and reduced to barely detectable levels upon imbibition (Figure 2.24, B, lanes 1 and 2, respectively). Expression of Rap2.4b was very low throughout early vegetative growth but increased slightly by the time seedlings were 3 weeks old (Figure 2.24, B, lanes 3 to 5).

The observed expression patterns suggest a diverged function for Rap2.4 and Rap2.4b. Even if the sets of target genes for Rap2.4 and Rap2.4b are overlapping, the two transcription factors are expressed in different tissues.

Rap2.4b expression in siliques and seeds of wild-type, *abi3-1* and *fusca3-8* *Arabidopsis* lines

Following the observation of low Rap2.4b mRNA levels in siliques and high expression in dry seeds, further experiments were undertaken in order to establish when and how the expression of Rap2.4b is induced during seed maturation.

RNA was extracted from *Arabidopsis* siliques at different desiccation stages and from dry and imbibed seeds. Rap2.4b expression levels were found to rise in parallel with increasing desiccation of siliques, reaching the highest levels in split siliques and dry seeds (Figure 2.25, A, lanes 1 to 4). Following imbibition in water, Rap2.4b mRNA levels were significantly reduced by 6 h and were undetectable after 24 h. We therefore looked at changes in Rap2.4b expression during the first three hours of imbibition. However, Rap2.4b mRNA levels decreased slowly during this time interval (Figure 2.25, B).

In order to explore the regulation of Rap2.4b expression in seeds, *abi3-1* and *fus3-8* mutant lines were used. RNA was extracted from siliques at different stages of maturation and from after-ripened seeds. Northern blotting revealed that in the wild-type control (*Arabidopsis* ecotype Landsberg) Rap2.4b was expressed at very low levels in green siliques and was induced in parallel with increasing desiccation of the siliques. *fus3-8* lines exhibited similar Rap2.4b expression patterns; however expression in green siliques of the mutant was enhanced compared to wild-type. In *abi3-1*, yellow siliques contained Rap2.4b mRNA in greater abundance than yellow siliques of wild-type or *fus3-8*. Also in open siliques of *abi3-1* Rap2.4b was expressed at higher levels than in similar tissue of other tested genotypes (Figure 2.25, panel C).

According to the results described above, Rap2.4b probably functions in establishment or maintenance of desiccation of *Arabidopsis* seeds but is not regulated by FUS3 or ABI3 transcription factors.

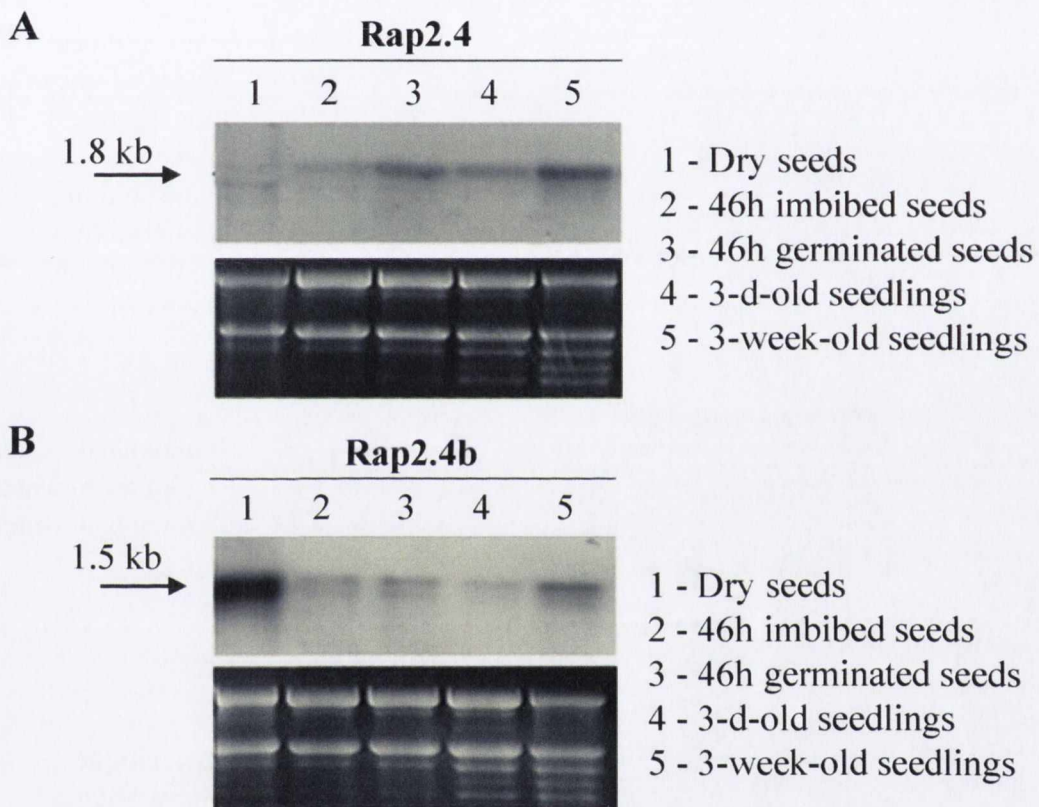


Figure 2.24. Expression of Rap2.4 and Rap2.4b in dry seeds and during germination. Total RNA was isolated from *Arabidopsis* seeds and seedlings and analysed by RNA gel blot hybridization. RNA from dry seeds was loaded to lanes marked 1, RNA from imbibed seeds to lanes marked 2, RNA from germinated seed to lanes marked 3, 3-day-old seedling RNA to lanes marked 4 and RNA from 3-week-old plants to lanes marked 5. A) Hybridization with Rap2.4 specific DIG-labelled probe detected a single band of 1.8 kb (top panel). B) Hybridization with Rap2.4b specific DIG-labelled probe detected a single band of 1.5 kb (top panel). Bottom sections on both A and B show RNA loading by ethidium bromide staining. Approximately 10 μ g of RNA was loaded into each lane.

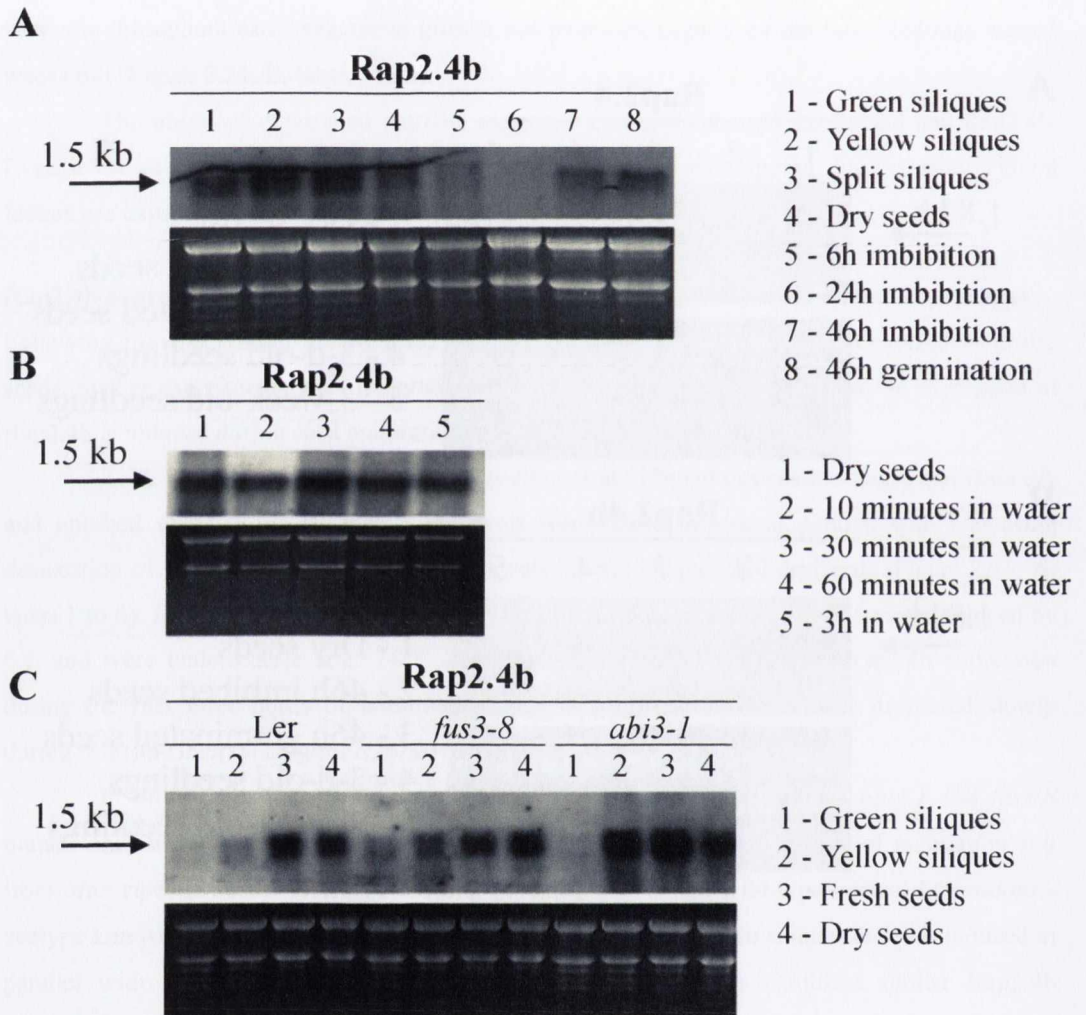


Figure 2.25. Expression of Rap2.4b in siliques, seeds and seedlings of WT, *abi3-1* and *fus3-8* lines. A) and B) 10 μ g of total mRNA from different tissues of WT was loaded onto gel according to the legends next to images. C) 10 μ g of total mRNA from different tissues of Landsberg WT, *fus3-8* and *abi3-1* were loaded onto gel according to the legends next to images. Hybridization with Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb on all blots. The bottom sections on A, B and C show RNA loading by ethidium bromide staining.

Rap2.4 and Rap2.4b expression in response to treatment with stress-related phytohormones.

The influence of different hormones on Rap2.4 and Rap2.4b transcription was assessed using 3 week-old whole *Arabidopsis* seedlings grown on MS medium. RNA was extracted 1 and 5 hours after treatments with 100 μ M abscisic acid (ABA), 5 μ M aminocyclopropane-1-carboxylic acid (ACC), 2 μ M salicylic acid (SA) or 5 μ M jasmonic (JA) acid. Plants used as control were sprayed with dH₂O.

Increased levels of Rap2.4 mRNA were observed in the sample collected 1 hour after ABA treatment but not in the sample collected 5 hours after ABA treatment. Following treatments with SA and the ethylene precursor ACC Rap2.4 transcripts were detected at the same levels as in control seedlings sprayed with water 1 h hour after treatment, but after 5 hours Rap2.4 mRNA levels displayed minor increases. In response to treatment with JA, higher levels of Rap2.4 mRNA were detected both 1 hour and 5 hours after treatment, compared to the sample from plants sprayed with dH₂O (Figure 2.26, A).

Interestingly Rap2.4b displayed a distinctively different induction pattern to Rap2.4. Rap2.4b expression was strongly induced by ABA treatment after 1 hour, but mRNA reduced to the levels similar to those observed in control plants after further 4 hours passed from the starting time of treatment. Spraying seedlings with the ethylene precursor ACC resulted in slightly increased Rap2.4b mRNA levels 1 hour following the treatment. As seen in the case of ABA application, 5 hours after spraying with an ACC solution, the levels of Rap2.4b mRNA were similar to the levels observed in control plants. Spraying seedlings with salicylic or jasmonic acid had no effect on Rap2.4b expression, the mRNA levels remained similar to those in control plants (Figure 2.26, B).

ABA is produced in plant tissues upon exposure to abiotic stresses and plays an important role in plant stress responses. Ethylene has many functions in plants, among them responses to biotic and abiotic stresses. Induction of Rap2.4 and Rap2.4b by ABA and ethylene precursor ACC could indicate that the two genes function in abiotic stress responses similarly to other DREB proteins.

Rap2.4 and Rap2.4b expression in response to treatment with development- and growth-related phytohormones and cycloheximide.

In addition to studying the effects of phytohormones generally associated with stress responses, the ability of growth- and development-related phytohormones to induce or repress Rap2.4 and Rap2.4b expression was investigated.

RNA was extracted from 3 week-old whole seedlings grown on MS plates 1 and 5 hours after treatments with 1 mM synthetic auxin (2, 4-D), 10 μ M protein synthesis inhibitor cycloheximide (CHX), 100 μ M gibberellic acid (GA) and 5 μ M synthetic cytokinin benzylaminopurine (BAP1). Plants used as controls were sprayed with dH₂O.

Application of gibberellic acid or cytokinin had no effect on the mRNA levels of either Rap2.4 or Rap2.4b (Figure 2.27, A and B).

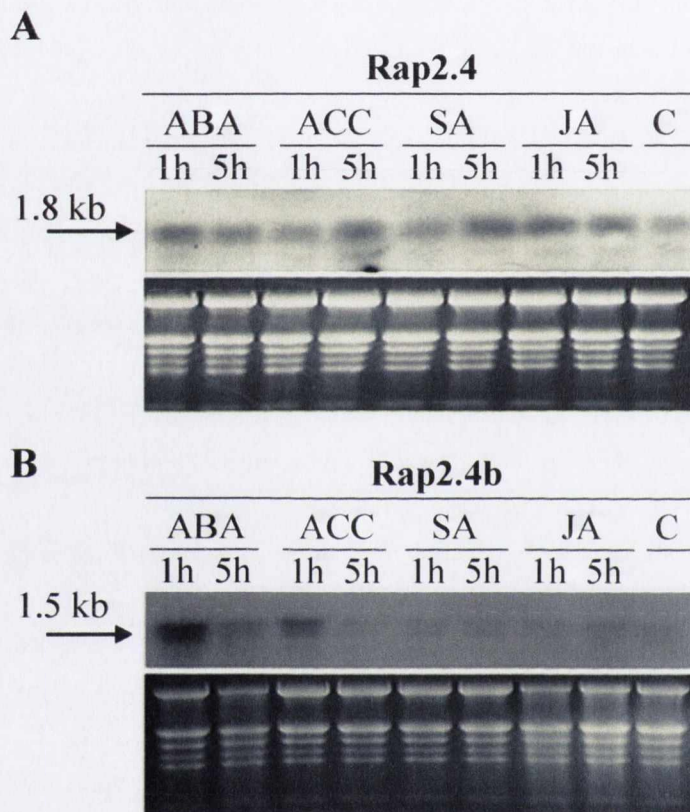


Figure 2.26. Expression of Rap2.4 and Rap2.4b following treatment with phytohormones. Total RNA was isolated from 3 weeks old seedlings treated with 100 μ M abscisic acid (ABA), 5 μ M aminocyclopropane-1-carboxylic acid (ACC), 2 μ M salicylic acid (SA) or 5 μ M jasmonic acid (JA) for 1 or 5 hours and analysed by RNA gel blot hybridization. RNA extracted from seedlings sprayed with dH₂O was loaded into lanes marked C. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 10 μ g of total RNA was loaded into each lane. Bottom sections on both images show RNA loading by ethidium bromide staining.

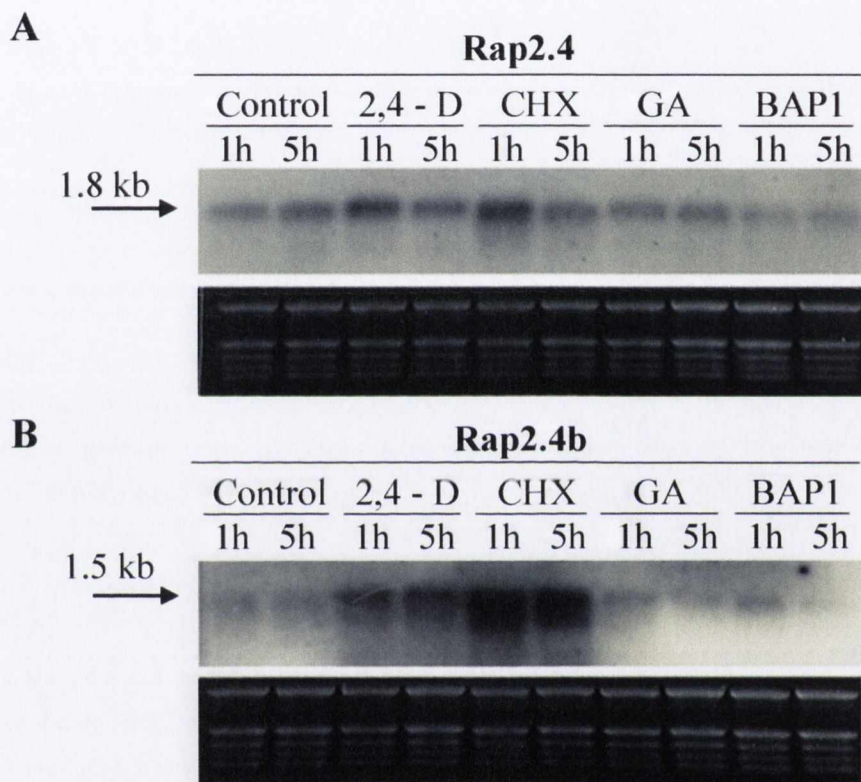


Figure 2.27. Expression of Rap2.4 and Rap2.4b following treatments with synthetic auxin (2,4-D), protein synthesis inhibitor cycloheximide (CHX), gibberellic acid (GA) and synthetic cytokinin benzylaminopurine (BAP1). Total RNA was isolated from 3 weeks old seedlings treated with 1 mM 2, 4-D, 10 μ M CHX, 100 μ M GA and 5 μ M BAP1 for 1 or 5 hours. Seedlings sprayed with dH₂O were used as control. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 8 μ g of total RNA was loaded into each lane. Bottom sections on both images show RNA loading by ethidium bromide staining.

In response to 2, 4-D application, Rap2.4 mRNA levels increased slightly 1 hour following the treatment but returned to levels observed in control plants after 4 hours. Rap2.4b expression was moderately increased at both 1 and 5 hour time-points after seedlings were sprayed with 2, 4-D (Figure 2.27, A).

Surprisingly, application of cycloheximide, which is a potent protein synthesis inhibitor, significantly increased Rap2.4b mRNA levels. The increase in Rap2.4b mRNA levels was observed at both 1 and 5 hours after application of this inhibitor. Rap2.4 mRNA levels were also increased following cycloheximide treatment but the extent of induction was smaller and the increase in Rap2.4 mRNA abundance was not sustained until the 5 hour time-point (Figure 2.27, B).

Given the high turn-over rates for the mRNAs of Rap2.4 and Rap2.4b described in literature, the observed induction by application of protein synthesis inhibitor CHX could indicate that the absence of normal *de novo* protein synthesis interferes with degradation of these transcripts.

Rap2.4 and Rap2.4b expression in response to abiotic stresses

The abiotic stress responsiveness of Rap2.4 and Rap2.4b was investigated by exposing 2 week-old *in vitro*-grown *Arabidopsis* seedlings to the following stress stimuli: drought, high salinity and cold. For the dehydration treatment seedlings were gently transferred from MS plates onto Whatman paper. The high salt environment was achieved by spraying seedlings on MS plates with 200 mM NaCl and for cold treatment MS plates with seedlings were placed in the 4° C cold room.

RNA was extracted from plant tissues following 1 and 6 hours of stress treatments. Untreated seedlings were used as controls.

Northern blotting with Rap2.4 and Rap2.4b specific DIG-labelled probes revealed that Rap2.4 mRNA was induced by dehydration and salt treatment whereas the Rap2.4b mRNA levels increased only following dehydration (Figure 2.28, A and B). The mRNA levels increased rapidly in response to drought with significant rises in transcript abundance observed only 1 hour after the onset of treatment for both transcription factors. By 6 hours following the onset of dehydration treatment the levels of Rap2.4 and Rap2.4b mRNA had decreased compared to levels at the 1 hour time-point, but still remained higher than those observed in untreated plants.

Spraying seedlings with 200 mM NaCl induced the expression of Rap2.4 but not Rap2.4b. Increased levels of Rap2.4 mRNA were observed at both 1 and 6 hour time points (Figure 2.28, A and B).

The effects of cold treatment on the expression of Rap2.4 and Rap2.4b were minor but showed interesting patterns. After 1 hour at 4° C the mRNA levels of both transcription factors were slightly elevated compared to those observed in untreated plants. At 6 hours, however, the levels of mRNA appeared reduced relative to untreated samples for both Rap2.4 and Rap2.4b (Figure 2.28, A and B).

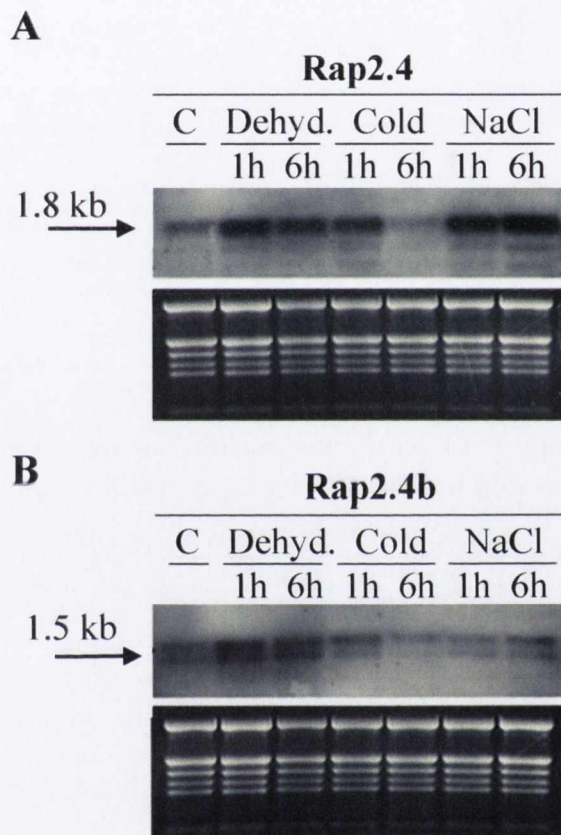


Figure 2.28. Expression of Rap2.4 and Rap2.4b following abiotic stress treatments. Total RNA was isolated from 2 week-old following dehydration, NaCl, ABA and cold treatments and analysed by RNA gel blot hybridization. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 10 μ g of total RNA was loaded into each lane. Bottom sections on both images show RNA loading by ethidium bromide staining.

Further abiotic treatments were carried out in order to determine whether the drought and salt responses observed earlier reflect the common aspects of these stresses such as hyper-osmolarity, or whether the increases in mRNA levels were specific to each stress. Also the effect of high temperature was investigated.

As an inducer of osmotic stress 300 mM mannitol solution was applied by spraying the seedlings. 100 mM KCl and 10 mM LiCl were used as a replacement for NaCl in order to determine whether the observed mRNA increase in response to 200 mM NaCl was due to toxicity of sodium ions rather than osmotic aspects of salt stress. Heat stress treatment was carried out by placing the seedlings on MS plates at 38° C. Untreated seedlings were used as controls and RNA samples were extracted 1 hour and 5 hours after the start of treatments (Figure 2.29, A and B).

Heat stress had no effect on Rap2.4 expression but rapidly induced the expression of Rap2.4b. Rap2.4 was moderately induced by mannitol treatment at both 1 and 5 h; Rap2.4b displayed a strong increase at 1 h. Spraying seedlings with LiCl and KCl solutions induced the expression of Rap2.4 but had no effect on the mRNA levels of Rap2.4b (Figure 2.29, A and B).

In summary, Rap2.4 responded to dehydration, high salinity, cold and application of mannitol, LiCl, KCl. Rap2.4b mRNA levels were altered in response to dehydration, cold, heat and mannitol application. Considering observed expression patterns Rap2.4 and Rap2.4b are probably involved in abiotic stress responses and have at least some non-redundant functions.

Time-course analysis of Rap2.4 and Rap2.4b expression during drought stress

Rap2.4 and Rap2.4b were both induced by drought stress. In order to gain further insight into the dehydration response mechanisms possibly mediated by these transcription factors, a time-course analysis of Rap2.4 and Rap2.4b expression following the onset of dehydration was conducted.

Drought stress treatment was carried out as described earlier by gently moving 2-3 week old *in vitro* grown seedlings onto Whatman paper disks. RNA was extracted at 1, 2, 3, 4 and 6 hours after the onset of dehydration treatment.

Hybridization with DIG-labelled probes revealed that Rap2.4 and Rap2.4b were both induced at maximum levels 1 hour into the drought treatment. However, the expression kinetics were slightly different for the two genes at the later time points. Two hours after the onset of dehydration Rap2.4b mRNA levels were considerably decreased compared to maximum induction but the expression of the gene remained noticeably higher than in un-treated seedlings until at least 6 hours into drought conditions. Rap2.4 mRNA on the other hand decreased only a little by the two hour time-point, remained elevated through the third hour of drought treatment but returned to levels similar to those observed in untreated seedlings by the 4th hour of dehydration (Figure 2.30, A and B).

Regulatory proteins such as transcription factors are normally induced early during the course of dehydration (Yamaguchi-Shinozaki and Shinozaki, 2006) and the induction patterns observed here for Rap2.4 and Rap2.4b are similar those described in literature for other transcription factors.

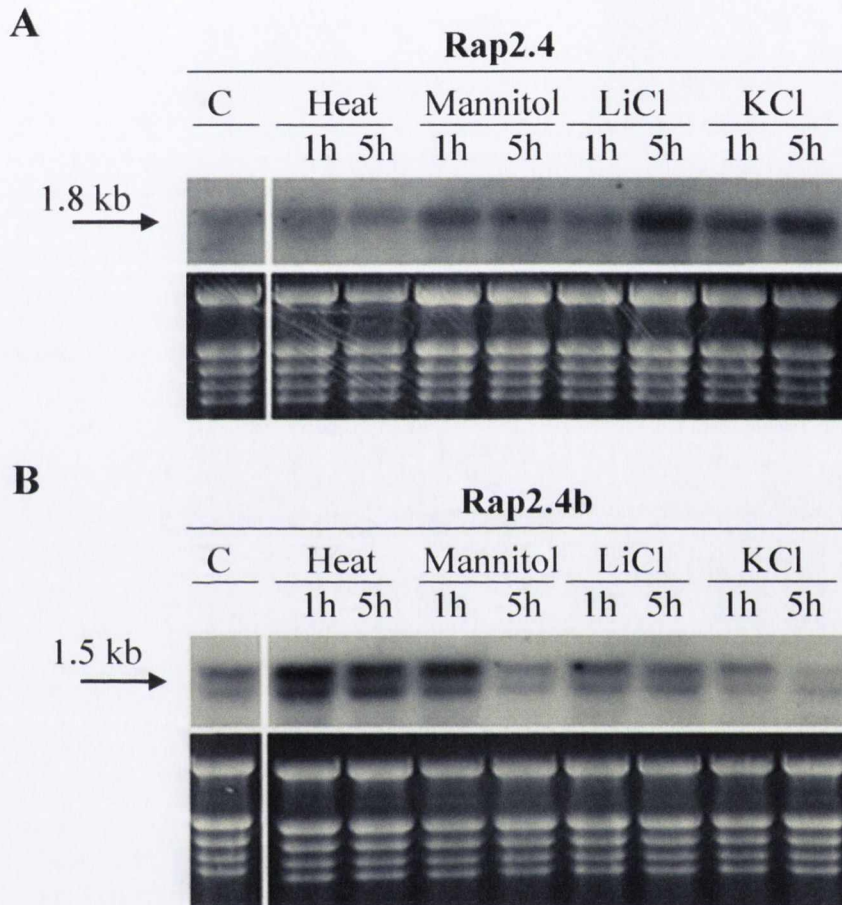


Figure 2.29. Expression of Rap2.4 and Rap2.4b following abiotic stress treatments. Total RNA was isolated from 3 weeks old seedlings following heat stress at 38° C, treatment with 300 mM mannitol, 10 mM LiCl, 100 mM KCl for 1 or 5 hours and analysed by RNA gel blot hybridization. RNA from untreated seedlings was loaded into lanes marked C. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 10 µg of total RNA was loaded into each lane. Bottom sections on both images show RNA loading by ethidium bromide staining.

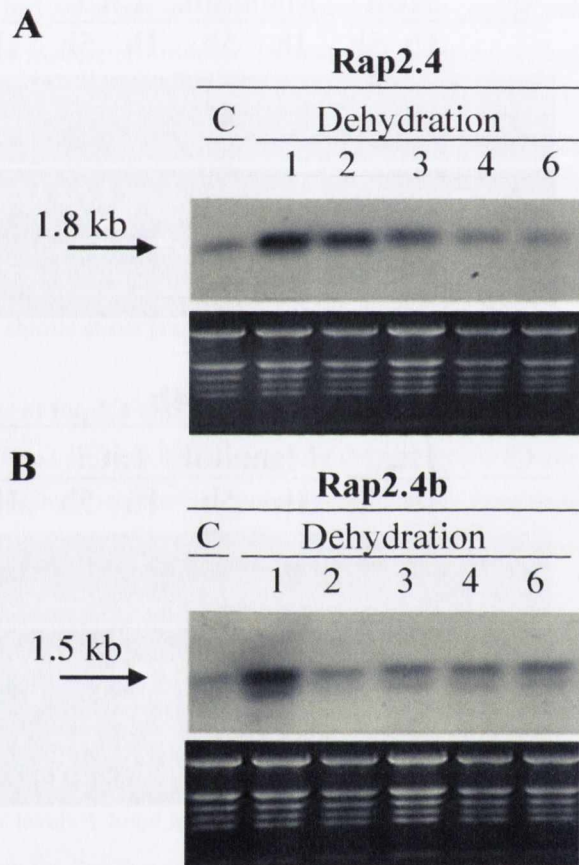


Figure 2.30. Time course analysis of Rap2.4 and Rap2.4b mRNA levels during drought treatment. Total RNA was isolated from 3 weeks old seedlings exposed to dehydration for 1, 2, 3, 4 or 6 hours and analysed by RNA gel blot hybridization. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 10 μ g of total RNA was loaded into each lane. Bottom sections on both images show RNA loading by ethidium bromide staining.

Time-course analysis of Rap2.4 and Rap2.4b expression in cold stress

Rap2.4 and Rap2.4b were both induced by cold treatment. Initial experiments had shown both transcription factors to be slightly induced at 1 hour and interestingly, reduced to levels lower than those observed in untreated plants following 6 hours of cold treatment.

A time-course analysis of Rap2.4 and Rap2.4b expression under cold conditions was conducted by placing MS plates with 2 weeks old seedlings at 4°C for up to 24 hours under continuous light. RNA was extracted at 1, 2, 4, 6 and 24 hours after the onset of cold treatment and analysed by gel blot hybridization.

Consistent with earlier experiments, Rap2.4 and Rap2.4b expression was slightly increased at 1 and 2 hour time-points. In the later time-points of cold treatment Rap2.4 and Rap2.4b mRNA accumulated to levels similar to unstressed conditions with the exception of Rap2.4 mRNA being almost undetectable at the 6 hour time-point (Figure 2.31, A and B).

Rap2.4 expression in *hos1* and *ice1* mutant lines under cold stress

In order to further investigate Rap2.4 expression under cold stress, *hos1* and *ice1* mutant seedlings were utilized. *HOS1* negatively regulates cold responses (Ishitani et al., 1998), while *ICE1* (*Inducer of CBF Expression 1*) is a well-characterized positive regulator of cold-responses (Chinnusamy et al., 2003). The cold stress experiments were carried out as described earlier, RNA was extracted and subjected to gel blot analysis with a Rap2.4 specific DIG-labelled probe.

In *ice1* lines the Rap2.4 cold-response was not changed compared to control plants indicating that ICE1 is probably not a regulator of Rap2.4 in cold conditions (Figure 2.32, panel A). Relative to wild-type, Rap2.4 mRNA levels were estimated to be 10-fold higher in *hos1* seedlings under non-stressed conditions (Figure 2.32, panel B, lane marked with C). Rap2.4 mRNA levels declined from the initial high values observed under no stress and at 1 hour of cold treatment when cold treatment was prolonged in both *hos1* and wild-type.

The higher accumulation of Rap2.4 mRNA in *hos1* seedlings suggests that HOS1 may be a repressor of Rap2.4 both in normal and cold conditions. The unchanged levels of Rap2.4 mRNA in *ice1* seedlings indicate that ICE1 is probably not a regulator of Rap2.4.

Rap2.4 and Rap2.4b expression in ABA-deficient plant lines in abiotic stress conditions

The experiments addressing the abiotic stress- and hormone-responsiveness of Rap2.4 and Rap2.4b expression revealed the two transcription factors to be differentially induced. The phytohormone ABA is an important regulator of abiotic stress responses to drought and hypo-osmolarity. These responses are often divided into two broad categories: ABA-dependent and ABA-independent. In an attempt to place the transcription factors under investigation into stress response pathways described in literature, experiments utilising mutant lines were conducted.

We used the ABA-deficient lines *aba1-5*, *aba2-4* and *aba3-1* in order to establish the role of endogenous ABA in Rap2.4 and Rap2.4b expression. The three lines carry mutations in genes coding for enzymes required for ABA biosynthesis.

Abiotic stress treatments were carried out as described earlier and RNA was extracted 3 hours after the onset of stress. Northern blotting revealed that following dehydration Rap2.4

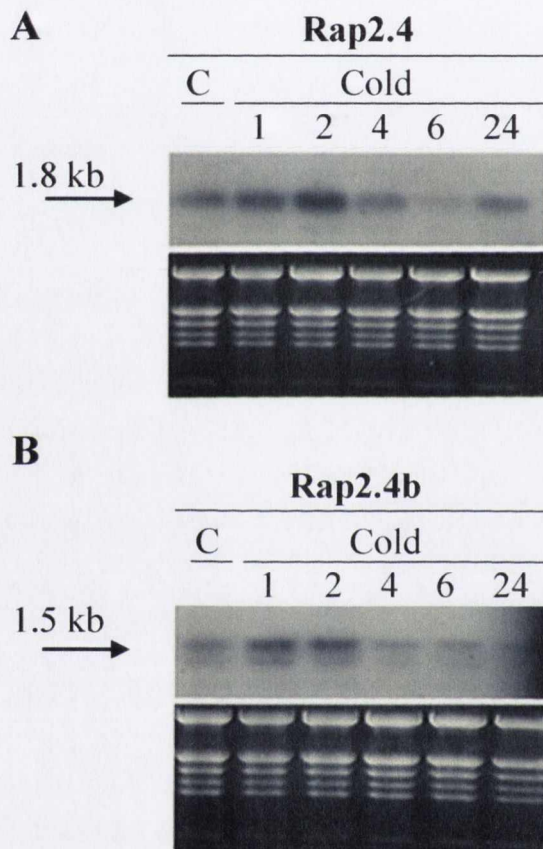


Figure 2.31. Time course analysis of Rap2.4 and Rap2.4b mRNA levels during cold treatment. Total RNA was isolated from 3 week old seedlings exposed to 4°C for 1, 2, 4, 6 or 24 hours and analysed by RNA gel blot hybridization. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 10 µg of total RNA was loaded into each lane. Bottom sections on both images show RNA loading by ethidium bromide staining.

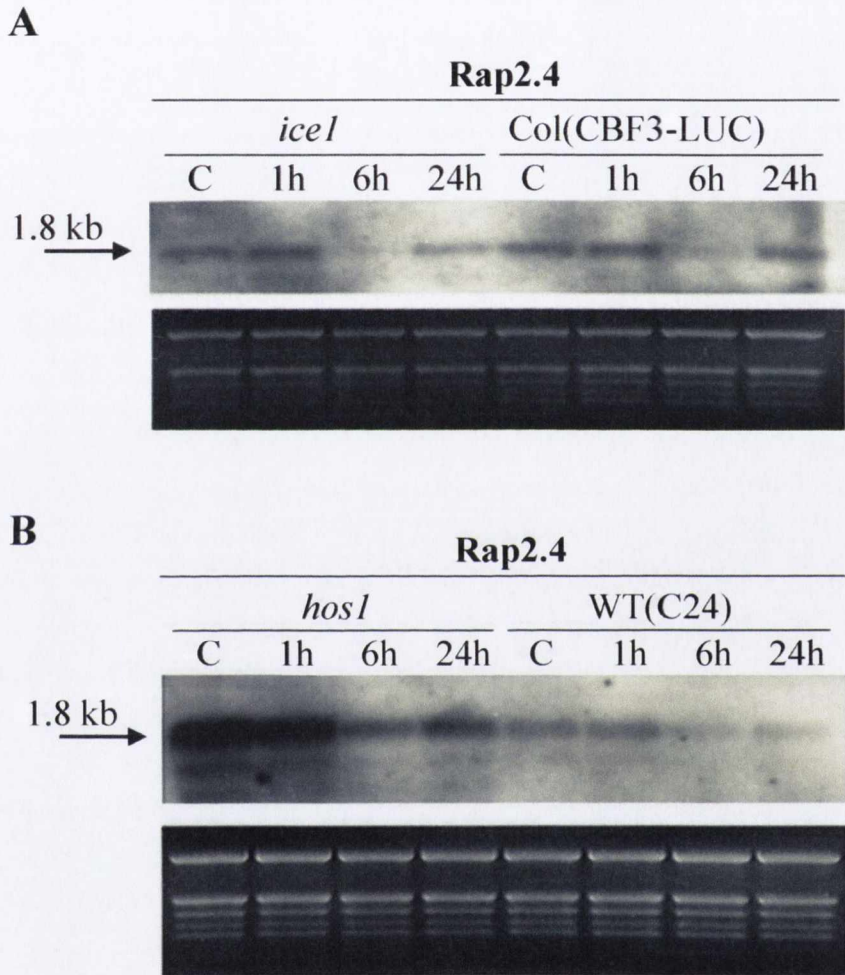


Figure 2.32. Time course analysis of Rap2.4 mRNA levels during cold treatment in *ice1* (A) and *hos1-1* (B) mutant lines. Total RNA was isolated from 3-week-old seedlings exposed to 4°C for 1, 6 or 24 hours and analysed by RNA gel blot hybridization. Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). Approximately 5 µg of total RNA was loaded into each lane on A and 10 µg of total RNA was loaded into each lane on B. Bottom sections on both images show RNA loading by ethidium bromide staining.

mRNA accumulated to significantly higher levels in all tested ABA deficient lines than in wild-type seedlings. However, Rap2.4 mRNA levels did not increase substantially in ABA deficient lines in response to salt stress. Interestingly Rap2.4 mRNA levels were higher in unstressed *aba1-5* seedlings compared to unstressed wild-type seedlings (Figure 2.33, A).

Rap2.4b induction in ABA deficient seedlings in response to abiotic stresses did not differ significantly from the response observed in wild-type seedlings with the exception of *aba1-5* mutant where Rap2.4b accumulation was enhanced compared to WT after drought treatment. In unstressed *aba2-4* and *aba3-1* seedlings the levels of Rap2.4b mRNA were higher than in unstressed wild-type. During dehydration Rap2.4b mRNA accumulated in *aba2-4* and *aba3-1* seedlings to levels similar to stressed wild-type but as the starting levels were higher the increase was not as marked (Figure 2.33, B).

The effect of a much higher concentration of NaCl was also tested in wild-type seedlings. Surprisingly when seedlings were sprayed with 1 M NaCl, the increases in both Rap2.4 and Rap2.4b mRNA levels were striking. In response to 1 M NaCl, both transcription factors were induced to higher levels than by any other abiotic stress treatment (Figure 2.33, A and B).

In summary, during dehydration mutant lines deficient in ABA biosynthesis accumulated Rap2.4 to higher levels than wild-type plants and Rap2.4b to levels mostly similar to wild-type plants. These results indicate that ABA is not required for the drought responsiveness of either Rap2.4 or Rap2.4b.

Rap2.4 and Rap2.4b expression in *fiery1* lines in abiotic stress conditions

The *Fiery1* (*Fry1/Hos2/SAL1*) gene encodes an inositol polyphosphate 1-phosphatase which acts in the degradation pathway of an important secondary messenger inositol 1,4,5-trisphosphate (IP₃). *Fry1/hos2* mutants display higher and more sustained levels of IP₃ under abiotic stress conditions, and enhanced expression of several stress-responsive genes (Wilson et al., 2009; Xiong et al., 2001c; Xiong et al., 2002b).

Abiotic stress treatments were carried out on *fry1* mutants and WT as described earlier and RNA was extracted 4 hours after the onset of stress.

Northern blotting revealed that Rap2.4 expression was induced to higher levels in *fry1* seedlings than in wild-type *Arabidopsis* seedlings in response to ABA as well as to all abiotic stresses tested. In unstressed plants Rap2.4 mRNA also accumulated to higher levels in *fry1* seedlings than in wild-type. Interestingly, cold treatment, which in wild-type seedlings down-regulated Rap2.4 had the opposite effect on Rap2.4 in *fry1* seedlings (Figure 2.34, A).

Rap2.4b expression on the other hand was not influenced by the *fry1* mutation in either unstressed plants or any of the tested stress conditions (Figure 2.34, B).

FRY1 is a general negative regulator of abiotic stress responses which may suppress Rap2.4 expression in stress conditions but has probably no effect on Rap2.4b expression.

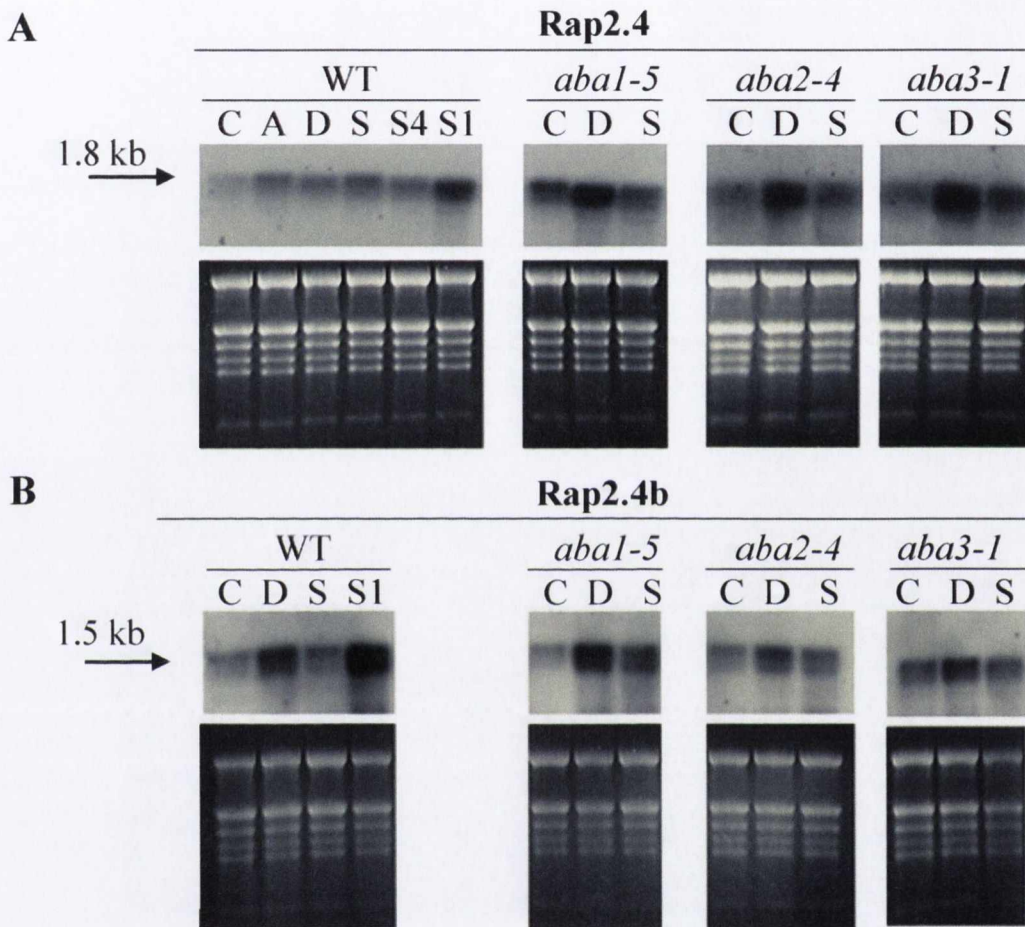


Figure 2.33. Expression of Rap2.4 and Rap2.4b under abiotic stress conditions in Columbia WT, *aba1-5*, *aba2-4*, and *aba3-1* mutant lines. Total RNA was isolated from 3-week-old seedlings and analysed by RNA gel blot hybridization. RNA was extracted from untreated seedlings (lanes marked C), drought-treated seedlings (lanes marked D), ABA-treated seedlings (lane marked A1) and seedlings treated with 0.2 M, 0.4 M and 1 M NaCl (lanes marked S, S4 and S1, respectively). Different genotypes are marked above the images. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 10 µg of total RNA was loaded into each lane Bottom sections on both images show RNA loading by ethidium bromide staining.

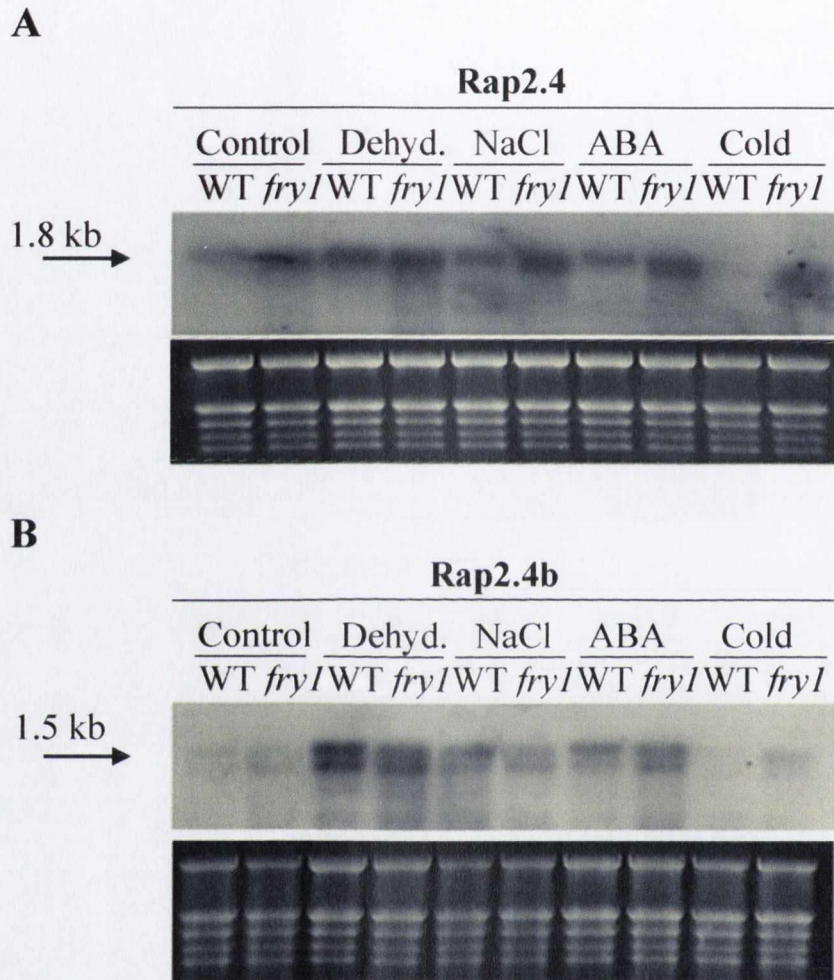


Figure 2.34. Expression of Rap2.4 and Rap2.4b following abiotic stress treatments in *fry1* and Columbia WT seedlings. Total RNA was isolated from 3-week-old seedlings following dehydration, NaCl, ABA and cold treatments; and analysed by RNA gel blot hybridization. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb (top section). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb (top section). Approximately 10 μ g of total RNA was loaded into each lane. Bottom sections of both images show RNA loading by ethidium bromide staining.

2.4 Discussion

2.4.1 Bioinformatic analysis of Rap2.4 and Rap.4b

The Rap2.4 and Rap2.4b proteins are AP2 DNA binding domain-containing transcription factors of 334 and 261 amino acids, encoded by mRNAs of 1825 and 1507 basepairs, respectively. The Rap2.4 and Rap2.4b mRNAs are expressed in relative abundance (Figure 2.10) despite having extremely short half-lives, according to reports in the literature. In a genome-wide analysis of mRNA decay rates conducted by Narsai et al. (2007), Rap2.4 was found to have a half-life of 0.2 hours and Rap2.4b of 0.3 hours. Measuring mRNA decay rates by treating samples with an inhibitor of transcription actinomycin D, the average mRNA half-life in *Arabidopsis* was found to be 5.9 hours (mean 5.9 hours and median 3.8 hours). An earlier study by Gutierrez et al. in 2002 also identified Rap2.4 as one of the 50 fastest decaying transcripts in the *Arabidopsis* transcriptome. The regulation of mRNA stability is an important factor regulating gene expression, and a short mRNA half-life may indicate that the gene may be involved in a process that requires a rapid transcriptional response. Thus, mRNA instability is thought to have particular importance in regulating processes associated with the circadian clock and stress responses (Gutierrez et al., 2002). Rap2.4 and Rap2.4b are intron-less genes, which might contribute towards fast turnaround of their transcripts, as introns have been demonstrated to confer stability to plant mRNAs (Narsai et al., 2007).

We found that Rap2.4 and especially Rap2.4b mRNA accumulated to high levels upon cycloheximide (CHX) treatment. CHX is a potent inhibitor of *de novo* protein synthesis and has been demonstrated to cause sustained superinduction of several plant hormone-responsive genes including the AP2/ERF genes ERF1-4 (Abel et al., 1995; Koshiha et al., 1995; Suzuki et al., 1998). The Rap2.4 and Rap2.4b response to CHX treatment could be due to either increased synthesis i.e. removal of a suppressor of transcription or decreased degradation. Considering the relative abundance of Rap2.4 and Rap2.4b mRNA even under non-inducing conditions and the quick turnover of transcripts in the plant cell, it is feasible that CHX treatment results in depletion of a pool of proteins that would normally degrade these transcripts. CHX treatment is known to interfere with RNA degradation by “freezing” the ribosomes on the mRNA and thereby halting the supply of substrate for certain RNA degradation pathways (Belostotsky and Sieburth, 2009).

Furthermore, in addition to the regulation of Rap2.4 and Rap2.4b at the transcriptional level, both mRNAs carry features that may indicate regulation at the level of translation. The 5'UTRs of both Rap2.4 and Rap2.4b mRNA are much longer than the average 130 bp in *Arabidopsis*, 542 bp and 463 bp, respectively, and the presence of longer than average 5' UTRs has been associated with poor ribosome loading (Kawaguchi and Bailey-Serres, 2005). The upstream un-translated region of Rap2.4 contains a short open reading frame (uORF), which encodes a phenylalanine-rich peptide of 27 amino acids. The uORFs are known to negatively regulate gene expression by impairing the ribosome loading of mRNAs (Kawaguchi and Bailey-Serres, 2005). Removal of a 405 bp region from the 5'UTR of *ABI3* mRNA containing three uORFs significantly enhanced the expression of a downstream GUS reporter gene (Ng et al.,

2004). Translation of the *Arabidopsis* bZIP11 transcription factor in response to high sucrose levels is controlled by a short peptide encoded by a uORF in the 5' leader sequence. The short peptide of 28 residues acts by stalling the ribosome on the mRNA when high concentrations of sucrose are present (Rahmani et al., 2009). Determining whether the Rap2.4 uORF has a role in regulating translation would require assessment of Rap2.4 expression at the protein level.

Regarding the Rap2.4 and Rap2.4b proteins, both contain several putative phosphorylation sites indicating that post-translational modifications might be important for protein activity. Rap2.4 and Rap2.4b contain identical AP2 domains of 58 residues and as expected of transcription factors, have putative nuclear localization signals in the N-termini of the AP2 domains. Both transcription factors feature serine-rich regions which could potentially act as transactivation domains. It is worth mentioning that Rap2.4 and Rap2.4b contain regions rich in glutamic acid, serine, threonine and proline which are recognised as PEST regions by the PESTfind tool. The scores of the PEST regions of Rap2.4 and Rap2.4b suggest that the proteins, and not only the corresponding mRNAs, might have a fast turnaround in plant cells (Rogers et al., 1986).

Phylogeny and duplication among DREB transcription factors

Based on the TIGR listing of genomic duplications (http://www.tigr.org/tdb/e2k1/ath1/duplication_listing.html) we found a large number of DREBs to have been generated by large-scale genomic duplications. Of the 56 DREBs 34 comprised 17 defined duplicated pairs, and a further 16 DREBs were located within large duplicated chromosomal segments but lacked a copy annotated as a DREB gene in the respective partner region. Unsurprisingly, Rap2.4 and Rap2.4b were found to comprise a pair of duplicated genes.

In the A-6 subgroup to which Rap2.4 and Rap2.4b belong to, two more duplicated gene pairs were found. Interestingly, 6 out of the 10 DREBs (Rap2.4, Rap2.4b, At1g36060, At4g39780, At2g22200 and At1g65130) of the A-6 subgroup were found to share elements of genomic context. In particular all 6 were either flanked by or in the immediate vicinity of a trehalose phosphate phosphatase (TPP) gene. There are only 10 TPPs in the *Arabidopsis* genome and a linkage to such a rare gene could suggest a common ancestor for the 6 DREBs. The TPP linked to Rap2.4 has been shown to be capable of complementing a yeast *tps2* mutant (Vogel et al., 1998).

The TPPs found in the *Arabidopsis* genome are divided into two classes which are thought to have risen before the divergence of monocot and eudicot lineages (reviewed in Paul et al., 2008). Five of the TPPs linked to DREBs belong to class I, but At1g65140 belongs to class II, suggesting that in the genome of the common ancestor of eudicots and monocots a progenitor gene(s) of the A-6 DREBs must have also been in close proximity to progenitor gene(s) coding for TPP. Consistent with this hypothesis, the *Arabidopsis* DREB A-6 subgroup equivalent in rice (Nakano et al., 2006) contains three AP2/ERF transcription factor genes that are also in the immediate vicinity of genes classified by Shima et al. (2007) as trehalose-6-phosphate phosphatases. The AP2/ERFs Os08g31580, Os09g20350 and Os06g11860 are found in the vicinity of Os08g31630 (TPP6), Os09g20390 (TPP7) and Os06g11840 (TPP8), respectively.

The position of Rap2.4-like genes in close proximity to TPPs might reflect common ancestry as mentioned above or a functional link between the two types of genes. Clustering of non-homologous genes with related functions is the norm in prokaryotes. Lee and Sonnhammer (2003) studied 318 *Arabidopsis* genes belonging to 79 distinct pathways and found over 40 % of genes in closer proximity to genes participating in the same pathways on the chromosomes than expected by chance. When tandem duplicates were included in the analysis over 60 % of genes were found clustered on the genome. Neighbouring genes are often co-expressed in *Arabidopsis* which might also indicate shared function (Williams and Bowles, 2004). Like the DREB genes, the genes encoding trehalose synthesis enzymes in *Arabidopsis* are involved in abiotic stress responses as demonstrated by their up-regulation in drought-tolerant *alx8* mutants (Wilson et al., 2009). The TPPs of *Arabidopsis* function in the last step of trehalose biosynthesis and are induced by abiotic stresses such as cold, high salinity and osmotic stress (reviewed in Paul et al., 2008). Trehalose is a rare disaccharide in plants and is found in significant quantities in only a few specialized resurrection plant species, where it is thought to play an important role in protecting cells against desiccation (Scott, 2000). Engineering for abiotic stress resistance by overexpressing components of the trehalose biosynthesis pathway or inhibiting trehalose breakdown has resulted in several tobacco, potato, tomato and rice lines with significantly enhanced tolerance to abiotic stresses (Almeida et al., 2007). In particular, overexpression of the TPP gene OsTPP1 in rice conferred salt and cold resistance (Ge et al., 2008). In a recent research paper, Lee et al. (2009b) observed co-expression pattern between a DREB transcription factor and a trehalose-6-phosphate synthase in rice, supporting a functional link between the two groups of genes.

Duplicated gene pairs

Gene duplications are regarded as a major potential source of evolutionary diversity. Immediately following duplication genes are identical but can subsequently acquire different fates by (i) one copy evolving into a non-functional pseudogene, (ii) neofunctionalisation i.e. divergence of the two copies in which one gene acquires a new function, (iii) subfunctionalisation i.e. the reduction in activity of both genes so that the ancestral gene function is maintained, or (iv) preservation of both copies in order to increase the robustness of a genetic network (Force et al., 1999; Gu et al., 2003). The acquiring of different functions can be realized by mutations in protein coding and/or regulatory regions. Mutations within the protein coding sequences can result in altered biochemical properties of the protein whereas mutations in the regulatory regions can potentially change the expression patterns (Haberer et al., 2004). In yeast, the expression patterns of duplicated genes diverged quickly with only a small fraction of duplicated gene pairs showing little or no variance (Gu et al., 2002). In *Arabidopsis* Blanc and Wolfe (2004) found that less than half of gene pairs formed in the most recent polyploidization event preserved significantly correlated expression patterns.

Based on the TIGR listing of genomic duplications, Rap2.4 and Rap2.4b comprise a pair of duplicated genes. The promoter regions of Rap2.4 and Rap2.4 show no discernible similarity but the AP2 DNA binding domains of the two proteins are identical. The proteins share an overall

68.5 % amino acid identity and a further 18.8 % of amino acids are conserved between these two transcription factors. Interestingly when the Rap2.4 and Rap2.4b proteins were aligned, the Rap2.4b coding region revealed long gaps in the coding sequence in comparison to Rap2.4, especially at the N-terminus of the protein. The long gaps in amino acid alignment could potentially alter the three-dimensional structure of the Rap2.4b protein relative to the Rap2.4 protein and therefore create a basis for different interaction patterns with other proteins. This is particularly intriguing because the DNA-binding domains of the two proteins are identical, and one might therefore expect that the two transcription factors recognise the same DNA elements and regulate matching sets of target genes. Whether Rap2.4 and Rap2.4b fulfil different, complementary or redundant roles in *Arabidopsis*, was further investigated by exploring their respective expression patterns and investigating putative targets of the two transcription factors.

Among the characterized DREB duplicated gene pairs, the individual genes might either play similar roles with one acting as the main regulator, or be significantly diverged and control quite different stress response pathways. A well-characterized example belonging to the former group is a pair of duplicated genes Dwarf and Delayed flowering 1 (DDF1) and DDF2. DDF1 (At1g12610) and DDF2 (At1g63030) proteins display a very high level of identity even outside the conserved DNA-binding domain (overall amino acid identity 69 %) with the exception of two deletions of 18 and 6 amino acids in the C-terminal part of DDF2. DDF1 and DDF2 are important regulators of endogenous gibberellic acid (GA) levels in *Arabidopsis* under high-salinity conditions and overexpression of either of the genes results in a dwarf phenotype due to reduced levels of GA. At the tissue level, the two genes display similar expression patterns but DDF2 is less responsive to salt treatment. DDF2 has been proposed to play a similar but minor role in the response to increased salinity (Magome et al., 2004).

DREB2A (At5g05410) and DREB2B (At3g11020) are also duplicated genes that share expression patterns. DREB2A and DREB2B are both induced by dehydration, heat and high salinity but not by low temperature. DREB2a induction by stresses is more than 10-fold stronger than that of DREB2B and therefore DREB2A is hypothesized to have a more important function in stress responses (Nakashima et al., 2000; Sakuma et al., 2006b).

The CBF cluster comprising CBF1, CBF2, CBF3 and CBF4 is, on the other hand, an example of duplicated genes that have significantly diverged. Haake et al. (2002) conducted a phylogenetic analysis which suggested that the common ancestor underwent a gene duplication event that gave rise to CBF4 and a homolog that subsequently underwent more recent duplication events that produced CBF1, CBF2 and CBF3 which lie in tandem on chromosome 4. CBF1/2/3 are involved primarily in cold responses (Gilmour et al., 1998) whereas CBF4 is up-regulated by drought stress (Haake et al., 2002). Consistent with involvement in different stress response pathways, the promoter sequences of CBF1/2/3 show significant sequence identity among themselves but share little similarity with the promoter of CBF4 (Haake et al., 2002).

Interestingly in the freezing-tolerant crucifer *Thlaspi arvense*, only one functional copy of CBF (TaCBF) is present in the collinear region shared with *Arabidopsis* CBF1/2/3. *T. arvense* is thought to have inherited three copies and subsequently lost two of the CBFs from the common

ancestor of the *Arabidopsis* and *Brassica* lineages. *T. arvense* is significantly more tolerant to freezing than *Arabidopsis* and TaCBF alone is sufficient for cold acclimation (Zhou et al., 2007). The maintenance of three CBF genes in *Arabidopsis* suggests that the cold stress signalling pathways are more tightly regulated in *Arabidopsis* than in *T. arvense*. Indeed, *Arabidopsis* CBF1 and CBF3 trans-activate the same target genes but are both required for full activation of the CBF regulon whereas CBF2 acts as a negative regulator of CBF1 and CBF3 expression (Novillo et al., 2004).

2.4.2 Expression studies of Rap2.4 and Rap2.4b

Expression of Rap2.4 and Rap2.4b in *Arabidopsis*

The expression patterns of Rap2.4 and Rap2.4b were investigated by utilizing the GUS reporter gene system and Northern blotting. Most strikingly the GUS gene under the control of the Rap2.4 promoter was found to be very strongly expressed in the radicle of germinating seeds and roots of both young seedlings and mature *Arabidopsis* plants. Rap2.4b-driven GUS reporter activity was also observed in roots of young seedlings. The promoter sequences of Rap2.4 and Rap2.4b revealed several TAPOX1 elements which are thought to confer high expression in roots (Elmayan and Tepfer, 1995). Interestingly, GUS expression remained strong even in roots of transgenic plants where only approximately 600 bp region upstream of the ATG of Rap2.4 without any TAPOX1 elements was controlling its expression. Northern blotting showed that in *Arabidopsis* mature tissues Rap2.4 was expressed at higher levels in stems and roots compared to other organs; in contrast, northern blots indicated that Rap2.4b was expressed mainly in stems. GUS transgene driven by the Rap2.4b promoter however accumulated in the rosette leaves of mature plants. Surprisingly, high levels of Rap2.4b mRNA were found in dry seeds. Consistently, the product of the GUS reporter gene driven by the Rap2.4b promoter accumulated in dry seeds. Publicly available microarray data supported the expression pattern observed for Rap2.4 and Rap2.4b.

Similar expression patterns in mature *Arabidopsis* have been observed for DREB2A which under normal growth conditions is strongly expressed in stems and roots, less expressed in leaves and is undetectable in flowers and siliques (Liu et al., 1998). The induction of DREB2A and DREB2B upon exposure to stresses such as drought and high salinity mainly takes place in roots and stems (Nakashima et al., 2000). Similarly, the maize DBF1 is expressed under unstressed conditions mainly in roots and stems but upon induction with various abiotic stresses can be detected in leaves, roots and stems. On the other hand, the soya bean GmDREBa/b/c are all expressed in stems, leaves, flowers and pods but are not detected in roots, however GmDREBc is induced in roots upon various abiotic stress treatments (Li et al., 2005).

Abiotic stresses such as drought and high salinity are manifested as soil stresses under normal growth conditions. Roots, which represent the interface between plant and its soil environment, perceive soil stresses and pass the message on to the rest of the plant (Jackson, 1997). The *Arabidopsis* histidine kinase ATHK1 that acts as an osmosensor is most abundantly expressed in roots (Urao et al., 1999) and accumulation of the stress hormone ABA in maize root

tissues is more sensitive to salt than ABA accumulation in leaf tissues (Jia et al., 2002). In the microarray analysis by Chen et al. (2002) 15 % of tested 402 transcription factors were root specific compared to 6 % leaf-specific and 3 % flower/siliques specific reflecting the unique role of roots. The abundant expression of Rap2.4 in roots probably reflects its role as a mediator of a soil-stress triggered response cascade.

Feng et al. (2005) surveyed tissue specific expression patterns of 145 AP/ERF genes utilizing a custom-made oligonucleotide macroarray. They found that in unstressed conditions 33 out of 57 tested DREB genes were not expressed and a further 10 genes were expressed in only one or two tissue types. Feng et al. (2005) found Rap2.4 and Rap2.4b to be expressed in most tested tissues whereas other genes of the A-6 subgroup were either expressed in very few types of tissue or considered non-expressed in unstressed conditions.

Expression of Rap2.4b in dry seeds

We found Rap2.4b to be highly expressed in dry seeds. In the microarray based profiling of stored mRNAs in *Arabidopsis* seeds conducted by Nakabayashi et al. (2005), Rap2.4b ranked among the 500 most highly expressed genes, and was found to be down-regulated after 24 hours of imbibition. Nakabayashi et al. also conducted experiments using seeds of *abi4* and *abi5* mutant lines in which Rap2.4b expression patterns both in dry seeds and following imbibition were not altered compared to wild type. *abi4* and *abi5* mutant lines are defective in the accumulation of seed storage reserves and seed dormancy (Finkelstein and Lynch, 2000; Finkelstein et al., 1998). ABI5 regulates transcription of target genes through the ACGT-containing ABRE element. However, although several ACGT-related sequences are present in the Rap2.4b promoter they probably do not mediate the regulation of Rap2.4b expression via *ABI5* in seeds.

The possible upstream regulators of Rap2.4b were investigated further after analysis of the Rap2.4b promoter revealed a quite well conserved RY-repeat element. The RY-repeat is the recognition sequence for *Arabidopsis* homologs of maize VP1, *ABI3* (*Abcisic Acid-Insensitive 3*) and *FUS3* (*Fusca 3*) transcription factors which regulate many aspects of seed maturation (Monke et al., 2004). We tested *abi3-1* and *fus3-8* mutant lines that carry mutations in the respective genes and display defects in late embryo development such as reduction in seed storage protein accumulation and lowered ability to acquire desiccation tolerance (Nambara et al., 2000, and references therein). Rap2.4b expression did not show significant alteration in desiccating siliques and seeds of the *fus3-8* line, although it was expressed at higher levels in green siliques of the mutant line than in the wild-type control. Rap2.4b expression was enhanced in the yellow and split siliques of *abi3-1* line, suggesting that neither *ABI3* nor *FUS3* are required for Rap2.4b expression during seed development and desiccation.

In *abi3* and *fus3* mutants a set of germination-specific genes is inappropriately activated during embryo development resulting in defects in seed maturation (Nambara et al., 2000). However, though we observed enhanced Rap2.4b expression in *abi3-1* line, Rap2.4b mRNA is reduced upon imbibition and therefore Rap2.4b does probably not fit in with the abovementioned group of germination-specific genes.

Conversely given the abundance of Rap2.4b mRNA in mature wild-type seeds, in seedlings of the *asill* mutant which display a shift in gene expression to a profile resembling late embryogenesis and de-repress many seed maturation-specific genes, Rap2.4b was down-regulated compared to wildtype seedlings. *ASILL1* codes for a transcription factor belonging to the *Arabidopsis* 6b-interacting protein-like family which interacts with the promoter of the embryogenesis-related 2S3 gene (Gao et al., 2009).

DREB proteins have previously been implicated in seed-specific processes. For example, the GhDBP2 protein from cotton which shares a 100 % identical DNA-binding domain with Rap2.4 and Rap2.4b is induced by abiotic stresses and ABA, but can also activate a late embryogenesis-specific LEA D113 gene via interaction with a DRE-like element in tobacco cells (Huang et al., 2008a). Maize DBF1 for which the most closely related gene in *Arabidopsis* is At4g39780 of the A-6 subgroup, accumulates to high levels in mature maize embryos and regulates the LEA gene RAB17 through a DRE-element (Kizis and Pages, 2002). As shown for many late-embryogenesis abundant proteins (reviewed in (Wise and Tunnacliffe, 2004) both D113 of cotton and RAB17 of maize are also induced by salt, high salinity and ABA. The activation of D113 and RAB17 by DREBs expressed during seed desiccation illustrates the common physiological aspects of dehydration in seeds and vegetative tissues and reveals the developmental role of DREBs in addition to their stress-related functions.

Induction of Rap2.4 and Rap2.4b by drought and osmotic stresses.

Northern blots revealed that Rap2.4 and Rap2.4b expression was induced by dehydration with maximum expression levels 1 hour after onset of the stress conditions. In order to investigate whether the effect of dehydration treatment was drought specific or due to general osmotic stress, 300 mM mannitol was applied to seedlings by spraying. Mannitol is a osmolyte whose effects mimic drought stress (Creelman and Zeevaart, 1985). Rap2.4 and Rap2.4b mRNA increased upon mannitol treatment and therefore it is likely that the effect of drought treatment on the expression of the two genes was due to osmotic aspects of the stress. The Genevestigator microarray database supported the observed expression patterns, with all osmotic stress experiments showing an increase in the mRNA levels of Rap2.4 and Rap2.4b but with some drought experiments failing to induce a response.

Drought responsive genes show large variations in the timing of induction. Regulatory proteins including transcription factors are induced rapidly, reach a maximum at several hours and then decrease. Functional proteins such as LEA proteins, detoxifying enzymes and proteins responsible for osmoprotectant biosynthesis are however induced more slowly and require up to ten hours for maximal accumulation (Yamaguchi-Shinozaki and Shinozaki, 2006). Indeed, Rap2.4 and Rap2.4b consistently accumulated at maximum levels as early as one hour after the onset of dehydration stress.

The role of DREB transcription factors in drought stress is well documented and many of them have been shown to enhance tolerance to drought when ectopically expressed in *Arabidopsis*. Overexpression of *Arabidopsis* DREB2A, DREB1A and CBF4 leads to enhanced drought

tolerance (Haake et al., 2002; Kasuga et al., 1999; Sakuma et al., 2006a). Overexpression of DREBs from other species, such as WXP1 and WXP2 from *Medicago truncatula* and TaAIDFa from wheat also confers enhanced drought tolerance in transgenic *Arabidopsis* (Xu et al., 2008b; Zhang et al., 2007b).

The possible mechanisms behind the Rap2.4 increase in response to drought

The increase in Rap2.4 mRNA under drought conditions could be contributed to either an increase in transcription or a decrease in degradation. Kim et al. (2008) showed that the increase in Rap2.4 mRNA abundance could be at least partly due to increased transcription. Kim et al. (2008) found that nucleosome density in the Rap2.4 genomic region was reduced in response to drought indicating that the Rap2.4 gene is organised into a structurally relaxed conformation under drought stress. The packaging of DNA into nucleosomes is important for regulation of transcription with nucleosomes working as general repressors. The nucleosome is the minimal unit of chromatin and tight packaging of DNA into chromatin inhibits transcriptional initiation by limiting access of the transcriptional apparatus to the DNA (Felsenfeld, 1992). Similarly to Rap2.4, the *Arabidopsis* ADH gene, which is induced by several abiotic stresses, has been shown to have a different nucleosome structure under non-induced versus induced conditions (Vega-Palas and Ferl, 1995). Kim et al. (2008) found the coding region of Rap2.4 to be enriched for RNAPII one to five hours following the start of drought treatment consistent with the idea that partially unfolding the chromatin results in derepression of transcription (Beato and Einfeld, 1997; Rombauts et al., 2003).

Regulation of the transcriptional status of chromatin via the modification of histones plays an important role in the regulation of gene activity. Histone modifications change the DNA-histone interactions and alter the accessibility of DNA to transcription factors. Histone modifications have been shown to be involved in abiotic stresses. For example, HOS15 controls gene expression through histone deacetylation and lack of HOS15 renders *Arabidopsis* hypersensitive to freezing (Zhu et al., 2008).

Chromatin at the coding regions of Rap2.4 showed an increase in tri-methylation of Lysine4 and acetylation of Lysine9 of the histone H3 N-terminus upon drought treatment. The enrichment of H3K4me3 and H3K9ac is considered to be a positive marker of histone modification associated with gene activation in a variety of organisms (Kim et al., 2008).

Induction of Rap2.4b by heat stress.

Expression of Rap2.4b but not Rap2.4 was induced by heat stress. Placing MS plates with 3 week-old seedlings at 38° C resulted in increased accumulation of Rap2.4b mRNA at both at 1 and 5 hour time-points. The Genevestigator microarray database however predicted induction for both transcription factors by heat stress.

Heat stress often occurs simultaneously with drought stress in field environments (Mittler, 2006), but has been demonstrated to elicit a transcriptional response that shares only a minor overlap with the response to drought. Rizhsky et al. (2004) found an overlap of 29 transcripts among 1075 transcripts up-regulated by drought and 262 transcripts up-regulated by

heat in *Arabidopsis*. An overlap of 48 transcripts was found among 496 transcripts down-regulated by drought and 279 transcripts down-regulated by heat. From separate studies DREB transcription factors of *Arabidopsis* have been shown to be induced by both heat and drought stresses. The DREB2 subfamily of genes (DREB2A/B/C/H) has been suggested to provide links that mediate cross-talk between heat and dehydration response pathways. Lim et al. (2006) found 4 DREB2 factors – DREB2A, DREB2B, DREB2C (At2g40340) and DREB2H (At2g40350) along with COR47/RD17 to be induced by heat treatment at 37° C for 1 and 6 hours in *Arabidopsis* cell suspension cultures. DREB2A and DREB2B are also well-characterized in respect of their drought responsiveness (Nakashima et al., 2000). In particular constitutive expression of active DREB2A protein confers an increase in both heat and drought tolerance (Sakuma et al., 2006a; Sakuma et al., 2006b). Constitutive expression of DREB2C also enhances *Arabidopsis* thermotolerance (Lim et al., 2007) and the gene has been shown to be induced by drought (Zhang et al., 2008a). In *Chrysanthemum* overexpression of *Arabidopsis* DREB1A confers increased salt, drought, cold and heat tolerance (Hong et al., 2009, and references therein).

The DREB2A/B/C/H transcription factors all belong to the A-2 subgroup and are closely related in terms of AP2 domain conservation. Rap2.4b belonging to the A-6 subgroup contains a more diverged DNA-binding domain. Rap2.4b could therefore have different DNA-binding preferences and might potentially regulate a different set of target genes during drought and heat stress.

Induction of Rap2.4 and Rap2.4b by high salinity.

In response to high salinity, Rap2.4 expression was induced whereas Rap2.4b mRNA levels remained similar to those observed in untreated *Arabidopsis* seedlings. The Genevestigator, however, showed up-regulation for both Rap2.4 and Rap2.4b.

Significant cross-talk between high-salinity and drought responses has been documented with over 50 % of drought-inducible genes being also induced by high salinity. Drought and high salinity share the aspect of osmotic stress, but high salinity also poses additional challenges such as interruption of metabolism by ionic imbalances and interference by salt ions in the uptake of essential micro- and macronutrients (Pasternak, 1987). Maintaining low level of Na⁺ ions and a high K⁺/Na⁺ ratio is vital for cellular metabolism in glycophytes such as *Arabidopsis* and a high Na⁺ concentration in plant cells is considered the most critical factor responsible for salt toxicity (Zhu et al., 1998).

LiCl and KCl solutions were applied to seedlings in order to check whether the effects of NaCl on Rap2.4 expression were caused by the osmotic aspects of high salt stress or were due to specific toxic effects of sodium ions on plant cells. As Rap2.4 was induced by LiCl, KCl and mannitol application as mentioned above, it can be concluded that the effect of NaCl treatment on Rap2.4 mRNA levels is more likely to be due to overall hyper-osmolarity rather than any specific effect Na⁺ ions might have.

Interestingly, the induction of Rap2.4 by LiCl only occurred 5 hours following the onset of treatment, whereas the effects of NaCl were already observed 1 h after application of the salt

solution. The different kinetics of Rap2.4 mRNA increases might be explained by the fact that Li^+ is thought to require more time to enter cells than Na^+ (Xiong et al., 2004).

Rap2.4 mRNA levels increased to higher levels after LiCl treatment compared with increases observed following NaCl or mannitol application indicating an additional effect of Li^+ . The lithium ion is known to interfere with phosphatidylinositol metabolism and signalling (Berridge and Irvine, 1989). The inositol tri-phosphate phosphatase FIERY1 (*Fry1*) is a general negative regulator of abiotic stress responses and ABA signalling which is known to be sensitive to Li^+ ions (Xiong et al., 2001c; Xiong et al., 2004). We have determined that Rap2.4 expression is moderately enhanced in *fry1* mutant lines upon stress treatments compared to wild-type plants whereas no change was observed in Rap2.4b expression. The microarray experiments conducted by Wilson et al. (2009) on *fry1* mutants are in agreement with the expression patterns observed by us – Rap2.4 was found to be significantly up-regulated and Rap2.4b expression was very moderately increased. The effect of LiCl on Rap2.4b expression is however larger than that of either NaCl or *fry1* mutation. It could be hypothesised that the effect of LiCl application is two-fold – on the one hand it elicits Rap2.4 expression as a part of the osmotic stress response but also at the same time it blocks an inhibitor of Rap2.4 induction, resulting in a higher total increase in mRNA levels.

The lack of Rap2.4b induction in our high salinity experiment cannot be explained easily. The Genevestigator reports on experiments in which Rap2.4b responds to salt stress with enhanced expression. On the other hand, mannitol application successfully induced Rap2.4b expression in our experiments confirming its responsiveness to osmotic stress. The failure of Rap2.4b to respond to the high salinity treatment, which includes a strong osmotic stress aspect, might therefore be due to experimental/technical problems.

Induction of Rap2.4 and Rap2.4b by cold stress.

Rap2.4 and Rap2.4b were induced by cold treatment in the early stages of cold treatment – 1 and 2 hours after placing the seedlings at 4°C the mRNAs of both transcription factors were more abundant than in seedlings maintained under normal growth conditions. However, after 6 hours of cold treatment, Rap2.4 mRNA was barely detectable in seedling tissues, returning to levels observed in unstressed plants after 24 hours of cold treatment. This result was in agreement with publicly available microarray data.

Recently published microarray experiments have demonstrated that in response to cold-treatments in addition to up-regulation, extensive down-regulation of gene expression also takes place (Oono et al., 2006). Most studies have concentrated on the genes that are up-regulated in response to cold and not much is known about the down-regulated genes. Investigating cold-responsive genes in the Rap2.4 overexpression lines might provide interesting new insights in relation to cold-response pathways.

Interestingly, when the cold treatment was carried out on *fry1* seedlings, the Rap2.4 mRNA levels were elevated compared to controls whereas lack of FRY1 did not affect Rap2.4b expression. Similarly, in *hos1* lines Rap2.4 expression was enhanced compared to control

seedlings both in unstressed and cold conditions. FRY1 (HOS2) is a general negative regulator of abiotic stresses and has been demonstrated to negatively regulate cold response pathways along with HOS1 (Ishitani et al., 1998; Lee et al., 1999).

HOS1 (High in Osmotic Stress 1) encodes a RING finger ubiquitin E3 ligase that also negatively regulates cold responses (Ishitani et al., 1998). In *hos1* plants the induction of cold-responsive genes such as RD29A, COR15A, KIN1, CBF2 and CBF3 is therefore enhanced compared to wild-type. *hos1* plants are considered to be constitutively vernalized as they express the central negative regulator of flowering *FLC* (*Flowering Locus C*) at considerably lower levels than wild-type plants. In consequence, *hos1* mutant lines, which lack sufficient levels of *FLC* to suppress the flowering pathways, flower early (Lee et al., 2001). Given that Rap2.4 is highly expressed in *hos1* seedlings, it might play a role in the physiological changes that occur during extended exposure to chilling temperatures i.e. vernalization.

The HOS1 gene regulates the cold response by mediating ubiquitination and degradation of ICE1 (Dong et al., 2006). ICE1 (Inducer of CBF Expression 1) is a well-characterized positive regulator of cold-responses. The *ice1* mutation blocks the induction of CBF3 during cold stress and reduces expression of several cold-responsive genes such as COR15A, COR47, RD29A, KIN1, ultimately resulting in a significant reduction in chilling and freezing tolerance. ICE1 is a MYC-like transcription factor that interacts with MYC recognition sequences in target gene promoters (Chinnusamy et al., 2003). Since the Rap2.4 promoter lacks MYC-like elements and the Rap2.4 expression patterns in the *ice1* lines were similar to control plants, ICE1 is probably not a regulator of Rap2.4 in cold conditions. However, only utilizing ICE1 overexpression lines would clarify whether it might act as an upstream regulator of Rap2.4.

Induction of Rap2.4 and Rap2.4b expression by phytohormones

Rap2.4 and Rap2.4b responded to treatments with various phytohormones in different ways illustrating the dissimilarities between the two transcription factors. Rap2.4 responded to almost all hormones tested – ABA, ACC, SA, JA, synthetic auxin 2, 4-D – but the increases were only slight. Neither gibberellic acid nor the synthetic cytokinin BAP1 altered Rap2.4 expression. Rap2.4b responded to ABA, ACC and 2, 4-D but the changes in expression levels were more striking than for Rap2.4.

In response to the ethylene precursor ACC, Rap2.4 expression was only slightly enhanced but Rap2.4b mRNA accumulated to considerably higher levels. Ethylene is a gaseous plant hormone with many functions during the plant life cycle including seed germination, leaf senescence, abscission and responses to abiotic and biotic stresses (Abeles et al., 1992). Overexpression or lack of Rap2.4 has been previously reported to alter the expression of ethylene-responsive genes and cause defects in ethylene and light-regulated developmental processes (Lin et al., 2008). While Rap2.4 is proposed to be a regulator of ethylene responses, the induction of Rap2.4b by ACC could possibly also be tied to abiotic stress responses. ACC can protect plant cells against heat-induced oxidative damage and has been implicated as a signalling molecule during heat stress (Larkindale and Knight, 2002). Rap2.4b is strongly induced by exposure to high

temperature and inducibility by ACC might therefore reflect its participation in the heat stress response pathways.

Induction by abscisic acid

Rap2.4 and Rap2.4b were both induced in response to treatment with ABA. Rap2.4 mRNA showed a small increase in abundance whereas Rap2.4b expression levels were markedly higher in treated plants than in untreated controls.

The phytohormone ABA is produced in plant tissues under abiotic stress conditions and plays an important role in establishing abiotic stress responses. Many stress-inducible genes are regulated by endogenous ABA that accumulates during drought and high-salinity stress (Bray et al., 2002; Seki et al., 2002a). AP2/ERF transcription factors have been implicated as early-response genes to either ABA treatment or high salinity or dehydration stress. Early-response transcription factors are typically constitutively expressed but are nevertheless also inducible and are proposed to be regulated at the posttranslational level (Zhu, 2002). Posttranslational phosphorylation has been shown to be necessary for the proper function of AP2/ERF proteins (Agarwal et al., 2007; Gu et al., 2000). Consistent with this scenario, Rap2.4 mRNA is indeed relatively abundant throughout the plant life-cycle, especially in root tissues, and the encoded protein contains several putative phosphorylation sites. Rap2.4b mRNA is expressed at lower levels in unstressed conditions but can still be detected in all tissues of mature *Arabidopsis* plants. The Rap2.4b protein also features a number of putative phosphorylation sites.

Rap2.4 and Rap2.4b promoter sequences contain ABA-responsive *cis*-element (ABRE) like elements and several recognition sites for MYB transcription factors. AtMYB2 has been shown to act as a transcriptional activator in ABA signalling (Abe et al., 2003) and might also mediate the induction of Rap2.4 and Rap2.4b in response to ABA.

Induction of Rap2.4 and Rap2.4b in ABA-deficient lines

In plant lines deficient in ABA biosynthesis, the induction of several stress-responsive genes is lower compared to wild-type. For example, in *los5* (*Low in Osmotic Stress 5*), expression of RD29A, COR15, COR47, RD22 and P5CS was reduced or even completely blocked under conditions of high salinity (Xiong et al., 2001b). In *los6* (*Low in Osmotic Stress 6*), osmotic stress treatment failed to elevate the expression of RD29A, COR15A, KIN1, COR47, RD19 or ADH to levels observed in wild-type plants (Xiong et al., 2002a).

We used the ABA-deficient lines *aba1-5*, *aba2-4* and *aba3-1* in order to establish the role of endogenous ABA in Rap2.4 and Rap2.4b expression. The three lines carry mutations in genes coding for enzymes required for ABA biosynthesis and display a variety of ABA and stress-related phenotypes. ABA1 is allelic to LOS6 and ABA3 to LOS5.

The seedlings of ABA-deficient lines expressed Rap2.4 at enhanced levels and Rap2.4b at the same levels compared to wild-type seedlings after drought treatment. This result indicates that even though both transcription factors respond to exogenous ABA, the phytohormone is not required for the drought responsiveness of Rap2.4 and Rap2.4b. Similarly maize DBF1 was found

to be induced independently by ABA and drought, utilizing ABA-deficient *vp2* maize embryos (Kizis and Pages, 2002).

CHAPTER 3

MOLECULAR CHARACTERIZATION OF RAP2.4 AND RAP2.4B

3.1 DETERMINATION OF THE NLS SIGNAL OF RAP2.4

3.1.1 Introduction

Proteins that function as transcription factors are synthesised by ribosomes in the cytoplasm of plant cells. However, transcription factors function within the cell nucleus and therefore must be transported across the double membrane that separates the nucleus from the cytoplasm. Nuclear pore complexes facilitate the interchange of molecules between the cytoplasm and nucleus. The “tunnels” within the complexes form passive diffusion channels for molecules up to 40 kDa; larger molecules are transported into the nucleus by active transport. Active transport across the nuclear membrane is selective and signal-dependent (Gorlich and Mattaj, 1996).

The sequences targeting proteins to the nucleus (termed nuclear localization signals (NLSs)) do not fit a tight consensus, but generally consist of short stretches rich in basic amino acids such as lysine and arginine. The NLSs are usually position independent, and some proteins contain more than one NLS (Smith and Raikhel, 1999).

The first characterized NLSs belonged to nucleoplasmin and to simian virus 40 large T antigen (SV40 T-ag). The nucleoplasmin NLS consists of two regions rich in arginine and lysine separated by a 10 amino acid spacer sequence. The NLS of SV40 T-ag is a cluster of positively charged residues PKKKRKV. Both types of abovementioned signal are very common and are called basic or classic nuclear localization signals (reviewed in Jans et al. (2000)).

In the AP2/ERF family the NLSs of several members have been characterized in detail. The nuclear targeting signal in the AtEBP (At3g16770, Rap 2.3) consists of a short peptide sequence KRRKRK at the N-terminus of the AP2 domain (Pan et al., 2001). Also AINTEGUMENTA (ANT, At4g37750) possesses a NLS consisting of the basic amino acids KKKR (Krizek and Sulli, 2006). The position of the NLS domain appears to be conserved to the proximity of the N-terminal end of the AP2 DNA-binding domain among the AP2/ERF transcription factors (Riechmann and Meyerowitz, 1998).

Although AP2/ERF transcription factors function in the nucleus, not all of them are constitutively localized there. Six CYTOKININ RESPONSE FACTORS (CRFs) belonging to the B-5 subgroup of the ERF subfamily, accumulate in the nucleus only upon cytokinin treatment (Rashotte et al., 2006). This suggests that nuclear import of AP2/ERF proteins in *Arabidopsis* may be responsive to developmental or environmental signals.

Several reporter gene systems have been developed for characterization of the subcellular localization of gene products in plants. One of the most widely used reporters is the green fluorescent protein (GFP) isolated from the jellyfish *Aequorea aequorea* (Chalfie et al., 1994). The use of the original GFP protein in higher plants was limited due to aberrant splicing of the corresponding mRNA, and protein insolubility. These problems were overcome by site-directed

mutagenesis which removed a cryptic splicing-site and generated a more soluble codon-optimised GFP (Davis and Vierstra, 1998; Haseloff et al., 1997). Since then GFP has been used in plants as a reporter of transcription, as a tracking device for endogenous proteins, and visualization tool in studying the dynamics of intracellular structures and cellular processes (for a recent review see Berg and Beachy (2008)).

The major advantage of GFP as a reporter gene is the possibility of making fluorescent translational fusion proteins that are stable in living plants cells. The ideal result of this approach is a fusion protein that maintains the normal functions and localization properties of the host protein but is fluorescent, providing the means to monitor the localization and fate of proteins of interest. GFP fused with the protein of interest can be expressed within intact plant tissues and monitored in a non-invasive manner by fluorescence microscopy without the disturbance caused by introduction of reagents. However GFP fluorescence does survive glutaraldehyde and formaldehyde fixatives if the need should arise for the use of fixed tissues (Chalfie et al., 1994).

GFP accumulates in the cytoplasm of cells and due to its small size (26 kDa) also diffuses freely through the nuclear pores. Fused with appropriate organelle targeting sequences, GFP can be retained in the plant cell in practically every major organelle including the plasma membrane, nucleus, endoplasmic reticulum, Golgi apparatus, secretory vesicles, mitochondria, peroxisomes, vacuoles and phagosomes (Cowles et al., 1997; Hanakam et al., 1996; Lang et al., 1997; Maniak et al., 1995; Presley et al., 1997; Rizzuto et al., 1996; Wiemer et al., 1997).

Of the disadvantages it is worth mentioning that GFP requires up to 2 hours to accumulate in plant tissues and more importantly as many biological molecules autofluoresce at the same wavelength as GFP, background noise can pose a significant concern, especially when expression of the gene of interest is relatively low (March et al., 2003).

Recently GFP and genetically modified versions such as yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) have been used to study protein-protein interactions. Bimolecular fluorescent complementation technique is based on tethering split YFP or other GFP variants to form a functional fluorophore. The non-fluorescent split fragments of fluorophore are fused to putatively interacting proteins. Only if the proteins interact, will the fragments of fluorescent protein re-associate and form a fluorophore that has the same properties as an intact GFP/YFP/CFP (Ohad et al., 2007).

Our aim in this section was first to determine whether Rap2.4 does indeed accumulate in the nucleus of plant cells and then to identify the relevant NLS. We approached the question by transiently expressing Rap2.4 translational fusions with a reporter gene in tobacco epidermis.

3.1.2. Materials and methods

3.1.2.1 Plasmids

RZ34b01 – accession AV544129 was obtained from Kasuza DNA Research Institute (Asamizu et al., 2000). It is a Bluescript II SK vector with the Rap2.4 cDNA cloned between EcoRI and XhoI restriction enzyme sites.

psmGFP – plasmid (Davis and Vierstra, 1998) containing codon- and soluble- modified GFP (accession U70495) under the control of CaMV35S promoter and NOS terminator was obtained from ABRC (clone CD3-326)

pFGC5941 – see section 2.2.3.1 for description and Figure 2.15 for outline.

pFGC-CaMV35S-nos – based pFGC5941; the EcoRI/HindIII fragment situated between the left and right border sequences has been replaced with a fragment containing the standard CaMV 35S promoter, multiple cloning site and NOS terminator from pROK2 vector. This vector allows the expression of cloned DNA fragments of interest under the control of the constitutive CaMV 35S promoter and NOS terminator. The T-DNA region contains the BAR marker for selection of transformed plant cells. Kanamycin marker which enables the selection in bacterial cultures is situated outside the T-DNA region and does not transfer to plant cells upon infection. The construction of the plasmid is outlined in Figure 3.1.2.

pROK2 - (Baulcombe et al., 1986) is a binary vector based on pBin19. The plasmid contains the 35S promoter from CaMV, a polylinker (restriction sites for XbaI, BamHI, SmaI, KpnI and SstI) and the NOS terminator. Two neomycin phosphotransferase genes confer kanamycin resistance as the selectable marker in both bacterial and plant cells.

3.1.2.2 Oligonucleotide primers

Primers used to amplify the Rap2.4 ORF or truncated versions of the Rap2.4 ORF.

Construct	Primer	Oligonucleotide sequence 5' to 3' orientation
pFGC-CaMV-GFP:: Rap2.4	R24orfFKpn	GCGGTACCATGGCAGCTGCTATGAATTTG
	R24orfRBamHI	<u>CGGGATCC</u> CTAAGCTAGAATCGAATCCCAA
pFGC-CaMV-GFP:: Rap2.4Δ1-158	Del1-158FKpn	GCGGTACCCACTGGGGAAAATGGGT
	R24orfRBamHI	as above
pFGC-CaMV-GFP:: Rap2.4Δ246-334	R24orfFKpn	as above
	Del246-334R	<u>CGGGATCC</u> CTACTGAGTCTCCGCCAT
	BamHI	
pFGC-CaMV-GFP:: Rap2.4Δ1-146	Del1-146FKpn	GCGGTACCAAGCCGACGAAGCTTTACAGA
	R24orfRBamHI	as above

Restriction sites added for cloning purposes are indicated by underlining.

Primers used to amplify GFP ORF for all the fusion constructs:

GFPorfFXba: 5'- GCTCTAGAATGAGTAAAGGAGAAGA ACTT - 3'

GFPorfnsKpn: 5'- GGGGTACCTTTGTATAGTTCATCCATGCC - 3'

Sequencing primers:

CaMV1: 5'- CCTCCTCGGATTCCATTGCCAGCTA-3'

GFPFC: 5'-ACTCCAATTGGCGATGGCCCTGTCCT -3'

3.1.2.3 Transient expression in *Nicotiana benthamiana* mesophyll cells

Constructs of interest were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation and the resulting plasmids were checked by restriction analysis. Glycerol stocks were streaked on LB plates and incubated at 28° C for two days. Liquid cultures of strains were grown from single colonies in LB broth with added 10 mM MES, 20 µM acetosyringone and suitable antibiotics at 28°C, 220 rpm. Once the culture reached OD₆₀₀=0.8, cells were collected by centrifugation (6000 rpm, 10 minutes) and resuspended in infiltration solution (10 mM MgCl₂, 10 mM MES and 100 µM acetosyringone). The infiltration solutions were incubated at room temperature with gentle shaking for at least 4 hours.

Leaves of 2-3-week old *N. benthamiana* were used for infiltration. The infiltration procedure was carried out using blunt disposable 1 ml syringes.

3.1.2.4 Fluorescent microscopy

Two days following infiltration, leaf tissue samples were transferred to glass slides and analysed using a Nikon Eclipse E600 microscope with a Y-LF Epi-fluorescence attachment at magnification of 200 times. The pictures were taken using a Spot RT CCD digital camera and processed with Adobe Photoshop 6.0 software.

3.1.3 Results

3.1.3.1 GFP and Rap2.4 translational fusion constructs

Analysis of the Rap2.4 protein sequence identified two arginine (R) and lysine (K)-rich regions that might act as nuclear targeting signals, even though no conserved NLS was revealed. In order to determine the sequence in the Rap2.4 open reading frame (ORF) that acts as an NLS, four GFP fusion constructs were made. The first featured a full length Rap2.4 ORF, while in the other three, truncated fragments of the transcription factor ORF were used. The first of the three truncated fragments (Δ 1-158) lacked the 158 amino acids from the N-terminus of the full-length Rap2.4 protein along with the putative NLS 'KPTKLYRGVRQR'. In the second fusion construct (Δ 246-334) 88 amino acids from the C-terminus were omitted along with the putative NLS 'KQDKSTKSSKKREKK'. In the third construct (Δ 1-146) GFP was fused with a shorter N-terminal deletion which resulted in amino acids 146 to 158 i.e. the putative N-terminal NLS sequence, being retained in the ORF (Figure 3.1.1).

When undertaking any subcellular localization experiments it is important to consider natural limitations of protein transport within the cell. GFP can diffuse passively through the nuclear pores due to its small size (26 kDa). The molecular mass of fusion constructs in all 4 cases was above the nuclear exclusion limit of 43 kDa so therefore the fusion proteins are expected to translocate into the nucleus only if specific NLSs are present.

3.1.3.2 Construction of plasmids

The plasmid used for the cloning was pFGC-CaMV-nos which is a modified version of the vector pFGC5941. In order to obtain the pFGC-CaMV-nos plasmid, the EcoRI/HindIII

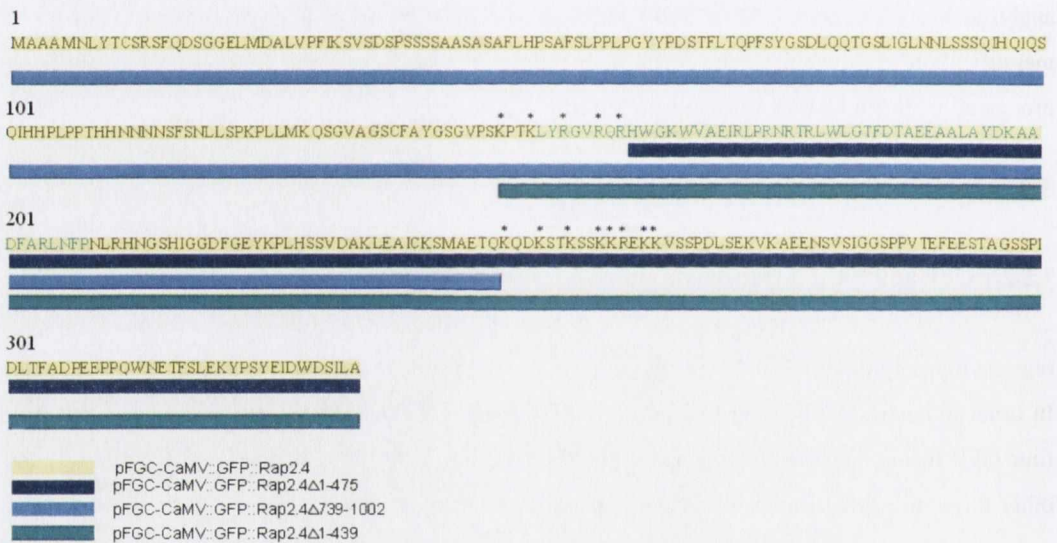


Figure 3.1.1. Schematic representation of Rap2.4 amino acid sequence and the deletions made for nuclear localization experiments. The AP2 domain is distinguished with green lettering. The basic amino acids comprising the putative nuclear targeting signals are marked with asterisks. The full and truncated versions of the Rap2.4 ORF used for constructing translational GFP fusions are marked with blocks in different colour.

fragment of the original pFGC5941 containing the CaMV 35S promoter followed by synthetic linker, a chalcone synthase intron from *Petunia hybrida* and the terminator sequence of the octopine synthase gene, was replaced with a fragment containing the standard CaMV 35S promoter, multiple cloning site and NOS terminator from pROK2 vector (Figure 3.1.2).

The GFP ORF for the various fusion constructs was amplified by PCR using the primers GFPorfFXba and GFPorfnsRKpn and the cDNA clone psmGFP as the template. The primers were designed to amplify the GFP ORF without the stop codon and to flank it with XbaI and KpnI restriction sites.

The Rap2.4 ORF and the truncated ORF fragments were amplified by PCR from the Rap2.4 cDNA clone RZ34b01 with primers Rap24orfFKpn and R24orfRBamHI for the full-length protein; Del1-158FKpn and R24orfRBamHI for the GFP::Rap2.4 Δ 1-158 fusion; R24orfFKpn and Del246-334RBamHI for GFP::Rap2.4 Δ 246-334 fusion; and Del1-146FKpn and R24orfRBamHI for GFP::Rap2.4 Δ 1-146 fusion.

The PCRs amplified a fragment of 1005 bp for the full length Rap2.4, a fragment of 530 bp for the Δ 1-158, a fragment of 742 bp for the Δ 246-334 and a fragment of 566 bp for the Δ 1-146 truncated Rap2.4 ORF versions. All the fragments above were flanked by KpnI and BamHI sites.

The amplified GFP ORF (717 bp in size) was digested with XbaI and KpnI restriction enzymes; the Rap2.4 ORF full-length and truncated versions of with KpnI and BamHI restriction enzymes. Triple ligations were used to clone GFP and all versions of Rap2.4 ORF into the XbaI and BamHI sites of pFGC-CaMV-nos vector. The resulting plasmids were called pFGC-CaMV-GFP::Rap2.4, pFGC-CaMV-GFP::Rap2.4 Δ 1-158, pFGC-CaMV-GFP::Rap2.4 Δ 246-334 and pFGC-CaMV-GFP::Rap2.4 Δ 1-146 (Figure 3.1.3).

The plasmids were checked by sequencing with CaMV1 and GFPFC primers. The expected size of the translational fusion protein is 63.5 kDa for pFGC-CaMV-GFP::Rap2.4, 46.8 kDa for pFGC-CaMV-GFP::Rap2.4 Δ 1-158, 53.9 kDa for pFGC-CaMV-GFP::Rap2.4 Δ 246-334 and 48.2 kDa for pFGC-CaMV-GFP::Rap2.4 Δ 1-146.

3.1.3.3 *Agrobacterium*-mediated transformation and transient expression in *N. benthamiana*

These vectors were electroporated into *Agrobacterium tumefaciens* strain GV3101; transformed strains were verified by restriction analysis (Figure 3.1.4). Following digestion with XbaI and BamHI, the pFGC-CaMV-GFP::Rap2.4 gave a fragment of 1731 bp (Figure 3.1.4, panel A, lane 1). Following digestion of pFGC-CaMV-GFP::Rap2.4 Δ 1-158 and pFGC-CaMV-GFP::Rap2.4 Δ 246-334 with HindIII, respective fragments of 1792 bp (Figure 3.1.4, panel B, lanes 1 and 2) and 2019 bp (Figure 3.1.4, panel B, lanes 3 and 4) were observed. Digestion of pFGC-CaMV-GFP::Rap2.4 Δ 1-146 with HindIII resulted in a fragment of 1584 bp (Figure 3.1.4, panel C, lane 1).

The *A. tumefaciens* cultures were infiltrated into three week-old *N. benthamiana* leaves in order to achieve transient expression. Following 48 hour incubation period, the GFP fluorescence was monitored by microscopy.

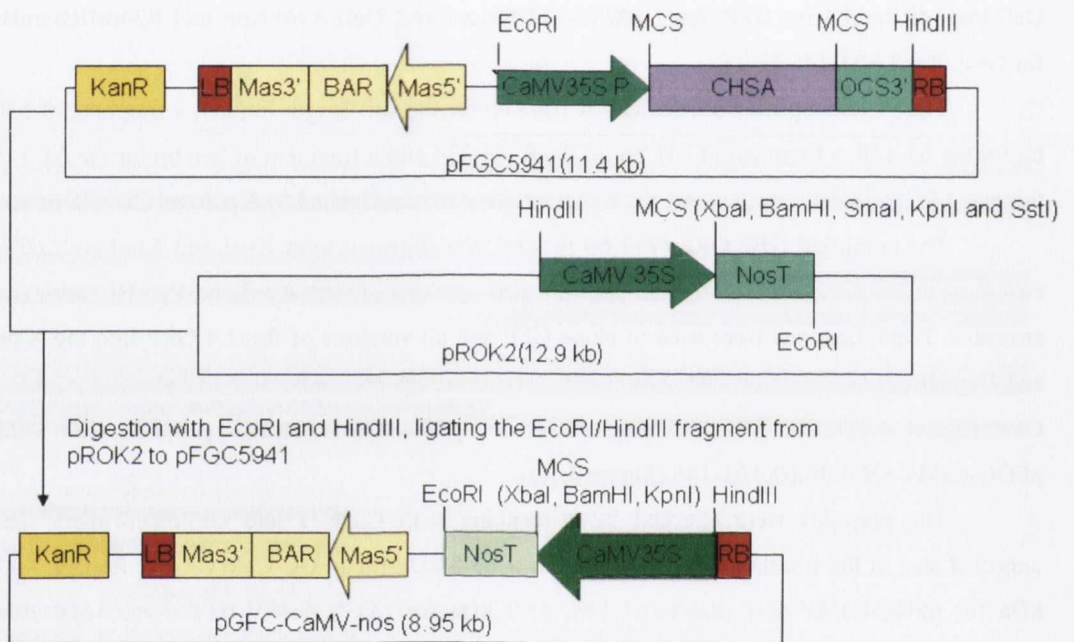


Figure 3.1.2. Construction of pGFC-CaMV-nos plasmid. The restriction sites shown were used for modifying the original pFGC5941.

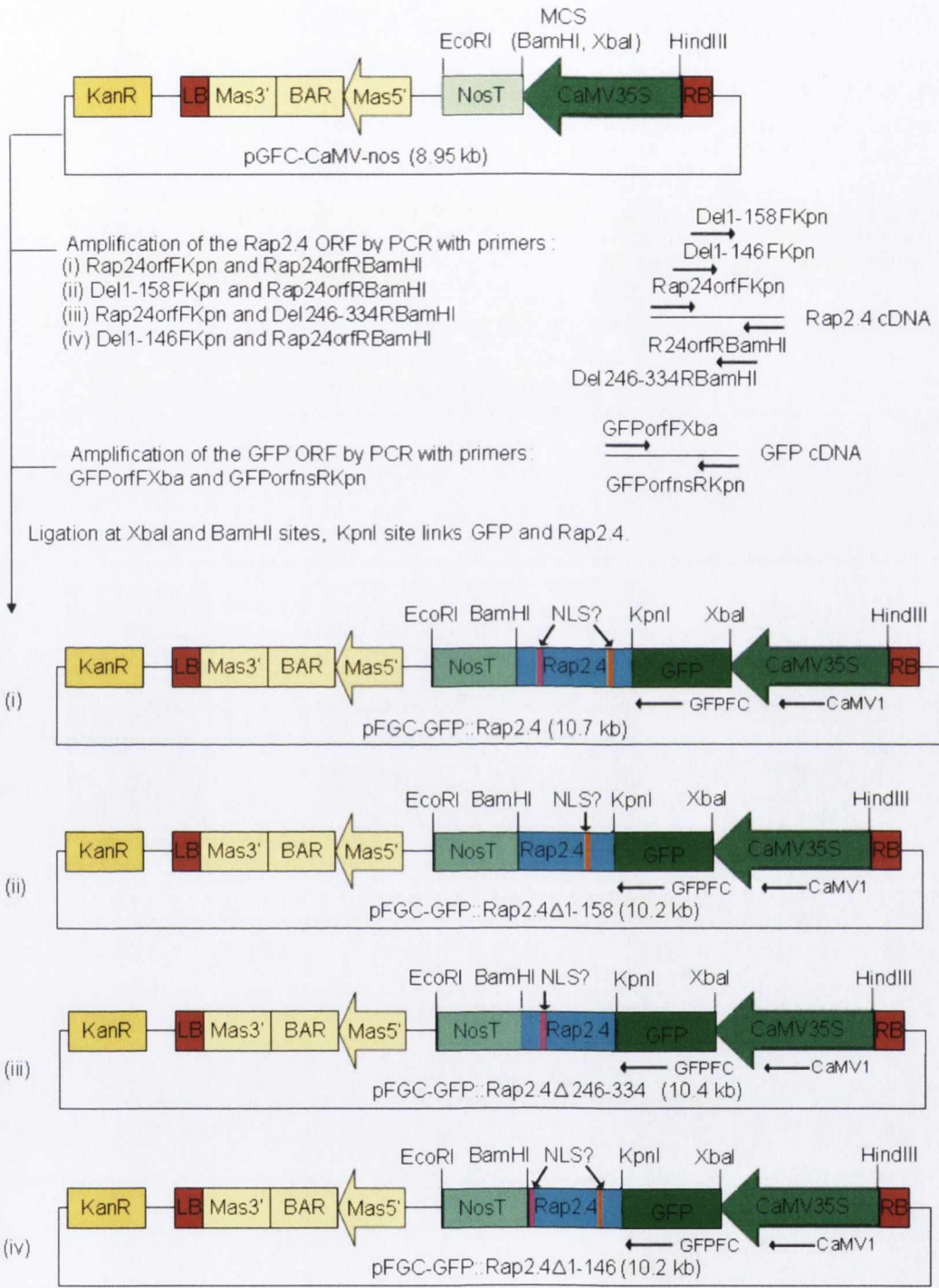


Figure 3.1.3. Construction of pFGC-CaMV-GFP::Rap2.4 (i), pFGC-CaMV-GFP::Rap2.4Δ1-158 (ii), pFGC-CaMV-GFP::Rap2.4Δ246-334 (iii) and pFGC-CaMV-GFP::Rap2.4Δ1-146 (iv) plasmids. The primers used for PCR amplifications and sequencing are indicated with black arrows. The pink and orange bars on the Rap2.4 ORF indicate putative N-terminal and C-terminal nuclear targeting signals, respectively.

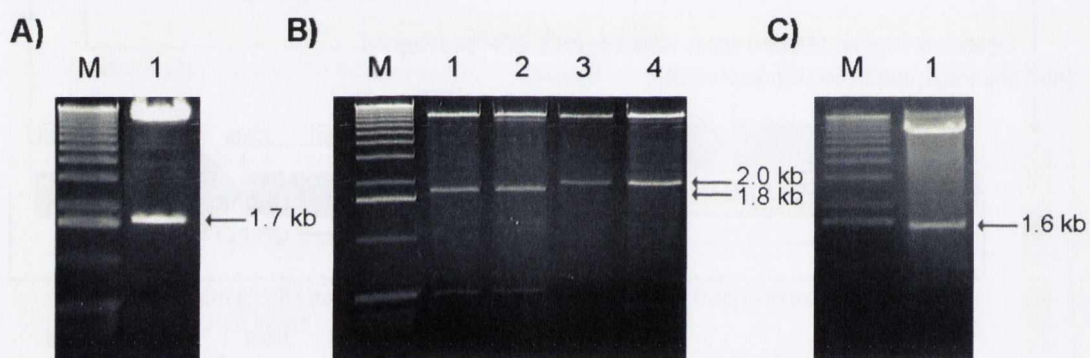


Figure 3.1.4. Verification of the constructs created for subcellular localization experiments. A) Plasmid DNA was isolated from pFGC-CaMV::GFP::Rap2.4 and digested with XbaI and BamHI (lane 1). B) Plasmid DNA was extracted from pFGC-CaMV::GFP::Rap2.4 Δ 1-158 and pFGC-CaMV::GFP::Rap2.4 Δ 246-334 and digested with HindIII (lanes 1 to 2 and 3 to 4, respectively). C) Digestion of pFGC-CaMV::GFP::Rap2.4 Δ 1-146 with HindIII (lane 1). HindIII sites are located at the 5' terminus of the CaMV promoter and within the Rap2.4 ORF, at 449 and 701 bp. M marks the lanes loaded with molecular weight marker.

3.1.3.4 Subcellular localization of the GFP::Rap2.4 fusion proteins

In the leaves infiltrated with full-length Rap2.4 ORF fused with GFP, the GFP fluorescence was observed in the nucleus of the *N. benthamiana* mesophyll cells. In contrast, GFP as a control was distributed throughout the cell and was detected both in the cytoplasm and nucleus. Leaves infiltrated with *A. tumefaciens* carrying pFGC-CaMV-GFP::Rap2.4 Δ 246-334 fusion, showed GFP fluorescence to be localized exclusively in the nuclei of mesophyll cells, indicating that the C-terminal putative NLS sequence is not necessary for targeting full-length Rap2.4 (Figure 3.1.5).

Infiltration with *A. tumefaciens* carrying the pFGC-CaMV-GFP::Rap2.4 Δ 1-158 however resulted in GFP accumulation in the cytoplasm. From the abolition of nuclear targeting of the fusion protein upon deletion of amino acids 1-158 in the N-terminal region of Rap2.4, it is possible to deduce that the deleted region must contain the sequence of amino acids responsible for the nuclear targeting of Rap2.4. Experiments with *A. tumefaciens* carrying the third fusion construct pinpointed the exact region responsible for the subcellular targeting of GFP-Rap2.4 fusion protein within the N-terminus. When *N. benthamiana* mesophyll cells were transiently transformed with pFGC-CaMV-GFP::Rap2.4 Δ 1-146 plasmid, GFP fluorescence was observed exclusively in the nuclei (Figure 3.1.5).

These results indicate that the biologically relevant NLS in the Rap2.4 is located between amino acid residues 147 and 158 and corresponds to the sequence KPTKLYRGVRQR. The sequence contains 5 positively charged amino acids – two lysins and three arginines – interspersed with non-polar amino acids.

3.1.3.5 Putative localization of the Rap2.4b NLS.

In Rap2.4b the KPTKLYRGVRQR amino acid sequence determined to be responsible for the nuclear localization of Rap2.4 is 100 % conserved. On this basis Rap2.4b can be expected to also localize in the nucleus.

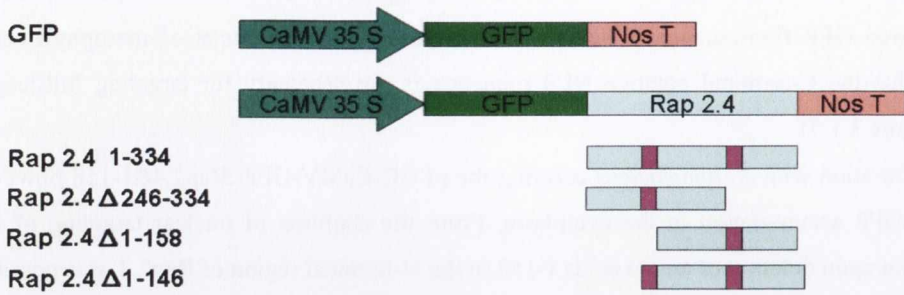
3.1.4 Discussion

Consistent with its putative role as a transcriptional regulator, we found the Rap2.4-GFP fusion protein to be localized to the nucleus in transient assays utilising tobacco mesophyll cells. Through deletion analysis we determined that the amino acids from position 147 to 158 (KPTKLYRGVRQR) are sufficient for nuclear targeting of Rap2.4. The position of the Rap2.4 NLS at the N-terminus of the AP2 domain is similar to that of characterized and putative NLSs of other AP2/ERF transcription factors such as AtEBP, ANT and PUCHI, HvRAF, respectively (Hirota et al., 2007; Jung et al., 2007; Krizek and Sulli, 2006; Pan et al., 2001).

The region from lysine¹⁴⁷ to arginine¹⁵⁸ is fully conserved in Rap2.4b, therefore it is reasonable to expect that Rap2.4b also localizes to the nucleus.

The amino acid sequence of Rap2.4 NLS does not resemble any well characterized nuclear localization signals. NLSs generally consist of one or more clusters of basic amino acids but do not always resemble consensus sequences. However it is not only the presence of positively

A



B

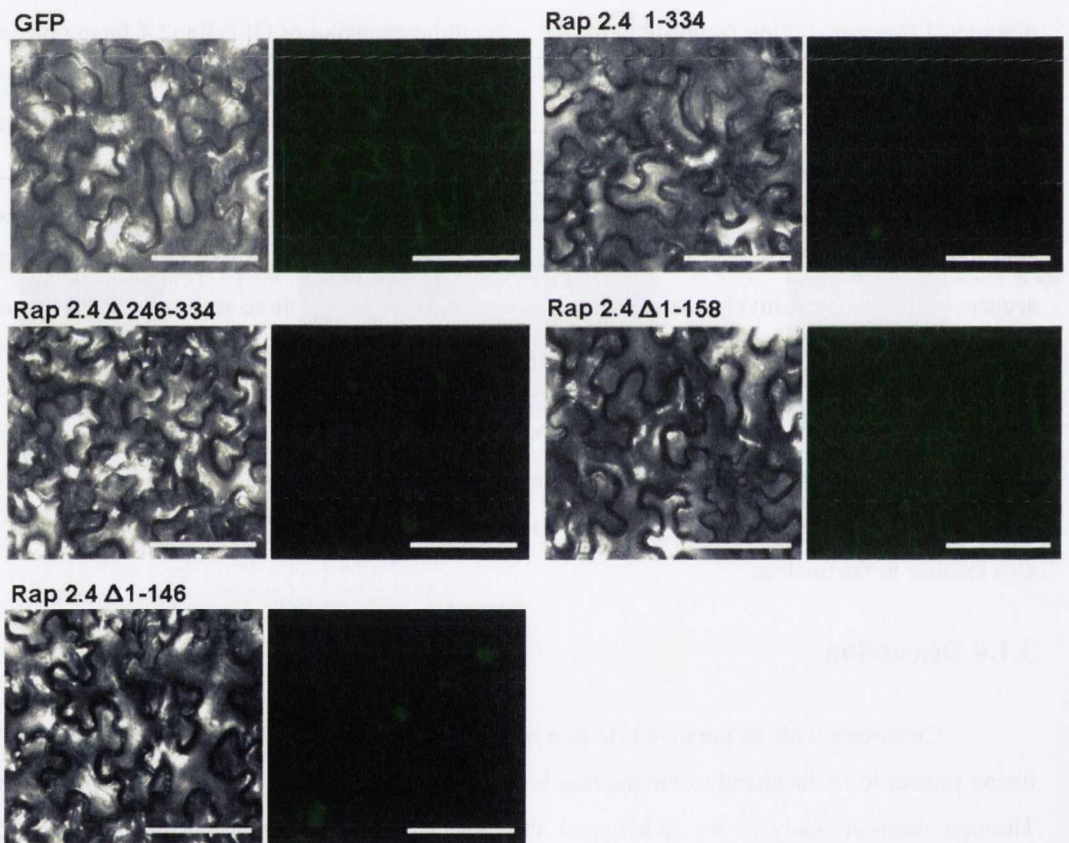


Figure 3.1.5. Determining the nuclear localization signal within the Rap2.4 open reading frame. A) Scheme illustrating the fusion constructs with GFP. Pink bars in the Rap2.4 ORF mark the putative NLS. B) Subcellular localization of GFP, GFP::Rap2.4, GFP::Rap2.4 Δ 246-334, GFP::Rap2.4 Δ 1-158 and GFP::Rap2.4 Δ 1-146, respectively. Images were analysed using fluorescent microscopy at 200X magnification in visible light and UV-light, left and right side image in each picture panel respectively. Scale bar in each image is 10 μ m.

charged residues that make up a functional NLS. In case of oncoprotein c-Myc, the NLS consists of nine amino acids out of which only three are positively charged (PAAKRVKLD). The surrounding neutral and even acidic residues play a crucial role in nuclear localization as demonstrated when the PAA and LD parts of the signal were cloned to flank an inactive basic cluster in the nucleoplasmin protein and turned it into a functional NLS sequence. The tripeptide PAA preceding the basic cluster was shown to be essential whereas the LD dipeptide enhanced the nuclear localization (Makkerh et al., 1996).

The conserved AP2 DNA-binding domain of Rap2.4 starts from the leucine 151 which is the 5th amino acid among the stretch of 12 determined to be sufficient for the nuclear localization. The three arginines of the Rap2.4 NLS are a part of the conserved AP2 DNA-binding domain and are therefore present in most AP2 transcription factors. The three arginine (R) residues in the N-terminus of the AP2 domain are not however sufficient for nuclear targeting. ANT possessing the three abovementioned conserved arginine residues, but carrying mutations in lysine residues located outside the AP2 domain, lost its ability to localize in the nucleus (Krizek and Sulli, 2006). Therefore it can be hypothesized that the two lysine residues (K¹⁴⁷ and K¹⁵⁰) within the twelve amino acids responsible for the nuclear localization of Rap2.4 and GFP fusion protein, are probably necessary but probably not sufficient.

3.2 DNA BINDING SPECIFICITY OF RAP2.4 AND RAP2.4B

3.2.1 Introduction

The majority of *Arabidopsis* transcription factors can be grouped into a few gene families based on the DNA-binding domain they contain. There are 11 major families of transcription factors in plants out of which 5 are considered plant lineage-specific. The plant-specific AP2/ERF transcription factor family consists of 147 members in *Arabidopsis*, all containing the conserved AP2 DNA binding domain of approximately 60 amino acids (Nakano et al., 2006). The ERF (ethylene-response factor) and DREB (DRE-binding protein) subgroups of the AP2/ERF family have one DNA-binding domain, while the AP2 subgroup possess two AP2 domains and the proteins the RAV subgroup have one AP2 domain and one B3 domain.

The AP2 DNA-binding domain of AtERF1 forms a three-stranded anti-parallel β -sheet and an α -helix packed approximately parallel to the β -sheet. According to Allen (1998), the arginine and tryptophan residues in the β -sheet contact 8 of the 9 consecutive base pairs in the major groove and at the same time bind to the sugar-phosphate backbone of DNA. In *Brassica napus* Ala37 of α -helix was found to be crucial for the DNA-binding of DREBIII-1 (Liu et al., 2006).

The DRE-element recognized by DREBs and the GCC-box to which ERF transcription factors bind to, are similar DNA sequences. The DRE-element was discovered in the promoter of drought-responsive gene RD29A. The 9 bp sequence TACCGACAT was determined to act as a *cis*-element essential for RD29Aa induction by dehydration, high salt or low temperature stress (Yamaguchi-Shinozaki and Shinozaki, 1994). The DRE element is responsible for the induction of COR15a and COR6.6 by cold (reviewed in Thomashow, 1999) and has been found to facilitate cold-responsive induction of *Brassica napus* BN115 – an ortholog of *Arabidopsis* COR15a (Jiang et al., 1996). The CCGAC core of the DRE-element was established as the minimal motif that ensures DNA binding of CBF1 (Hao et al., 2002). The bases at positions 1(C), 2(C), 3 (G) and 5 (C) of the CCGAC core have been reported to be essential for specific interactions with DREB proteins whereas substitutions at the position 4 (A) are tolerated (Sakuma et al., 2002).

The GCC-repeat was first found to be an important *cis*-acting element in tobacco ethylene- and salicylic acid-inducible β (1,3)-glucanase and chitinase promoters (Ohme-Takagi and Shinshi, 1990). The 11-bp sequence TAAGAGCCGCC was identified as an essential element for ethylene responsiveness of promoters in tobacco (Ohme-Takagi and Shinshi, 1995). In the GCCGCC core of the GCC-repeat, mutations in the bases 1 (G), 4 (G) and 6 (C) cause the greatest decrease in recognition by ERFs (Hao et al., 1998).

The DREB and ERF proteins recognize similar sequences – the CCGNC motif, where N denotes any nucleotide, could be a potential binding site for proteins from either subgroup – yet largely regulate distinct physiological processes. In the common core, the G and C at positions 3 and 5, respectively, are crucial for interactions with both DREBs and ERFs. However the ERFs require additional 5' G flanking the CCGAC-element and DREBs strictly need a cytosine residue

in the 2nd position of the core sequence. These small differences create a basis for separation between the abiotic and biotic stress signalling pathways (Sakuma et al., 2002).

Generally ERFs and DREBs are thought to function in biotic or abiotic stress pathways, respectively, due to binding to different *cis*-elements. However, recent work has revealed that ERF proteins can often bind multiple *cis*-acting elements and some have been shown to activate the expression of genes containing either of those *cis*-elements. For example, the tobacco ERF gene Tsi1 can bind to both GCC- and DRE-elements. Overexpression of Tsi1 enhances the tolerance to pathogens and salt indicating that the transcription factor is functioning in abiotic and biotic stress response pathways (Park et al., 2001). Tomato JERF3 was also shown to bind to DRE- and GCC-boxes and the overexpression of JERF3 enhanced the tolerance to salt stress and activated the transcription of pathogenesis-related genes (Wang et al., 2004). Other ERF proteins such as NtCEF1 (Lee et al., 2005), and JcERF (Tang et al., 2006) have been demonstrated to bind to both DRE- and GCC-elements but function only in defence or abiotic stress related pathways, respectively. The TaERF1 from wheat binds to DRE and GCC elements and is induced by drought, salinity, low temperature, ethylene and *Blumeria graminis* f. sp. *tritici* infection (Xu et al., 2008b).

One of the aims of the present project was to determine the DNA binding specificity of Rap2.4 and Rap2.4b. The DNA-binding AP2 domain is 100 % conserved between the two proteins suggesting that these transcription factors could share at least some targets. The DNA-binding domains of the other 8 transcription factors in the A-6 subgroup in the DREB subfamily differ from the AP2 domains of Rap2.4 and Rap2.4b by 3-11 amino acids (Chapter 2, Figure 2.8).

Based on amino acid sequence comparison with well characterized members of the AP2/ERF transcription factor family, both proteins are likely to control transcription of target genes containing DRE/CRT *cis*-element in their promoters. Rap2.4 and Rap2.4b contain valine and leucine residues in the conserved 14th and 19th positions of the AP2 domain which are known to be characteristic of DRE-binding proteins (Sakuma et al., 2002). In order to clarify the specific sequences these transcription factors bind to, electrophoretic mobility shift assays (EMSA) were performed.

The EMSA assays are based on the observation that protein-oligonucleotide complexes move slower in non-denaturing poly-acrylamide gels than free oligonucleotides. We carried out the EMSA experiments using a system which employs two fluorescent dyes (Jing et al., 2003): the SYBRGreen dye is used to visualise nucleic acids and SYPRORuby is used to stain proteins in the polyacrylamide gels following electrophoresis. As the SYBRGreen dye stains all the nucleic acids present in the acrylamide gel, this method does not facilitate competitor assays. However this was not a significant disadvantage for our experiments.

The purified transcription factor proteins required for the gelshift assays were obtained using an expression system based on pET vectors in *Escherichia coli* (Novagen). The pET system facilitates inducible high-level production of recombinant proteins with various tags for later purification steps. Protein coding sequences of Rap2.4 and Rap2.4b were cloned to pET-32a(+) vectors and the resulting vectors were used to transform the *E. coli* BL21 (DE3) host strain. The

pET-32a(+) plasmid is designed to produce proteins fused with the Trx-tag which enhances the solubility of the expressed protein. Expressed proteins also contained one or two His₆-tags for the subsequent purification and detection steps.

3.2.2 Materials and methods

3.2.2.1 Plasmids

AV544129 - was obtained from Kasuza DNA Research Institute (Asamizu et al., 2000). It is a Bluescript II SK vector with the Rap2.4 cDNA cloned between EcoRI and XhoI restriction enzyme sites (clone RZ34b01).

pET-32a(+) is a commonly used protein expression vector by Novagen (Cat. No.69337-3). The pET-32a(+) plasmid is designed for cloning and high-level expression of proteins under the control of T7 promoter and terminator. The expressed proteins are fused with the thioredoxin tag in order to enhance solubility. Cloning sites are available for expressing proteins fused with cleavable His₆ tag and S-tag for detection and purification. The vector carries an ampicillin resistance conferring gene for selection.

3.2.2.2 Oligonucleotide primers

A) To amplify the Rap2.4 ORF for construction of pET32-Rap2.4:

R24orfFNco: 5'-TGCCATGGCAGCTGCTATGAATTTGTA-3'

R24orfnsRXho: 5'-CCCTCGAGAGCTAGAATCGAATCCCAATCG-3'

B) To amplify the Rap2.4b ORF for construction of pET32-Rap2.4b:

Rap2.4borfFEcoRI: 5'-CGGAATTCATGACAACCTTCTATGGATT-3'

Rap2.4bnsRXho:5'-CCCTCGAGATTTACAAGACTCGAACACT - 3'

D) To sequence the pET32-Rap2.4 and pET-Rap2.4b:

T7: 5' TAATACGACTCACTATAGG-3'(available on the MWG-Biotech AG).

Underlining indicates sequences added in order to create restriction sites.

3.2.2.3 Protein expression in *E. coli*

Transformed BL21-DE3 strains containing plasmids for protein expression were streaked from glycerol stocks onto selective plates and incubated at 37°C overnight. A single colony was inoculated into 2 ml LB broth with added antibiotics and glucose (carbenicillin 50 mg/l, 1 % glucose) for each strain. Precultures were grown at 37°C, 250 rpm to mid-exponential stage (OD₆₀₀=0.4-0.6), cells were collected by centrifugation at 4000 rpm for 10 minutes and resuspended in fresh LB broth. The preculture was subcultured (1/100 dilution) to 50 ml fresh LB with high antibiotic content (Carbenicillin 500 mg/l, 1 % glucose) to prepare the culture for induction of protein expression. When the culture reached OD₆₀₀=0.4-0.6, cells were centrifuged and resuspended in same volume of fresh medium (LB with carbenicillin 500 mg/l, no glucose). Expression of target genes was induced by adding isopropyl β-D-thiogalactoside (IPTG) to final concentration of 0.4 mM to the culture. Induction was carried out at 28°C, 250 rpm. Samples were collected 4 h after induction and stored as cell pellets at -20°C.

3.2.2.4 Protein extraction and SDS-PAGE

Soluble and insoluble proteins were separated following sonication by centrifugation. The cell pellets of induced cultures were suspended in CelLytic B buffer (Sigma) to extract recombinant proteins under non-denaturing conditions. Lysozyme and the protease inhibitor PMSF were added to a final concentration of 0.1 mg/ml and 1 mM, respectively, and lysates were incubated for 15 minutes at room temperature with gentle shaking. Sonication was carried out with Labsonic U tipsonifier by Braun, Germany (five 15 second bursts, 30 second breaks). During the procedure samples were kept on ice to prevent heating. Following centrifugation at 16 000 rpm 4°C, supernatant containing soluble proteins was removed and the pellet containing insoluble proteins suspended in 1X SDS-PAGE loading buffer (50mM Tris-HCl, pH 6.8; 2 % SDS, 10 % glycerol, 0.1 % β -mercaptoethanol, 0.1 % bromophenol blue). For assessing the yield of expression, integrity of recombinant proteins and the amount of soluble proteins, samples of total lysates, soluble protein and insoluble protein were separated on precast 10 % polyacrylamide gels (NuPAGE® Bis-Tris Gels, Invitrogen). Proteins were visualised by GelCode Blue (Pierce) staining or by Western blotting.

3.2.2.5 Western blotting

Protein samples were separated by SDS-PAGE and transferred onto Protrans nitrocellulose membrane (Scheicher & Schuell) using a Scie-Plas semidry blotter V10-SDB (60 mA, 2 hours, transfer buffer: 25 mM Tris pH=7.5, 75 mM glycine, 1 mM SDS in 20 % methanol). The transfer efficiency was checked by staining with Ponceau S solution (Sigma). The nitrocellulose membranes were blocked for 20 minutes in 3 % non-fat dry milk (Marvel, Chivers Ireland Ltd.) solution in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10mM Na₂HPO₄, 1.8 mM KH₂PO₄). The membrane was incubated with primary mouse anti-His6 antibody (Boehringer Mannheim) 1:5000 dilution in blocking solution overnight at 4°C, washed twice with water and incubated in secondary antimouse IgG-alkaline phosphatase conjugated antibody (Sigma) 1:10 000 dilution in blocking buffer for 2 hours. Non-hybridized antibody was removed by washing the blot once with PBS with added 0.05 % Tween 20 and several times with water. The blot was incubated for 30 minutes in APB buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl) to achieve suitable pH for alkaline phosphatase mediated detection. For visualisation BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) and NBT (Nitro-blue tetrazolium chloride) mix by Roche was added to APB buffer. Images were taken with Sony DCS-P32 digital camera.

3.2.2.6 Protein purification and concentration

Proteins expressed in *E. coli* with added His6 tags were purified by His6-Bind Quick 300 Cartridges by Novagen. The columns were equilibrated prior to protein purification with a buffer containing 0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9. The solutions containing only soluble proteins were applied to Ni²⁺-affinity columns and the columns were washed twice (first with equilibration buffer and then with 0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9) to remove non-tagged proteins. Proteins with 6-His tags were eluted with buffer containing 1 M imidazole in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. Protein samples were concentrated and

desalted with Microcon Centrifugal Filter Devices YM-10 (Millipore) by applying elute to the filter device and spinning at 12 000 rpm in the cold room. The integrity and concentration of proteins was assessed by running samples on on pre-cast 10 % polyacrylamide gels (NuPAGE® Bis-Tris Gels by Invitrogen) and staining gels with GelCode Blue (Pierce).

3.2.2.7 EMSA assay

The protocol for the assay was adapted from Lee and Kim (Lee and Kim, 2003). The single basepair substitutions to the DRE/CRT and GCC-element core were based on the results of Sakuma et al. (2002). The oligonucleotide primer sequences used for making the probes for EMSA assay are described in the following table.

<i>Cis</i> -element	Primer	Primer sequence 5' to 3'
DRE	DREfor DRErev	ATTTTCATGG CCGAC CTGCTTTTAAGCTTTT AAAAGCTTAAAAGCAG GTCG GCCATGAAAT
m1DRE	m1DREfor m1DRErev	ATTTTCATGG T CGA CTGCTTTTAAGCTTTT AAAAGCTTAAAAGCAG GTCG A CCATGAAAT
m2DRE	m2DREfor m2DRErev	ATTTTCATGG CTG A CTGCTTTTAAGCTTTT AAAAGCTTAAAAGCAG GTC A GCCATGAAAT
m3DRE	m3DREfor m3DRErev	ATTTTCATGG CC A CTGCTTTTAAGCTTTT AAAAGCTTAAAAGCAG GTT IG GCCATGAAAT
m4DRE	m4DREfor m4DRErev	ATTTTCATGG CCG GC CTGCTTTTAAGCTTTT AAAAGCTTAAAAGCAG GC CG GCCATGAAAT
m5DRE	m5DREfor m5DRErev	ATTTTCATGG CCGA A CTGCTTTTAAGCTTTT AAAAGCTTAAAAGCAG TT CG GCCATGAAAT
ΔDRE	ΔDREfor ΔDRErev	ATTTTCATGCTGCTTTTAAGCTTTT AAAAGCTTAAAAGCAGCATGAAAT
GCC	GCCfor GCCrev	CATAAGAG CCGCC ACTAAAAT ATTTTAGT GGCGG CTCTTATG
mGCC	mGCCfor mGCCrev	CATAAGAT TCCT CCACTAAAAT ATTTTAGT GGAGG ATCTTATG

The DRE- or GCC-elements within the oligonucleotides are shown in bold lettering; underlining marks single basepair substitutions in the core of *cis*-elements.

Oligonucleotide annealing was carried out by mixing the forward and reverse single-stranded oligonucleotides in 0.2 X SSC (30 mM NaCl, 3 mM sodium citrate, pH 7.0) buffer. The mix was incubated at 95°C for 5 minutes and then cooled down to room temperature over several hours. Binding reactions contained 150 nM annealed oligonucleotides, 10 mM Tris-HCL, 1 mM EDTA, 1mM DTT, 50 mM NaCl, 5 % glycerol and 2 µg of protein of interest. Binding-reaction was incubated on ice for 40 minutes and then loaded to 6 % native polyacrylamide gel (6 % bis-acrylamide 29:1, 45 mM Tris-HCl pH 8.3, 45 mM boric acid, 1 mM EDTA, 0.075 % TEMED, 0.075 % ammonium persulfate). The gel was run at 4°C in TBE buffer (45 mM Tris-HCl pH 8.3, 45 mM boric acid, 1 mM EDTA) at 120V for 25 minutes.

Detection was carried out using the EMSA kit supplied by Molecular Probes Inc. and the accompanying protocol. Nucleic acids were visualised by staining the gel with SYBRGreen I

nucleic acid dye (1: 10 000 dilution in TBE) for 20 minutes in the dark. For detecting proteins gel was incubated in SYPRORuby solution overnight in the dark and destained in 10 % methanol, 7 % acetic acid before capturing the image. Images were recorded using the UV transilluminator (UVP Inc.).

3.2.3 Results

3.2.3.1 Construction of plasmids for protein expression in *E. coli*

Protein coding sequences of Rap2.4 and Rap2.4b excluding the stop codons were amplified by PCR using the oligonucleotide primers R24orfFNco and R24orfnsRXhoI for Rap2.4 ORF; Rap2.4borfFEcoRI and Rap2.4bnsRXho for Rap2.4b. The primers were designed so that the PCR fragments were flanked by NcoI and XhoI sites for Rap2.4 and by EcoRI and XhoI sites for Rap2.4b. The cDNA clone RZ34b01 was used as a template for amplifying the Rap2.4 protein coding sequence. Rap2.4b was amplified using plant chromosomal DNA as the template. The PCR resulted in amplified fragments of 1002 bp for Rap2.4 and 783 bp for Rap2.4b.

The amplified Rap2.4 ORF was digested with NcoI and XhoI, the Rap2.4b ORF with EcoRI and XhoI and cloned into corresponding sites of the pET-32a(+) vector. The Rap2.4 ORF was in frame with both N-terminal and C-terminal His₆ tags (Novagen), and the Rap2.4b ORF with only the N-terminal His₆ tag. The resulting plasmids were called pET32-Rap2.4 and pET32-Rap2.4b (Figure 3.2.1, panel A). The plasmids were verified by restriction enzyme analysis and by sequencing using the T7 primer available on the MWG Biotech AG website. The restriction digest of pET32-Rap2.4 with NcoI and XhoI and pET32-Rap2.4b with EcoRI and XhoI, gave fragments of 1002 and 783 bp, respectively (Figure 3.2.1, panel B).

3.2.3.2 Analysis of protein expression in *E. coli*

The pET32-Rap2.4 and pET32-Rap2.4b plasmids were transformed into the expression host *Escherichia coli* strain BL21 (DE3). Protein expression was induced by adding IPTG to the culture and the soluble protein fraction was recovered after sonication and centrifugation. Western blot analysis with anti-His antibody revealed that no Rap2.4 or Rap2.4b protein was expressed prior to induction (lanes marked with 1 on Figure 3.2.2, panel A). Following 4h induction with IPTG at 28°C, Rap2.4 and Rap2.4b proteins with added tags accumulated up to 20-30 µg and 75-80 µg per 1 ml of induced culture, respectively (lanes marked with 2 on panel A on Figure 3.2.2 were loaded with samples of total lysis, each lane was loaded with sample representing 0.1 ml of culture).

In different expression batches 50-100 % of Rap2.4 protein was in a soluble form (compare lanes 3 and 4 - soluble and insoluble fractions, respectively - on the left hand sides of Coomassie gel and Western blot on part A of Figure 3.2.2). For Rap2.4b, soluble protein also accounted for around 50 % of total target protein (compare lanes 3 and 4 - soluble and insoluble fractions, respectively - on the right hand sides of Coomassie gel and Western blot on part A of Figure 3.2.2).

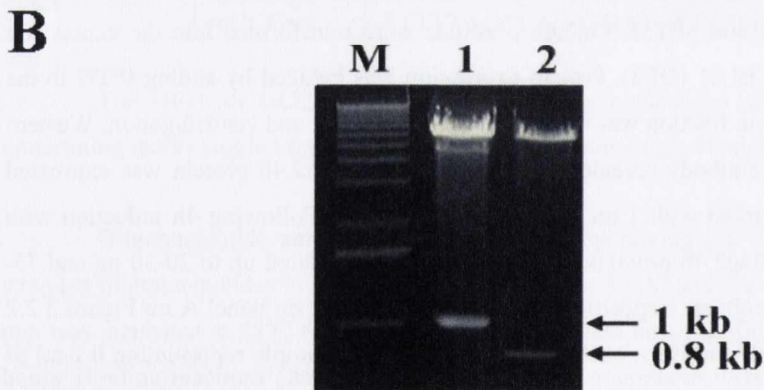
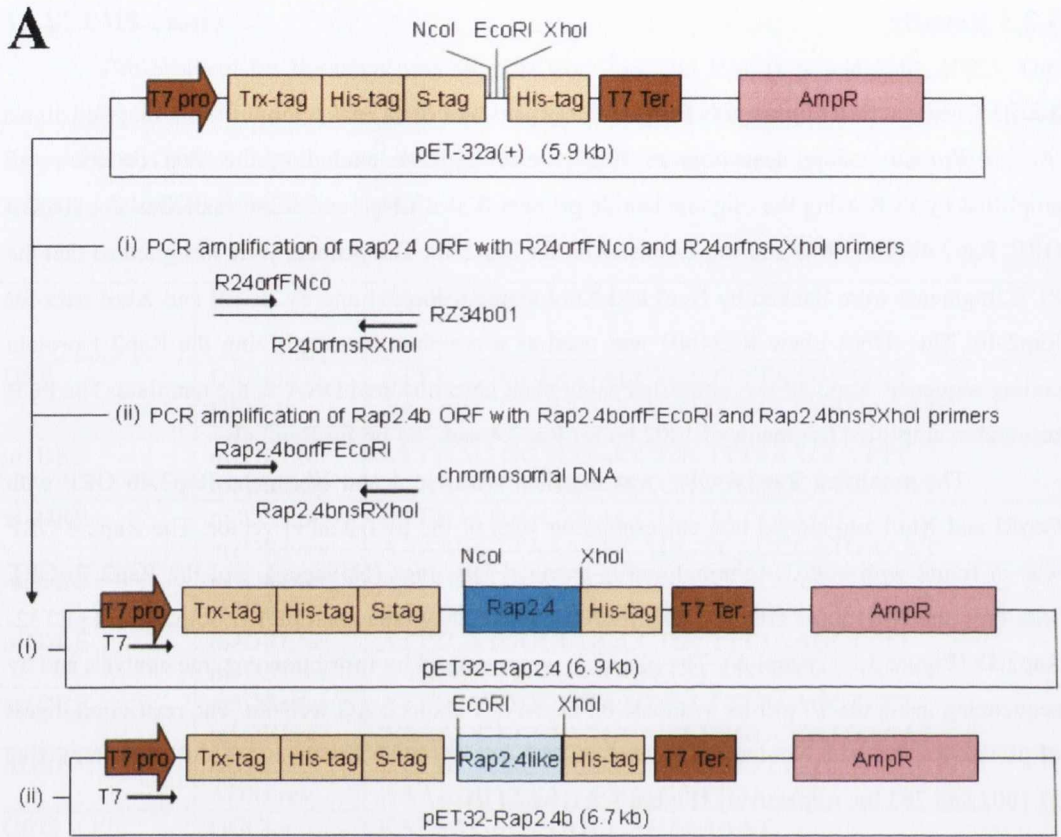


Figure 3.2.1. Construction of pET32-Rap2.4 and pET32-Rap2.4b plasmids. A) Cloning plan. Restrictions sites shown were used for cloning fragments into the pET32a(+) vector. The primers used for PCR amplifications and sequencing are indicated with black arrows. B) Plasmid DNA was isolated and digested with XhoI and NcoI or XhoI and EcoRI restriction enzymes for pET-Rap2.4 and pET-Rp2.4b, respectively. Digestion of pET32-Rap2.4 gave a fragment of 1002 bp (lane 1), digestion of pET32-Rap2.4b a fragment of 783 bp (lane 2). M - molecular weight marker.

The fusion tags in the pET-32a(+) vector add 20.4 kDa to the molecular weight of the expressed protein. Rap2.4 protein has molecular weight of 36.61 kDa and Rap2.4b of 29.14 kDa; the fusion proteins are expected to have molecular weights of 57.01 kDa and 49.5 kDa, respectively. The size of the His-tagged protein bands observed in the Western blot suggests that Rap2.4 and Rap2.4 recombinant proteins carry appropriate tags and are stable in *E. coli* cells.

3.2.3.3 Protein purification

The Rap2.4 and Rap2.4b proteins fused with appropriate tags and the tags alone expressed in *E. coli* were purified using His-Bind Quick Cartridges from Novagen. The eluted fractions were concentrated and desalted with Microcon Centrifugal Filter Devices. As seen from Figure 3.2.2 panel B, the purification procedure successfully removed soluble non-His-labelled proteins. On the average up to 30 % of total soluble protein was recovered after purification and concentration steps with the final concentration of protein of approximately 0.5-1 µg/µl.

3.2.3.4 The EMSA assay

Binding of the Rap2.4 and Rap2.4b to the DRE/CRT- or GCC-boxes was examined by using gel-retardation assay using an EMSA kit from Molecular Probes Inc. (Invitrogen).

Initially, the assay was optimized using LPI (*Capsicum annuum* DREB-like protein1) protein obtained from Woo Taek Kim in Yonsei University and a 24 bp double-stranded DRE/CRT-element oligonucleotide that has been shown to bind to LPI as a positive control for the assay (Hong and Kim, 2005). It was found that at least 0.8 µg of LPI protein and 300 ng of double-stranded oligonucleotides were necessary to see a bandshift using the abovementioned detection system. Regarding the DNA-binding ability of Rap2.4, at least 1 to 1.5 µg of purified protein was needed to observe a bandshift (Figure 3.2.3, panels A and B, respectively).

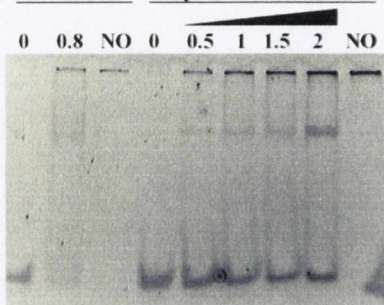
In order to confirm that the observed band-shift is specific to Rap2.4 and Rap2.4b proteins, the 20.4 kDa tags of pET32a vector were purified and used for a gel-shift assay. As expected, the tags alone could not bind to the wild type DRE/CRT *cis*-element containing oligonucleotides (Figure 3.2.3 panel C, lane 2). Rap2.4b purified protein was used a positive control (Figure 3.2.3 panel C, lane 1).

In further experiments Rap2.4 and Rap2.4b binding specificities to DRE/CRT element were further investigated using oligonucleotides where single bases in the *cis*-element core CCGAC were substituted (Figure 3.2.4).

It was determined that the most crucial nucleotides for Rap2.4 binding in the core sequence of the DRE/CRT are the C1 and C5. Replacing C1 with T (oligonucleotide m1DRE) reduced the binding with Rap2.4 to residual levels, while substituting C5 with A (oligonucleotide m5DRE) abolished binding completely. Rap2.4 bound weakly to an oligonucleotide where C2 was replaced by T (m2DRE). Altering G3 to A (m3DRE) and A4 to G (m4DRE) had less influence on the binding with Rap2.4 (Figure 3.2.4, panel A). The 4th basepair in the DRE/CRT-core has been demonstrated to be replaceable with any base in the case of DREB1A and DREB2A, members of the DREB subfamily closely related to Rap2.4 (Sakuma et al., 2002). Panel B of Figure 3.2.4

A

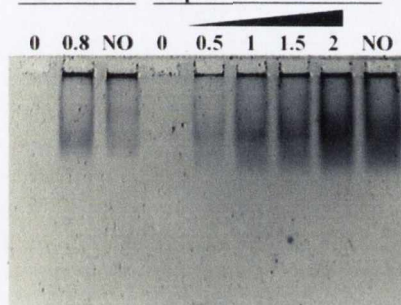
SYBRGreen staining of nucleic acids
CaDREBLP1 Rap2.4



Oligo - DRE/CRT - ATTCATGG CCGAC CTGTTTTAAGTTTT

B

SYPRORuby staining of proteins
CaDREBLP1 Rap2.4

**C**

SYBRGreen staining of nucleic acids



1 Rap2.4b
2 20.4 kDa fusion tag of
pET32a

Oligo - DRE/CRT - ATTCATGG CCGAC CTGTTTTAAGTTTT

D

Coomassie staining of proteins



1 Rap2.4b
2 20.4 kDa fusion tag of
pET32a

Figure 3.2.3. Experiment optimization process for gel-shift assays. A) Increasing concentrations of LP1 and Rap2.4 protein were used for optimising the assay. Numbers indicate protein amounts in micrograms. NO – no oligonucleotides in the binding mix, negative control. The oligonucleotide sequence used in this bandshift was the WT DRE (oligonucleotide sequences are shown in underneath panel A. B) SYPRORuby staining of proteins in gel shown on panel A. C) DRE/CRT containing oligos were mixed with Rap2.4b purified protein (lane 1) and 20.4 kDa tags of pET32a vector (lane 2). Nucleic acids were stained with SYBERGreen. D) Proteins loaded onto gel shown in panel C were stained with Coomassie Blue.

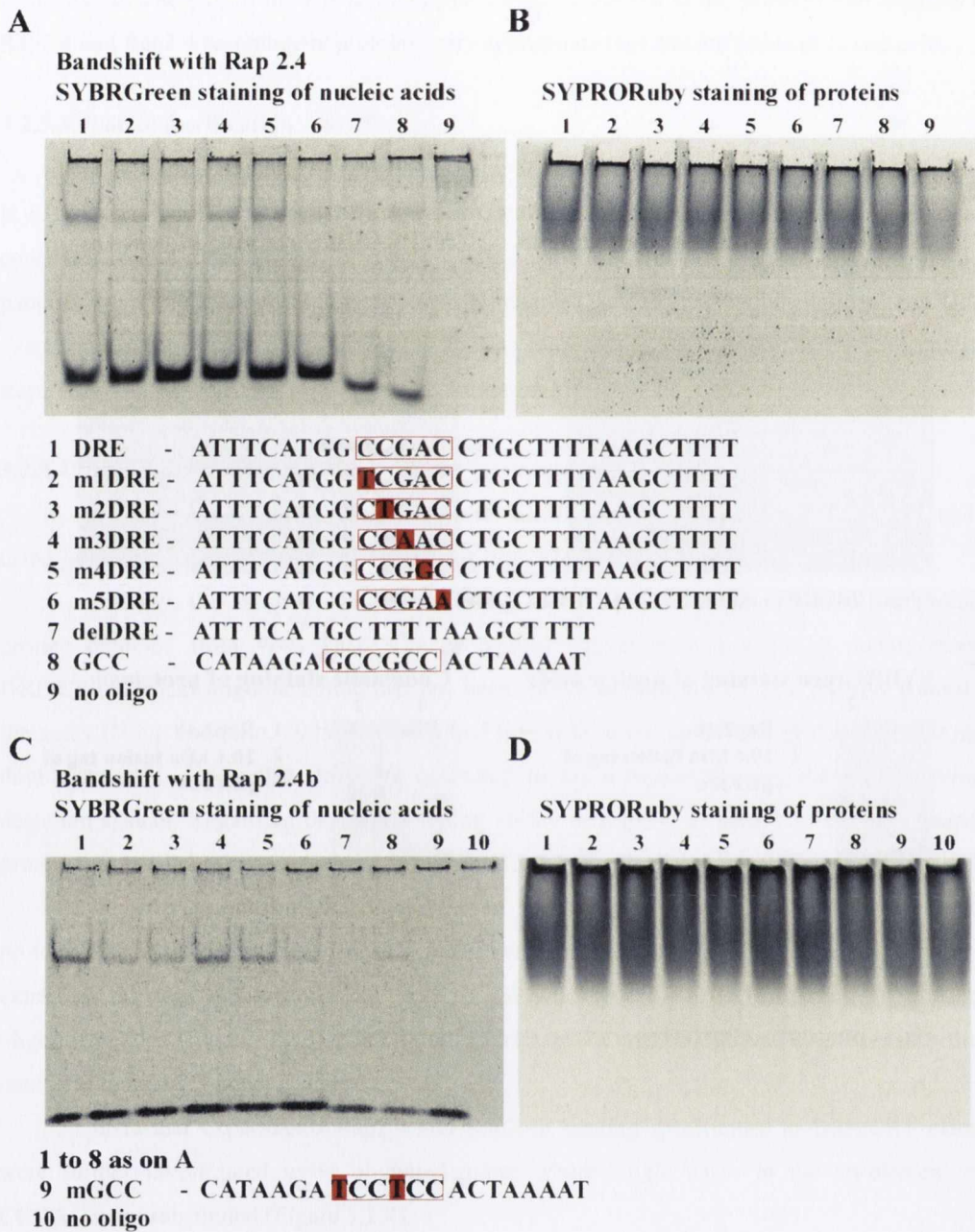


Figure 3.2.4. Gel-shift analysis of Rap2.4 and Rap2.4b DNA-binding specificity. A) and C) Rap2.4 and Rap2.4b binding specificities to DRE/CRT-element were investigated by using double-stranded oligonucleotides with single basepair substitutions. Numbers indicate different oligonucleotides, the sequences of which are shown underneath the panels A and C. *Cis*-element cores are outlined by red boxes and substituted basepairs are marked by red background. Nucleic acids were stained with SYBERGreen (Panels A and C), SYPRORuby staining of Rap2.4 and Rap2.4b proteins is shown in B and D, respectively.

shows that equal amounts of Rap2.4 proteins were used for the binding reactions with different oligonucleotides.

Rap2.4b was found to have a binding specificity similar to Rap2.4. Mutations in the C5 (m5DRE) in the DRE-element core eliminated the binding of Rap2.4b to the oligonucleotides. Replacing C1 (m1DRE) or C2 with T (m2DRE) weakened the binding of Rap2.4b considerably as was previously observed in case of Rap2.4. Altering G3 or A4 had little effect (Figure 3.2.4, panel C). Panel D of Figure 3.2.4 shows that equal amounts of Rap2.4b proteins were used for the binding reactions with different oligonucleotides.

Both Rap2.4 and Rap2.4b showed residual binding to the GCC-box (lanes marked with 8 on panels A and C on Figure 2.2.4).

3.2.4 Discussion

In the case of the MADS transcription factors AG, AGL1 and AGL2 it was shown that the amino acid sequence similarity of the DNA-binding domain correlated with similarity for preferred binding sites. The binding affinities towards particular recognition sequences differed between proteins thereby creating a basis for functional specialization through target site selection (Huang et al., 1996).

The DNA-binding domains within the DREB subgroup are very similar and all the proteins are either known or expected to bind the CCGAC core of the DRE *cis*-element. However the physiological roles, under abiotic stress conditions, carried out by this subgroup of transcription factors vary from increasing tolerance to heat, drought, cold or high salinity. At the cellular level these stresses have some common aspects such as water deficit, but also require activation of additional specific sets of response genes. The appropriate regulation of target genes could be due to variations in DNA binding site selection between DREBs, especially regarding the sequences in the immediate vicinity of the CCGAC core, between DREBs.

In the DREB subgroup in *Arabidopsis*, DNA binding specificity has been studied in detail for only a limited number of proteins and the best studied examples from the subfamily are the DREB1A and DREB2A transcription factors. Both are involved in abiotic stress response pathways; the former in cold, and the latter in heat and drought signalling. The two proteins share 78 % similarity in the AP2 domain and were both shown bind to the CCGAC core tolerating substitutions at the 4th position (Sakuma et al., 2002). DREB2A and DREB1A regulate an overlapping set of cold and drought responsive target genes, such as RD29A, COR6.6 and COR15A, through the DRE-element in the respective promoters in addition to specific targets for each transcription factor. DREB1A and DREB2A recognise their specific targets due to subtle differences in their preferences regarding the basepairs flanking the CCGAC core (Sakuma et al., 2002).

Rap2.4 and Rap2.4b share 100 % amino acid identity in the DNA-binding domain and display similar DNA-binding preferences. The importance of single basepairs within the core recognition sequence was similar to that of other DREBs with C5 being crucial and A4 being dispensable for successful binding. Both Rap2.4 and Rap2.4b showed residual binding to the

GCC-box. Dual binding has been reported previously for a number of ERF-proteins. In addition to examples mentioned in the introduction, the LP1 protein of hot pepper (Lee et al., 2004) and Tsi1 of tobacco (Park et al., 2001) have all been shown to bind to both GCC- and CRT/DRE elements. GCC-elements differ functionally from DRE/CRT elements in that they are generally present in promoters of genes participating in biotic stress signal transduction networks as opposed to abiotic stress pathways. The dual binding observed for some DREF and ERF transcription factors might indicate that these stress networks overlap.

Based on their DNA-binding specificity, Rap2.4 and Rap2.4b target gene sets should be at least somewhat overlapping. However the two DREBs are not identical outside the DNA-binding domain and could therefore interact in a dissimilar manner with other proteins (Figure 2.7). In addition, the different expression patterns reported earlier for Rap2.4 and Rap2.4b suggested distinct physiological functions. Rap2.4 might mediate abiotic stress responses in root tissues whereas Rap2.4b might function under similar conditions in seeds.

3.3 TRANSACTIVATION ABILITY OF RAP2.4 AND RAP2.4B

3.3.1 Introduction

Transcription factors are described as modular proteins containing a variety of domains for DNA binding and interaction with other molecules such as signalling factors or modifying proteins and components of the transcription complex. Such modularity facilitates the evolution of transcription factors with distinct properties from a relatively small number of component domains.

Transcription factors can function as activators or repressors, depending on the effect they have on transcription of target genes. The regulatory effect is often mediated by the binding of TFs to specific sequences (called the *cis*-elements) in the promoters of the target genes. When the DNA-binding domains of TFs are highly conserved, the activation/repression domains are often highly divergent. For this reason it has been relatively easy to characterize the former; whereas less attention has been paid to the latter. In the case of most eukaryotic activation domains defined functionally, the consensus sequences are not obvious (Schwechheimer and Bevan, 1998). Studies in yeast have shown activation domains often to be enriched in acidic amino acids, glutamine, proline or serine/threonine residues although this is not always important for function. Functional domains outside the DNA-binding domain conveying properties such as protein-protein interactions, nuclear localization etc.; are often conserved among proteins belonging to a specific subgroup in large TF families (Liu et al., 1999) and determining the activation domain of Rap2.4 could provide valuable information regarding the function of other DREB proteins.

The best characterized transcriptional regulation domain present among the AP2/ERF proteins contains the [L/F]DLN[L/F]xP motif. The motif is found in five ERF transcription factors and referred to as ERF-associated amphiphilic repression (EAR) domain (Ohta et al., 2001). The EAR domain gives Arabidopsis transcription factors AtERF3 and AtERF4 the ability to actively repress GCC-box mediated transcription (Fujimoto et al., 2000; Ohta et al., 2001). Similar xLxLxL

motifs are found within the repression domains of SUPERMAN and other TFIII-type C2H2 zinc-finger proteins where they are also important in conferring repression activity (Hiratsu et al., 2004).

However, the majority of DREBs investigated for their transactivation properties, have been shown to act as activators. CBF1 activated transcription by binding to DRE and GCC elements in transient assays carried out in *Arabidopsis* leaves (Hao et al., 2002). *Arabidopsis* DREB2A and TINY were demonstrated to act as transcriptional activators in *Arabidopsis* protoplasts and tobacco BY2 cells, respectively (Sakuma et al., 2006a; Sun et al., 2008). DREBs from various other plant species such as DmDREBa and DmDREBb of chrysanthemum, TaAIDFa of wheat, CaDREBLP1 of hot pepper and PpDBF1 from non-vascular plant *Physcomitrella patens* were all demonstrated to function as transactivating factors in yeast one-hybrid experiments (Hong and Kim, 2005; Liu et al., 2007; Xu et al., 2008b; Yang et al., 2009b).

In *Brassica napus* two distinct groups of DREBs were found – Group I consisting of two and Group II comprising five proteins. The transcription factors of both groups were able to bind the DRE-element but only the transcription factors in Group I possessed transactivation activity. In response to stress treatments the expression of Group I preceded that of Group II. These observations suggested that Group I proteins open the DRE-mediated signalling pathway and Group II factors close it in a competitive manner (Zhao et al., 2006).

In soya bean GmDREBa and GmDREBb were able to activate the expression of reporter genes whereas the GmDREBc could not. GmDREBa and GmDREBb were induced by stress treatments whereas GmDREBc was expressed constitutively. All three were shown to specifically bind to the DRE-element and it could be speculated that GmDREBc acts similarly to Group II DREB proteins from *Brassica napus* (Li et al., 2005).

In characterizing Rap2.4 and Rap2.4b, one of the aims was to determine if the proteins function as transcriptional activators or repressors by using a transactivation assay based on the interaction between the AP2 domain and the DRE *cis*-element. DRE-elements taken out of their native context retain the ability to mediate inducibility in the context of a synthetic promoter consisting of tandem repeats of DRE element and a minimal CaMV 35S promoter which has been shown to respond to abiotic stresses (Rushton et al., 2002). In our experiments a construct containing the GUS reporter gene linked to 4 repeats of DRE and a minimal TATA-box element (Rushton et al., 2002) was used to test the ability of Rap2.4 to transactivate transcription and to elucidate which region of the Rap2.4 open reading frame contains the activation domain. A scheme of the GUS reporter construct is shown in Figure 3.3.2, panel B. We also carried out the experiments with Rap2.4b in order to investigate whether the transcription factor functions as an activator of reporter gene expression.

The agroinfiltration technique was chosen for transient expression in *Nicotiana benthamiana* leaves. The NAN reporter gene (Kirby and Kavanagh, 2002) under the control of the constitutive CaMV 35S promoter was included in the experiment as an internal control (Figure 3.3.2, B). NAN reporter gene expression was not expected to be affected by the transcription

factors under investigation and could therefore be used as an internal standard by which to normalise GUS expression data.

The NAN gene is a codon optimised version of a *nanH*, a cytoplasmic sialidase from *Clostridium perfringens*. The NAN reporter gene is efficiently expressed in both transiently and stably transformed plant cells and does not require extraction or assay buffers that differ from those used with the GUS reporter gene system. Upon addition of suitable substrates both GUS and NAN activities yield fluorescent products that can be measured simultaneously (Kirby and Kavanagh, 2002).

3.3.2 Materials and methods

3.3.2.1 Plasmids used

AV544129 - was obtained from Kasuza DNA Research Institute (Asamizu et al., 2000). It is a Bluescript II SK vector with the Rap2.4 cDNA cloned between EcoRI and XhoI restriction enzyme sites (clone RZ34b01).

pGPTV-4DRE – (Rushton et al., 2002) is a binary vector based on pBT10-GUS (Sprenger-Haussels and Weisshaar, 2000) that contains 4 copies of the DRE/CRT-box located upstream of CaMV minimal promoter, followed by GUS gene and the NOS terminator. The plasmid was a kind gift from Dr. Somssich in the Max Planck Institute for Plant Breeding Research, Cologne, Germany. See Figure 3.3.2, B.

pROK2-NAN – (Kirby and Kavanagh, 2002) contains the NAN reporter gene under the control of the CaMV35S promoter and NOS terminator in the pROK2 binary vector (see section 3.1.2.1). The pROK2-NAN is available in the Kavanagh lab. The pROK2-NAN vector is shown in Figure 3.3.2, B.

pFGC-CaMV-nos – see section 3.1.2.1

3.3.2.2 Oligonucleotide primers

Oligonucleotide primers used for making constructs for the transactivation assay.

Construct	Primer	Oligonucleotide sequence 5' to 3' orientation
Rap2.4 FL	Rap24orfFXbaI	GCTCTAGAAATGGCAGCTGCTATGAATTTG
	Rap24orfRBamHI	CGGGATCCCTAAGCTAGAATCGAATCCCAA
Rap2.4ΔN	Del 1-146FXbaI	GCTCTAGAAATGAAGCCGACGAAGCTTTACA
	Rap24orfRBamHI	CGGGATCCCTAAGCTAGAATCGAATCCCAA
Rap2.4ΔC	Rap24orfFXbaI	GCTCTAGAAATGGCAGCTGCTATGAATTTG
	Del261-334RBamHI	CGGGATCCCTACTTCTTCTCACGTTTCTTCG
Rap2.4ΔN/ΔC	Del 1-146FXbaI	GCTCTAGAAATGAAGCCGACGAAGCTTTACA
	Del261-334RBamHI	CGGGATCCCTACTTCTTCTCACGTTTCTTCG
Rap2.4b	R24borfFXbaI	GCTCTAGATGACAACCTTCTATGGATTTTAC

	R24borfRBamHI	<u>CGGGATCCCTAATTTACAAGACTCGAACA</u>
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Restriction sites added for cloning purposes are indicated by underlining. The start and stop codons are marked by bold lettering.

Sequencing primer: CaMV1: 5'- CCTCCTCGGATTCCATTGCCAGCTA-3'

3.3.2.3 Quantitative fluorometric GUS/NAN enzymatic activity assay

Agroinfiltration was carried out as described in section 3.1.2.3.

GUS and NAN activity was quantified as described by Jefferson et al. (1987). Approximately 50-200 mg of plant tissue was homogenised in a double volume of GUS extraction buffer (50 mM NaH₂PO₄, 10 mM EDTA, 0.1 % Triton X-100, 0.1 % sodium lauryl sarcosine, 10 mM β-mercaptoethanol). 2 µl of plant extract was mixed with 100 µl GUS assay (50 mM NaH₂PO₄, 10 mM β-mercaptoethanol, 0.1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide) or NAN assay (50 mM NaH₂PO₄, 10 mM β-mercaptoethanol, 0.04 mM 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid) buffer. The reaction was incubated at 37 °C for one hour and stopped by adding 100 µl 0.4 M Na₂CO₃. Fluorescence was measured using a FLUOstar OPTIMA microplate based multi-detection reader by BMG Labtech (excitation at 365 nm, emission at 455 nm).

3.3.3 Results

3.3.3.1 Construction of plasmids

The full-length and truncated versions of the Rap2.4 open reading frame were amplified by PCR using primers outlined in section 3.3.2.2. The PCRs amplified fragments of 1005 bp for the full-length Rap2.4, 570 bp for Rap2.4Δ1-146; 786 bp for Rap2.4Δ261-334 and 351 bp for Rap2.4Δ1-146/Δ261-334. For Rap2.4b, PCR gave a fragment of 786 bp.

All the fragments amplified were flanked by XbaI and BamHI sites. XbaI and BamHI were used for cloning the amplified fragments into respective sites of the pFGC-CaMV35S-nos vector. The resulting effector plasmids were called pFGC-Rap2.4 (full-length Rap2.4), pFGC-Rap2.4ΔN (Rap2.4Δ1-146), pFGC-Rap2.4ΔC (Rap2.4Δ261-334), pFGC-Rap2.4ΔN/ΔC (Rap2.4Δ1-146/Δ261-334) and pFGC-Rap2.4b (Figure 3.3.1).

The plasmids were checked by restriction digests and sequenced with the CaMV1 primer. Digestion with XbaI and BamHI gave following fragments: 1005 bp for pFGC-Rap2.4, 570 bp for pFGC-Rap2.4ΔN, 351 bp for pFGC-Rap2.4ΔN/ΔC, 786 bp for pFGC-Rap2.4ΔC and 786 bp for pFGC-Rap2.4b (lanes 1 to 5 on Figure 3.3.2, panel A)

3.3.3.2 Transactivation assay by transient expression in *N. benthamiana*

All effector and reporter constructs (schemes are provided on Figure 3.3.2, panel B) were electroporated into *Agrobacterium* strain GV3101 and rechecked prior to carrying out the transactivation experiments. The transactivation assay compared GUS enzymatic activity in plant protein extracts following either co-infiltration of *Agrobacterium* strains carrying an effector

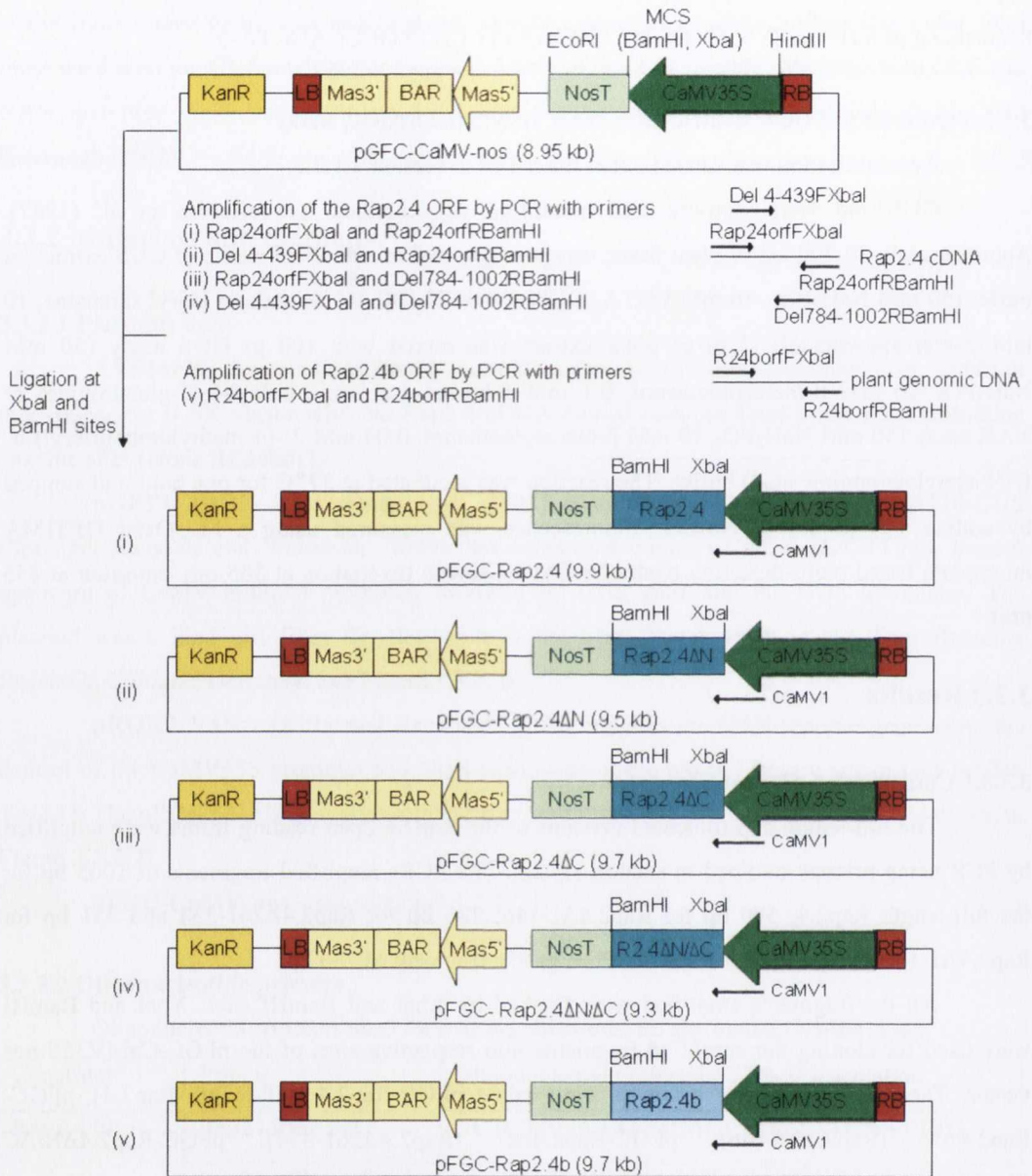


Figure 3.3.1. Construction of pFGC-Rap2.4, pFGC-Rap2.4ΔN, pFGC-Rap2.4ΔC, pFGC-Rap2.4ΔN/ΔC and pFGC-Rap2.4b plasmids. Restrictions sites shown were used for cloning fragments into the pFGC-CaMV-nos plasmid. The primers used for PCR amplifications and sequencing are indicated with black arrows.

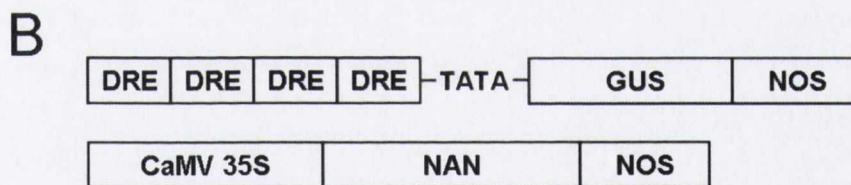
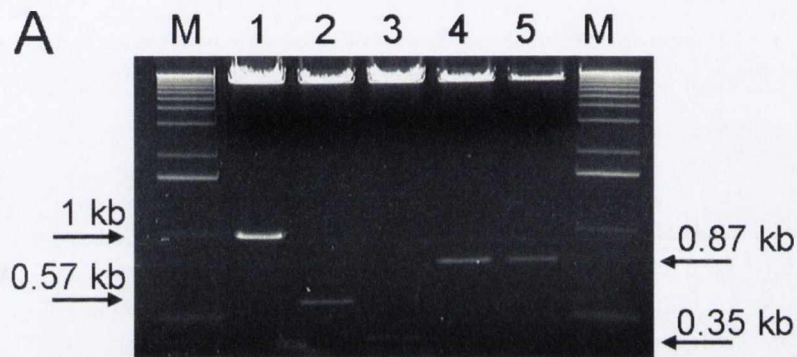


Figure 3.3.2. Transactivation assay constructs. A) Verification of the effector constructs generated for the transactivation experiments. The plasmid DNA was isolated and digested with XbaI and BamHI. Following fragments were observed: 1005 bp for pFGC-Rap2.4 (lane 1), 570 bp for pFGC-Rap2.4 Δ N (lane 2), 786 bp for pFGC-Rap2.4 Δ C (lane 4), 351 bp for pFGC-Rap2.4 Δ N/ Δ C (lane 3) and 786 bp for pFGC-Rap2.4b (lane 5). The molecular weight marker was loaded to lanes marked with M. B) Schematic representation of 4XDRE::GUS and CaMV 35S::NAN reporter constructs.

construct and both reporter genes or co-infiltration of only the two reporter genes. For the co-infiltration, equal amounts of *Agrobacterium* cultures (all at $OD_{600}=0.8$) carrying the desired plasmids were mixed and injected into the abaxial side of 3-4 week-old *N. bethamiana* leaves using a 1ml syringe without a needle.

In order to eliminate differences between samples occurring due to experimental procedures (variable infiltration volume, different efficiency of transient expression among samples), the GUS activity was calculated as a ratio of the GUS to NAN enzymatic activity.

Co-infiltration of full-length Rap2.4 and reporters resulted in significantly elevated levels of GUS enzymatic activity (p-value <0.001) compared to samples extracted from leaves infiltrated with only the reporter constructs (Figure 3.3.3, panel A, a vs b). Transient expression of truncated versions of Rap2.4 (Rap2.4 Δ N, Rap2.4 Δ C and Rap2.4 Δ N/ Δ C) did not produce significantly enhanced levels of GUS compared to the reporter-only infiltrated tissues (Figure 3.3.3, panel A). These results suggest that Rap2.4 acts as a transcriptional activator and the whole intact protein is required for its activation ability.

Transactivation experiments with Rap2.4b suggest that it might also act as a transcriptional activator as co-infiltration of Rap2.4 with reporter genes tends to enhance GUS enzymatic activity compared to infiltration with reporter genes alone (Figure 3.3.3, panel B). However, due to variability between individual samples, the results from Rap2.4b transactivation experiments are not statistically significant.

3.3.4 Discussion

Nakano et al. (2006) conducted a thorough analysis of motifs conserved outside the DNA-binding domain among the AP2/ERF genes. A number of the motifs identified were enriched for amino acids hypothetically characteristic of transactivation domains according to Liu et al. (1999). In the cluster of 10 DREBs, to which Rap2.4 and Rap2.4b belong to, four conserved motifs were identified. Rap2.4 and Rap2.4b share an N-terminal serine-rich region, an acidic C-terminal region and show enrichment for glutamine in the vicinity of the C-terminus of the AP2 domain. In order to explore which regions of Rap2.4 and Rap2.4b mediate transcriptional regulation of target genes, four constructs were made and used in transient expression assays in combination with a DRE-driven GUS reporter gene.

The transactivation experiments showed that Rap2.4 acts as a transcriptional activator and although the Rap2.4b experiments were not conclusive, the results still suggested that it might also act as an activator.

The results obtained are consistent with data from other characterized DREBs. Most DREBs in *Arabidopsis* and other plant species have been shown to act as activators with few exceptions such as the trans-inactive BnDREBs of Group II which depress DRE-mediated signalling in a concentration-dependent manner or the *Arabidopsis* transcriptional repressors ABR1, ERF3 and ERF4 (Fujimoto et al., 2000; Pandey et al., 2005; Zhao et al., 2006).

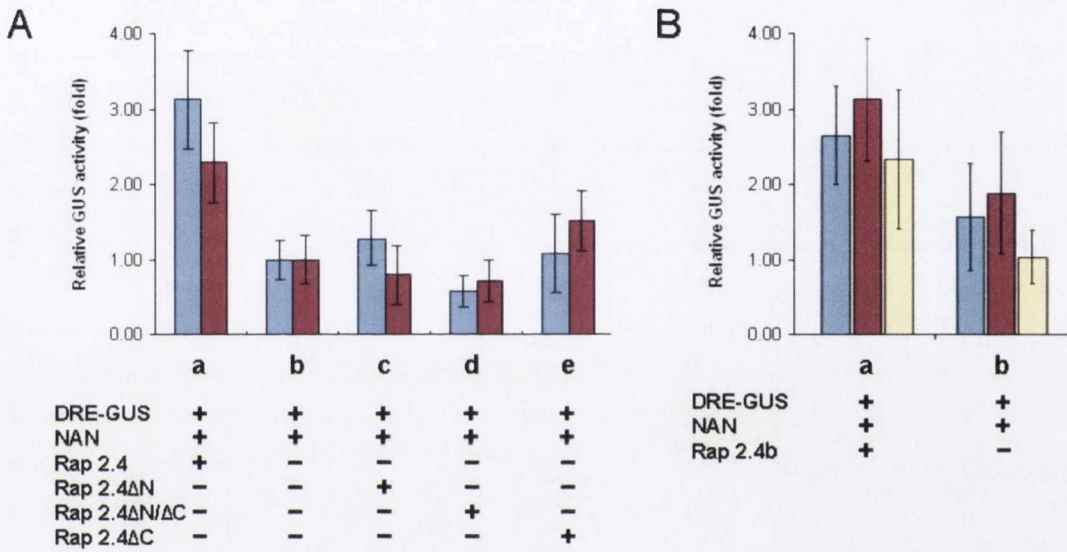


Figure 3.3.3. Transactivation assay. A) GUS/NAN enzymatic activity ratios in *N. benthamiana* leaves transiently expressing Rap2.4 full-length or truncated versions and GUS and NAN reporter genes. B) GUS/NAN enzymatic activity ratios in *N. benthamiana* leaves transiently expressing Rap2.4b and GUS and NAN reporter genes. The different colours on each chart represent independent experiments. Each bar represents the average GUS/NAN ratio of 8 samples extracted from individual infiltrated leaves. The average ratios were normalised so that the reporters-only average equalled 1.

Our experiments showed only small increases in reporter gene expression when effector-strains were added to the infiltration mixture. This could be due to non-optimal *cis*-element recognition by Rap2.4 and Rap2.4b or lack of necessary co-factors such as protein kinases

The promoter and *cis*-element sequences driving reporter gene expression i.e. the minimal CaMV promoter and DRE-core repeats contained in 4 copies of the motif TCTAGTCTACCGACATAGAGGACCCAGAAT might not be optimal for the specific TFs used in our experiments. The EMSA assays performed with Rap2.4 and Rap2.4b showed that both proteins recognise the oligonucleotide ATTTTCATGGCCGACCTGCTTTTAAGCTTTT. Both abovementioned oligonucleotides do contain the DRE-core (CCGAC) but the flanking sequence context differs significantly. The nucleotides in the immediate vicinity of the DRE-core have been shown to affect the binding ability of DRE proteins. For example, DREB1A and DREB2A have different DRE-box preferences depending on the nucleotide immediately preceding the core CCGAC. The interaction between DREB2A and the DRE-box is only possible when it is preceded by G or A, but DREB1A tolerates all four nucleotides in the -1 position relative to the DRE-core (Sakuma et al., 2002). It could be hypothesized that the sub-optimal DRE-element context for activation of reporter gene expression by Rap2.4 and Rap2.4b is behind the small increases upon activation observed in our experiments.

Finally, in our system only the Rap2.4 or Rap2.4b proteins were transiently overexpressed. Some stress-related transcription factors are known to require additional post-translational modifications in order to bind to DNA and regulate the transcription of target genes. For example, phosphorylation of the rice ERF protein OsEREBP1 by mitogen-activated protein kinase BWMK1, enhances its binding activity to the GCC box *in vitro* and co-expression of OsEREBP1 and BWMK1 in *Arabidopsis* protoplasts lead to elevated expression of a GCC box-driven reporter gene compared to experiments where only OsEREBP1 was expressed (Cheong et al., 2003). PgDREB2A from pearl millet on the other hand is subject to negative regulation by phosphorylation. The abiotic stress-induced PgDREB2A lost its ability to interact with DRE *in vitro* following phosphorylation at threonine residues by whole cell extracts (Agarwal et al., 2007).

Our experiments revealed that probably the whole Rap2.4 protein is required for the activation ability as none of the truncated versions retained the level of transactivation activity shown by the entire Rap2.4.

The activation domains of DREB2A, TINY and CaDREBLP1 have been mapped to the C-terminal part of the protein. In successful mapping experiments C-terminal domains of each of these transcription factors were linked to the Gal4 DNA-binding domain and the reporter gene was fused to Gal4 *cis*-element (UAS) recognition sites (Lim et al., 2007; Sakuma et al., 2006a). Our experiments however relied on the native DNA-binding domain of Rap2.4 protein for the interaction with the 4 tandem DRE repeats driving GUS reporter gene expression. While constructing the effector plasmids carrying the truncated versions of Rap2.4, care was taken to keep the AP2 domain and the nuclear targeting signal intact. The lack of success in mapping the activation domain of Rap2.4 could be due to a reduced ability of the truncated derivatives to

recognise the DRE-sequence or to interact with other proteins in the transcription machinery owing to the loss of native conformation of the Rap2.4 protein.

CHAPTER 4

RAP2.4 AND RAP2.4B FUNCTION

4.1 Introduction

Plant transformation technologies provide scientists with tools for investigating hypotheses that are difficult to resolve using biochemical methods or conventional breeding techniques. In *Arabidopsis*, *Agrobacterium*-mediated T-DNA transfer enables two powerful approaches for investigating gene function. The first consists of ectopic overexpression of candidate genes followed by monitoring the obtained transgenic lines for phenotypic changes. The second is based on the creation of large collections of transgenic lines in which T-DNA is exploited as an insertional mutagen. Using T-DNA in this way offers the advantage of very simple methods (e.g. PCR) for identifying knockout alleles of known genes. Several very large collections of T-DNA insertion mutants are available to the *Arabidopsis* research community.

4.1.1 Overexpression of genes of interest in *Arabidopsis*

Generation of stably-transformed transgenic lines overexpressing a gene of interest is a standard tool in plant genetics, used in the hope that any resulting phenotypes will reflect the normal role of the gene under investigation. As an alternative strategy to that of knockout/knockdown analysis, overexpression is potentially less affected by functional redundancy (Zhang, 2003).

When designing expression constructs, several features such as appropriate promoters and terminators, the 5' and 3' untranslated regions (UTRs), the presence/absence of introns, and required targeting signals need to be considered in order to achieve the desired patterns and levels of expression. The choice of promoter used to drive overexpression requires careful consideration. Placing a gene of interest under the control of a strong constitutive promoter is the most commonly used approach for obtaining gain-of-function alleles. The *Cauliflower Mosaic Virus* 35S promoter (CaMV 35S) is the constitutive promoter of choice in the majority of overexpression studies in dicotyledonous plants (Zhang, 2003) while the maize ubiquitin promoter has been widely used in monocot species (Gurr and Rushton, 2005a). Strong endogenous plant promoters used to drive constitutive transgene expression often originate from highly expressed actin and ubiquitin genes (reviewed in Potenza et al., 2004).

High level ectopic expression of certain genes can have a dramatic impact on plant development and growth, making it often difficult to interpret phenotypes. In the case of constitutive expression leading to detrimental consequences, utilizing tissue-specific or inducible promoters offers a method for controlling the spatial and/or temporal expression of introduced transgenes and can help to avoid unwanted phenotypic changes. Several tissue-specific and chemically-inducible promoters have been described in *Arabidopsis* (reviewed in Padidam, 2003; Potenza et al., 2004; Zhang, 2003).

For overexpression of abiotic stress-responsive genes, endogenous stress-inducible promoters have been demonstrated to work better, in some cases, than promoters conferring constitutive expression. For example, overexpression driven by a strong constitutive promoter, of the DREB transcription factors DREB1B (CBF1), DDF1 and DDF2, TINY, DREB1A and DREB2A resulted in enhanced abiotic stress tolerance but also caused growth retardation in transgenic *Arabidopsis* plants (Kasuga et al., 1999; Liu et al., 1998; Magome et al., 2004; Sakuma et al., 2006a; Sun et al., 2008). However, using the stress-inducible promoter of RD29A solved the problematic dwarf phenotypes while retaining the enhanced stress tolerance for DREB1A and DREB2A overexpression lines (Kasuga et al., 1999; Sakuma et al., 2006a). The RD29A promoter also been used in other species such as tobacco and potato to drive DREB1B and DREB1A genes in order to increase tolerance to abiotic stresses and minimize negative effects on plant growth (Kasuga et al., 2004; Pino et al., 2007).

The 5' and 3' UTRs also play an important role in determining gene expression levels. De Loose et al. (1995) tested several synthetic and naturally occurring 5' leader sequences in combination with the CaMV35S promoter and found that the inclusion of a 5' UTRs from the gene encoding chlorophyll a/b-binding protein, the satellite RNA of *Tobacco Necrosis Virus*, or the coat protein gene of *Tobacco Mosaic Virus* significantly enhanced reporter protein accumulation compared with inclusion of short random synthetic leader sequences. Similarly, the 5' UTR of the tobacco alcohol dehydrogenase (ADH) gene acts as an effective translational enhancer in *Arabidopsis* and tobacco cell cultures and stable tobacco transformants; and the 5'-UTR of the *Oryza sativa* ADH gene functions as a translational enhancer in cells of monocot plants (Satoh et al., 2004; Sugio et al., 2008). Furthermore, the 5' leaders of several *Arabidopsis* heat shock protein genes and the ACT1 gene have been shown to work as translational enhancers (Dansako et al., 2003; Vitale et al., 2003). On the other hand, short open reading frames present in some 5' UTRs, termed upstream ORFs (uORFs), are known to impair translation in *Arabidopsis*. The 5' uORFs of ABI3, of a vacuolar metal transporter AtMHX and of a bZIP transcription factor AtbZIP11 all repress translation of the respective downstream genes (David-Assael et al., 2005; Ng et al., 2004; Wiese et al., 2005).

Sequence elements that influence mRNA stability have also been identified in 3' UTRs. In particular AU-rich elements have been associated with unstable transcripts (Ohme-Takagi et al., 1993). The 3' UTR of the SAUR-AC1 gene, which encodes one of the most short-lived transcripts identified in *Arabidopsis* has been shown to contain a specific instability determinant (Gil and Green, 1996).

4.1.2 Reverse genetics approach to elucidating gene function – knockout lines

Knockout/knockdown lines are a valuable reverse genetics tool for establishing gene function. In *Arabidopsis*, targeted gene disruption is laborious and inefficient, involving the generation of large numbers of transgenic plants (Kempin et al., 1997). However some methods for creating knockout/knockdown pant lines, particularly insertional mutagenesis employing *Agrobacterium* transferred DNA (T-DNA) or transposons, are appropriate for large-scale reverse

genetics screens (Krysan et al., 1999). The integrated DNA sequence serves two purposes in the mutant collections – first, it interrupts genes at random generating loss-of-function mutants and secondly, it serves as a marker for identifying interrupted loci by PCR methods.

In this study we have made use of the largest collection of T-DNA insertion mutant lines available. The Salk T-DNA insertion mutant collection available from the NASC consists of over 150 000 individual transgenic *Arabidopsis* lines which represent over 225 000 independent T-DNA insertion events. The precise locations have been determined for more than 88 000 T-DNA insertions, which has resulted in the identification of mutations in more than 21 700 of the 29 454 predicted *Arabidopsis* genes. The collection was created by transforming *Arabidopsis* ecotype Columbia-0 with *Agrobacterium tumefaciens* carrying the vector pROK2 (Baulcombe et al., 1986) which carries a neomycin phosphotransferase genes conferring kanamycin resistance for selection of transformed plant lines (Alonso et al., 2003).

Although such large populations of insertion mutants theoretically contain a knockout line for almost every gene, the numbers of informative phenotypes reported in the literature is quite low, because only a small proportion of mutants exhibit distinguishable phenotypes (Cutler and McCourt, 2005; Radhamony et al., 2005). The lack of phenotypes in knockout lines is usually attributed to genetic redundancy. As mentioned in Chapter 2, over 80 % of the *Arabidopsis* genome is made up of duplicated sequence blocks (Bowers et al., 2003; Vision et al., 2000) and a significant portion of duplicated genes have maintained correlated expression patterns (Blanc and Wolfe, 2004). Examples of fully redundant homologous genes in *Arabidopsis* include the fruit dehiscence-associated SHATTERPROOF MADS-box genes SHP1 and SHP2 and the auxin-response factors ARF10 and ARF16 involved in the control of root cap development (Liljegren et al., 2000; Wang et al., 2005a). Examples of partially redundant genes are more numerous and include the photomorphogenesis-related SPA1 and SPA2 (*SUPPRESSOR OF PHYA*), and three growth-related receptor-like kinases ER, ERL1 and ERL2 (*ERECTA* and *ERECTA-LIKE1/2*) (reviewed in Briggs et al., 2006)

While structural redundancy of proteins, due to sequence similarity, can explain functional redundancy to some extent, the buffering ability of signalling networks also contributes to the difficulty in obtaining mutant lines with obvious phenotypes (Cutler and McCourt, 2005). One approach for dealing with redundancy issues is to generate lines where multiple closely-related and/or functionally-associated genes have been knocked out. For example, knocking out any one of the three AGAMOUS-like MADS-box genes SEPALLATA1/2/3 led to no particular phenotype, while generation of a triple knockout resulted in transformation of all floral organs into sepals (Pelaz et al., 2000). Similarly, single knockouts of the Type-A *Arabidopsis* response regulators (ARRs) were indistinguishable from wild-type plants whereas generation of double and triple knockouts resulted in a progressive increase in sensitivity to cytokinin (To et al., 2004). However, considering the amount of labour and time involved in generation of lines carrying multiple mutations, scaling up this approach to knock out whole gene families or functional networks of genes is not a simple task.

Upon encountering mutant lines with no obvious phenotype, in addition to redundancy issues, it is important to consider that a more detailed analysis, such as transcriptional profiling, might reveal changes in the expression of other genes that might in turn enable the gene under study to be placed in a specific pathway. Also exposing aphenotypic mutants to environmental stresses might reveal latent phenotypes.

Rap2.4 and Rap2.4b are duplicated genes that share 100 % amino acid identity in the conserved DNA-binding domain and 69 % amino acid similarity in the regions outside the conserved DNA binding domain. Based on the conserved AP2 domain and their documented similar DNA-binding preferences, Rap2.4 and Rap2.4b might be expected to share similar target genes, at least to some extent. Although the two transcription factors display different expression patterns in mature plants, they were both shown to be induced by ABA and dehydration in seedlings in our earlier experiments (see Chapter 2). The similarities in expression patterns might reflect overlapping functions for the two genes. Studying double knockouts of Rap2.4 and Rap2.4b could potentially provide a means for avoiding problems in assigning functions caused by redundancy.

4.1.3 Using microarrays for elucidating stress-response pathways

The phenotypes of mutant lines overexpressing or lacking expression of the gene(s) under investigation can provide important clues regarding the role of the gene in the plant life-cycle, but elucidating the exact role requires identification of other components of the specific pathway. The most efficient method for identifying the targets of transcription factors is monitoring changes in global expression patterns in plants in which the expression of the gene of interest has been altered. Expression profiling can also yield valuable information for genes for which altering expression levels does not result in informative phenotypes. For example, although deletion of 70 % of duplicated genes and 49 % of singleton genes in *Saccharomyces cerevisiae* resulted in either no or only weak phenotypes (Gu et al., 2003), more than 95 % of 300 gene deletion strains studied by whole-genome transcriptional profiling displayed changes in expression for at least one more gene besides the mutated gene (Hughes et al., 2000).

In the past few years microarray-based transcriptional profiling has become a feasible approach for searching for differentially regulated transcripts in mutants of *Arabidopsis*, with a wealth of commercial options available. For *Arabidopsis*, Agilent Technologies and Affymetrix offer commercially-produced DNA microarrays containing 43,800 and 22,500 probe sets, respectively. The main differences between the two commercial arrays are the probes printed on the chip and the detection methods. The Affymetrix microarray contains sets of 25-nucleotide probes per gene; the Agilent microarray utilizes one 60-mer oligonucleotide probe per gene/transcript. Agilent arrays are two-channel microarrays with ratio-based analysis whereas Affymetrix platform is one-channel array that estimates the absolute levels of gene expression.

The microarray approach has been utilized in *Arabidopsis* to identify genes responding to changing environmental conditions and for finding targets for specific regulatory genes. The response to drought has been investigated in several microarray studies but the set of genes

commonly induced or repressed in different experiments is surprisingly small. Bray (2004) compared three microarrays exploring the drought response and found that only 1.4 % and 0.2 % of the genes analysed in common, were commonly induced or repressed, respectively. However, the three microarrays utilized different experimental procedures in order to achieve drought stress and none of them covered the whole genome. Seki et al. (2002b) analysed 3-week-old seedlings that were desiccated on filter paper, Kreps et al. (2002) treated seedlings in liquid culture with mannitol to mimic drought stress and Kawaguchi et al. (2004) utilized soil-grown plants exposed to gradual water-deficit. Genes commonly induced in all experiments include several LEA proteins, protein phosphatases, transporters and transcription factors (Bray, 2004).

Regarding functional investigations of individual genes, the microarray approach has also been used to identify genes regulated by stress-responsive transcription factors such as AtMYB44 (Jung et al., 2008), zinc finger homeodomain protein ZFHD1 (Tran et al., 2007a), ZmDREB2A (Qin et al., 2007), DREB2A and DREB1A (Maruyama et al., 2004; Maruyama et al., 2009; Sakuma et al., 2006a). Microarrays have also been used to analyse mutant lines lacking enzymes with stress-related roles such as inositol polyphosphate 1-phosphatase deficient *sal1/fry1/hos2* plants (Wilson et al., 2009) and receptor histidine kinase *ahk1* mutants (Tran et al., 2007b).

In this chapter, transgenic lines overexpressing Rap2.4 or Rap2.4b were generated and analysed. In addition, Rap2.4 and Rap2.4b single knockout lines carrying T-DNA insertions at the respective loci were identified and characterized. Rap2.4/Rap2.4b double knockout lines were also constructed. Finally, the overexpression and knockout lines were used in transcriptional profiling experiments using Agilent microarray technology.

4.2 Materials and methods

4.2.1 Rap2.4 and Rap2.4b overexpression lines

4.2.1.1 Generation of *Arabidopsis* lines overexpressing Rap2.4 and Rap2.4b

Plasmids used

RZ34b01 – accession AV544129 was obtained from Kasuza DNA Research Institute (Asamizu et al., 2000). It is a Bluescript II SK vector with the Rap2.4 cDNA cloned between EcoRI and XhoI restriction enzyme sites.

pFGC-CaMV35S-nos – described in section 3.1.2.1 and outlined in figure 3.1.2.

Oligonucleotide primers for making the Rap2.4 and Rap2.4b cDNA overexpression constructs

A) To amplify Rap2.4 cDNA for pFGC-CaMV::Rap2.4 overexpression construct:

Rap24cDNAFXba: 5'-GCTCTAGAATCGGTGAGGTTGAGAGTAATTCAC-3'

Rap24cDNABamHI: 5'-CGGGATCCGCATTATAGATGAAGAACTGAAG-3'

R24bcDNABamHI: 5'-CGGGATCCAACAAGAGCCAAGCTAATGAGA-3'

R24bcDNARKpnI: 5'-GGGGTACCCGTTCTTTTGTGCCAATTTAACTTGAAC-3'

Underlining indicates sequences added in order to create restriction sites.

B) To sequence the finished constructs:

CaMV1: 5'- CCTCCTCGGATTCCATTGCCAGCTA-3'

NosR: 5' – CCGAATTCCCGATCTAGTAACATAGATGAC – 3'

4.2.1.2 Identification of overexpression lines

Arabidopsis plants were transformed by the floral dip method (described in Chapter 5). BASTA resistant putative transgenic lines were subjected to expression analysis by Northern blotting (described in Chapter 5).

The Rap2.4-specific 244 bp DIG-labelled probe was generated using following primers:

Rap24P2F: 5'-ATCTCACATCGGAGGCGATTTCCGG-3'

Rap24P2R: 5'-TCCAGCGGTGGACTCTTCAAACCTCCGT-3'

The Rap2.4b-specific 285bp DIG-labelled probe was generated using following primers:

Ftdna591: 5'- TGAGACGACGCAGAAACAGG – 3'

R24orfRBamHI: 5' – CGGGATCCCTAATTTACAAGACTCGAACA- 3'

4.2.2 Rap2.4 and Rap2.4b knockout lines

4.2.2.1 Identification of T-DNA insertion lines

Seed stocks for identification of Rap2.4 knockout line

SALK_020767 (Alonso et al., 2003), segregating T3-generation T-DNA line generated by vacuum infiltration of *Arabidopsis* Columbia 0 (Col-0) plants with *Agrobacterium* binary vector pROK2 (NASC stock identifier N654090).

SALK_149156 (Alonso et al., 2003), segregating T3-generation T-DNA line generated by vacuum infiltration of *Arabidopsis* Columbia 0 (Col-0) plants with *Agrobacterium tumefaciens* vector pROK2 (NASC stock identifier N149156).

Oligonucleotide primers for PCR analysis of Rap2.4 knockout lines

TDNA2F: 5' – GAGAGTAATTCACACTACACACACACA – 3'

TDNA2R:5' – GAGTTGTTGTTGTTGTGATGCGTCCGAGG – 3'

TDNA3F:5' – AATTCGGTTTCGATCGGTGGATCTCC – 3'

TDNA3R:5' – CAAGCTGGACCGACACAGAGAGACTAATAC – 3'

LB: 5'– GGCAATCAGCTGTTGCCCGTCTCACTGGTG – 3'

Seed stocks for identification of the Rap2.4b knockout line

SALK_139727 (Alonso et al., 2003), segregating T3-generation T-DNA line generated by vacuum infiltration of *Arabidopsis thaliana* Columbia 0 (Col-0) plants with *Agrobacterium tumefaciens* vector pROK2 (NASC stock identifier N639727).

SALK_091654 (Alonso et al., 2003), segregating T3-generation T-DNA line generated by vacuum infiltration of *Arabidopsis thaliana* Columbia 0 (Col-0) plants with *Agrobacterium tumefaciens* vector pROK2 (NASC stock identifier N591654).

Oligonucleotide primers for PCR analysis of Rap2.4b knockout lines

Ftdna639: 5' - CGATTCATCCGCGTTTGC GTTCTCT - 3'

Rtdna639: 5' - GACGGAGATCAGGGAAATTAAG - 3'

Ftdna591: 5' - TGAGACGACGCAGAAACAGG - 3'

Rtdna591: 5' - GCTTGGACCGACGCAGAGATTAACAC - 3'

LB: as above.

4.2.2.2 Generation of double knockouts

Crosses were performed using the protocols from the *Arabidopsis: A Laboratory Manual* (Weigel and Glazebrook, 2002).

Oligonucleotide primers for PCR analysis of double knockout lines

Primers for detecting interruption of the Rap2.4 ORF

Rap24orfFXbaI: 5' - GCTCTAGAATGGCAGCTGCTATGAATTTG

TDNA2R: 5' - GAGTTGTTGTTGTTGTGATGCGTCGGAGG - 3'

Primers for detecting interruption of the Rap2.4b ORF

R24bcDNAFBamHI: 5'-CGGGATCCAACAAGAGCCAAGCTAATGAGA-3'

Rtdna639: 5' - GACGGAGATCAGGGAAATTAAG - 3'

4.2.3 Analysis of obtained transgenic lines

4.2.3.1 Northern analysis

RNA extraction and Northern analysis were carried out as described in Chapter 5.

4.2.3.2 Southern analysis

Extraction of genomic DNA for Southern blotting

Genomic DNA was extracted from mature *Arabidopsis* plants grown in the greenhouse under normal conditions. 50 mg of plant tissue per sample was homogenised in 0.15 ml extraction buffer (0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, 50 mM NaCl, 2 % SDS, 0.75 % β -mercaptoethanol). Following centrifugation at 12 000 rpm at 4°C the aqueous phase was extracted once with an equal volume of phenol and once with equal volume of chloroform. RNase treatment was carried out for 10 minutes at 37°C followed by extraction with equal volume of 1:1 mix of phenol and chloroform and then with equal volume of chloroform. The collected aqueous phase containing DNA was put through a homemade Sepharose (CL6B) minicolumn. The integrity of DNA was assessed in 0.8 % agarose gels and the concentration of DNA was measured with ND-1000 spectrophotometer.

Southern blotting

Approximately 2 μg DNA was used for each restriction digestion. Restriction fragments were separated on 0.8 % agarose gels. Gels were immersed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes and then neutralised for 30 minutes in neutralising buffer (1 M Tris-HCl, 1.5 M NaCl, pH 7.5). Blotting, cross-linking, hybridisation with DIG-labelled probes, washing and detection were carried out as described for Northern blotting in Chapter 5 with the exceptions that the hybridisation buffer used was DIG-Easy buffer (Roche) instead of the 7 % SDS buffer and hybridisation was carried out at 40°C.

Primers used for generating Rap2.4, Rap2.4b and BAR specific probes for Northern and Southern analyses

The Rap2.4-specific 244 bp DIG-labelled probe was generated as above.

The Rap2.4b-specific 285bp DIG-labelled probe was generated as above.

Primers used for generating a DIG-labelled BAR-specific probe:

BARF: 5' – CATCCGCCGTGCCACCGAGGCGGACAT – 3'

BARR: 5' – CCGGCAGGCTGAAGTCCAGCTGCCAGA – 3'

4.2.3.3 Investigating phenotypes of mutant lines under abiotic stress conditions

High salinity – Seeds were sown on MS plates supplemented with 200 mM NaCl. The control plates (MS, no supplements) were prepared in an identical way. Plates were vernalized for 48 hours prior to transfer to standard growth conditions (See section 2.2.3.2). Three replica plates were prepared for each condition tested.

Drought – seeds of mutant lines and wildtype were sown on trays of peat-based compost (General Potting Medium from Shamrock), vernalized for 48 hours and transferred to the greenhouse where they were kept under standard growth conditions. Water was withheld for two weeks from 14 days-old seedlings.

ABA - Seeds were sown on MS plates supplemented with 1 μM ABA. Plates were vernalized for 48 hours prior to transfer to the growth conditions. Three replica plates were prepared for each condition tested.

4.2.3.4 Abiotic stress treatments prior to RNA extraction

The stress treatments were carried out as described in Section 2.2.4.

4.2.4 Microarray analysis

4.2.4.1 Microarray sample preparation

RNA was extracted from drought treated 14-days-old seedlings of mutant lines and wild-type controls. Seedlings were grown on MS agar plates supplemented with 1 % sucrose at 22-24°C under 16 h light/8 h dark growth conditions. Drought treatment was carried out as previously described in section 2.2.4. The standard RNA extraction protocol, as described in Chapter 5, was

followed with the exception of re-suspending the precipitated RNA in nuclease-free water instead of the TE buffer.

For the microarray 4 biological repeat samples were sent for each genotype. Biological repeat samples were grown on separate MS plates and drought-treated at the same time on separate Whatman paper disks.

4.2.4.2 Microarray experiment

Microarray analyses were performed by the VIB MicroArray Facility (www.microarrays.be). At VIB two of the biological repeat samples for each genotype were labelled with cyanine-3-CTP (Cy3) and two with cyanine-5-CTP (Cy5). A mixture of equal quantities of Cy3-labelled wild-type and Cy5-labelled mutant RNA was hybridized to two of four individual arrays on an Agilent slide, the two other arrays were hybridized with an equal quantity mixture of Cy5-labelled wild-type and Cy3-labelled mutant RNA. Expression values were obtained using Agilent Feature Extraction Software version 10.1.1.1 and the data analysis was performed by VIB with the *Limma* package (Smyth, 2004; Smyth, 2005).

4.2.4.3 Microarray validation by Northern analysis

The microarray results were confirmed by checking the expression of several genes reported to be differentially regulated by Northern analysis.

Oligonucleotides used for making DIG-labelled probes against differentially regulated genes identified in both microarray experiments were as follows:

Gene	Primer	Oligonucleotide sequence 5' to 3'	mRNA
At3g53420 PIP2;1	PIP21for	GTATACAAACAAATTAACAAGAAGGAGAG	1374 bp
	PIP21rev	AAATCCTTCTCCGGGAACGGCTTCC	
At2g37180 PIP2;3	PIP23for	GTGTGCCTACAATTATGTAGAAAC	1047 bp
	PIP23rev	AGGGTATATAATCGAAGAAAAAGTTGTC	
At2g36830 TIP1;1	TIP11for	CATCAAATCCATCTCCCACTCTTC	1059 bp
	TIP11rev	TGGTGGCTCCGTTTTCAAGTGTGCTT	
At4g17340 TIP2;2	TIP22for	CACACAAATAGAAATAACTTGTAATCAAC	978 bp
	TIP22rev	GGATGTGAGCTTGGCAAAAGCAAGAGCAG	
At2g20880	20880for	GAGGACATCGTTCGTTGACTCCTCTTC	1291 bp
	20880rev	GTCGGTCAAAGGTTCTAGTCTTCCTAAG	
At4g28140	28140for	CTCTCTCCTCTCAAATTCTTCCATTGC	1218 bp
	28140rev	GGACGACTAGGACTCTTCATTTT	

4.3 Results

4.3.1 Rap2.4 and Rap2.4b overexpression lines

4.3.1.1 Generation of overexpression constructs

Rap2.4 cDNA was amplified by PCR from Rap2.4 cDNA clone RZ34b01 with primers Rap2.4cDNAFXba and Rap2.4cDNARBamHI. A DNA fragment of 1825 bp flanked by XbaI and BamHI restriction sites was obtained, digested with XbaI and BamHI and cloned into the corresponding sites of the pFGC-CaMV-nos vector. pFGC-CaMV-nos is a binary vector based on the pFGC5491 plasmid (the original vector is described in section 2.2.3.1 and Figure 2.15; the modified vector is described in section 3.1.2.1 and outlined in Figure 3.1.2).

Rap2.4b cDNA was amplified by PCR from *Arabidopsis* genomic DNA with primers R24bcDNAFBamHI and R24cDNARKpnI. A DNA fragment of 1507 bp flanked by BamHI and KpnI restriction sites was obtained, digested with KpnI and BamHI and cloned into the corresponding sites of pFGC-CaMV-nos vector.

The resulting plasmids were called pFGC-CaMV::Rap2.4 and pFGC-CaMV::Rap2.4b (Figure 4.1, A). Constructs were verified by sequencing with CaMV1 and NosR primers.

4.3.1.2 *Arabidopsis* transformation and analysis of putative transgenic plants

The obtained pFGC-CaMV::Rap2.4 and pFGC-CaMV::Rap2.4b plasmids were electroporated into *Agrobacterium* strain GV3101 and transformed strains were verified by restriction analysis. Digestion of pFGC-CaMV::Rap2.4 with PstI restriction enzyme gave fragments of 2.4 and 1.5 kb, digestion of pFGC-CaMV::Rap2.4b with BamHI and KpnI restriction enzymes gave fragments of 1.5 and 1.2 kb (Figure 4.1, B and C, respectively).

The constructs were then introduced into *Arabidopsis* by the “Floral Dip” transformation method. Transgenic plants were identified by BASTA selection on soil and the levels of Rap2.4 and Rap2.4b expression were assessed in rosette leaves of 4-week-old plants by Northern blotting.

Hybridization with the DIG-labelled Rap2.4-specific probe revealed that Rap2.4 was expressed at considerably higher levels than in wild-type controls in 6 out of 8 BASTA resistant plants (Figure 4.2, A). Rap2.4b was overexpressed in 9 out of the 11 BASTA resistant plants tested (Figure 4.2, B).

Southern blotting was carried out in order to verify that identified overexpressers of Rap2.4 were independent transformants and to determine the number of T-DNA insertion events in each plant line. DNA was extracted from rosette leaves of plants which had been confirmed to overexpress Rap2.4 by Northern blotting. Approximately 800 to 1000 ng of genomic DNA was digested with NcoI for each line. Hybridization with a DIG-labelled probe targeted against the BAR gene (conferring BASTA resistance) revealed that all four tested Rap2.4 overexpression lines carried multiple T-DNA insertions. No recurring band patterns were observed in the Southern blot which indicated that the transgenic lines tested represent individual transformation events (Figure 4.3, A).

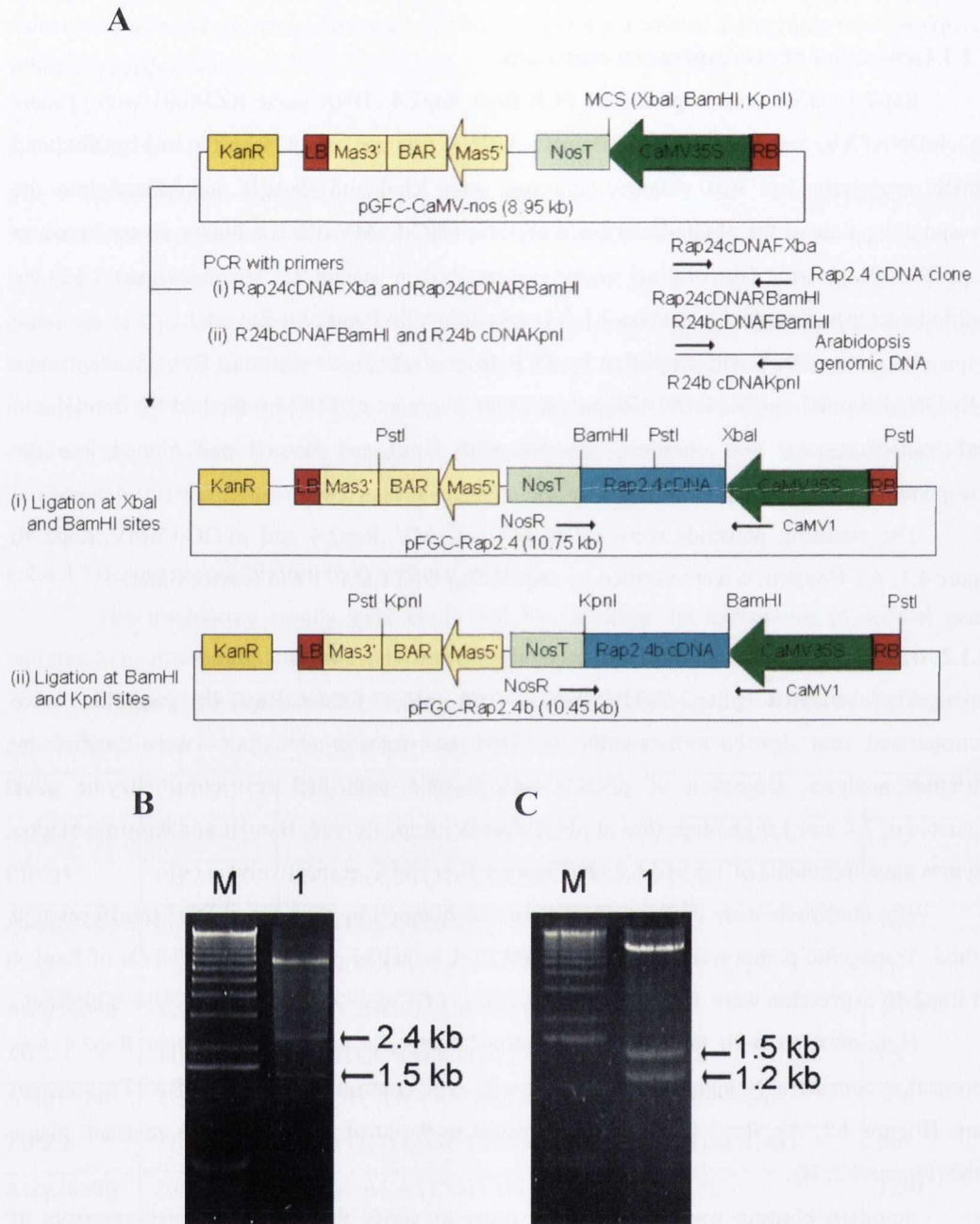


Figure 4.1. Construction of pFGC-CaMV::Rap2.4 and pFGC-CaMV::Rap2.4b plasmids. A) Schematic illustration of construction of pFGC-CaMV::Rap2.4 and pFGC-CaMV::Rap2.4b plasmids. Restriction sites shown were used for cloning the PCR fragment into the vector or verifying the plasmid by restriction digest. The primers used for PCR amplifications and sequencing are indicated with black arrows. B) Verification of obtained pFGC-CaMV::Rap2.4 plasmid by restriction digest. Digestion of with PstI enzyme resulted in fragments of 2.4 kb and 1.5 kb. C) Verification of obtained pFGC-CaMV::Rap2.4b plasmid by restriction digest. Digestion of with BamHI and KpnI enzymes resulted in fragments of 1.5 kb and 1.2 kb.

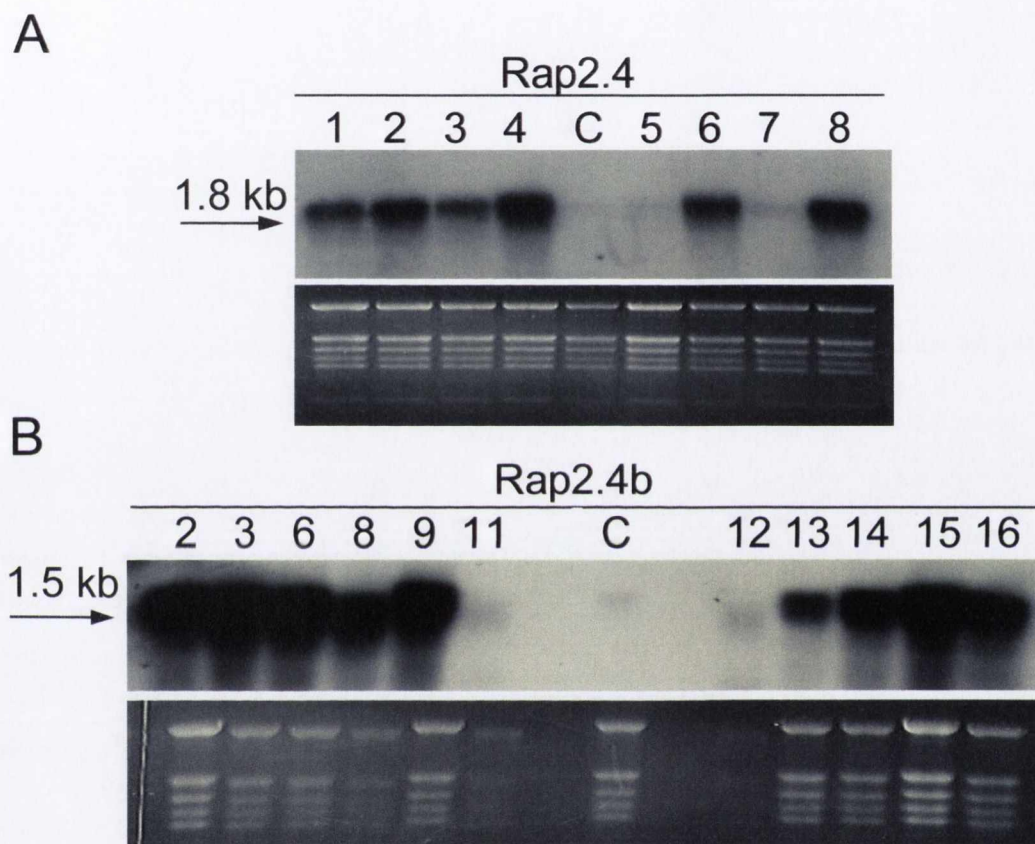


Figure 4.2. Rap2.4 and Rap2.4b expression in putative over-expression lines. A) Hybridization with DIG-labelled Rap2.4 specific probe detected a single band of 1.8 kb. B) Hybridization with DIG-labelled Rap2.4b specific probe detected a single band of 1.5 kb. A and B) RNA extracted from putative transgenic plants was loaded to lanes marked with numbers, RNA from WT plant to lanes marked C. Approximately 5 μ g of total RNA was loaded into each lane on A and 2-3 μ g of total RNA was loaded into each lane on B. Loading control is shown in the bottom panels of A and B by ethidium bromide staining.

A



B



Figure 4.3. Southern blotting with a probe targeted against the BAR gene within the T-DNA. 800-1000 ng of genomic DNA was extracted from rosette leaves of plants confirmed to over-express Rap2.4 (A) or Rap2.4b (B) and digested with *Nco*I restriction enzyme. The BAR probe detected multiple bands in all DNA samples with no recurring patterns, indicating that plants tested are independent transformants. The numbers refer to individual over-expressor plants, as in Figure 4.2.

Southern blotting analysis was also carried out on the progeny of plants confirmed to overexpress Rap2.4b. Hybridization with the BAR-specific DIG-labelled probe revealed that the four tested Rap2.4b overexpressing lines all carried multiple T-DNA insertions. The band patterns observed were different for each line suggesting that the transgenic lines tested represent individual transformation events (Figure 4.3, B)

4.3.1.3 Phenotypic characterization of Rap2.4 overexpression lines

None of the identified Rap2.4 overexpression lines had any discernible aberrant phenotypic characteristics when grown on soil under normal growth conditions. Two independent lines (OE6 and OE8) were chosen for further study and investigated for phenotypes that could potentially appear under abiotic stress conditions.

The effect of high salinity was investigated by sowing seeds of Rap2.4 overexpression lines and wild-type on MS plates supplemented with 200 mM NaCl. Radicle emergence and subsequent root growth were assessed as measures of growth. No discernible phenotypes emerged during the two week observation period in the Rap2.4 overexpression line.

The effect of dehydration was assessed under greenhouse conditions by withholding water for two weeks from 14-day old seedlings. No differences in drought tolerance or recovery were observed between Rap2.4 overexpression lines and wildtype. However, Rap2.4 overexpression has been reported to confer enhanced tolerance to dehydration on soil (Lin et al., 2008). Failure to detect the reported phenotypic increased tolerance here may be due to differences in the experimental conditions used.

The effect of the plant hormone abscisic acid (ABA) was investigated by sowing seeds of the Rap2.4 overexpression lines, Rap2.4 knockout lines and wild-type *Arabidopsis* on MS plates supplemented with 1 μ M ABA. In a small preliminary test (total of 60 seeds tested for each genotype for each condition) germination of Rap2.4 overexpression lines was inhibited by ABA to a greater extent than the germination of wild-type and Rap2.4 knockout lines (Figure 4.4, A).

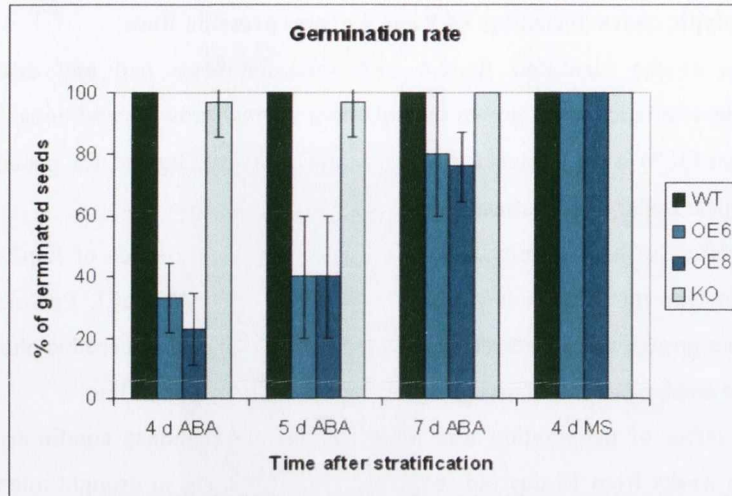
When the experiment was repeated with a larger number of seeds (160 for WT, 311 for OE 6, 389 for OE8 on ABA supplemented MS plates, >150 for each genotype on MS without ABA), clear ABA hypersensitivity was observed in the Rap2.4 overexpression lines. Namely, while exogenous ABA inhibited germination of seeds of all tested genotypes, radicle emergence was considerably more delayed in both Rap2.4 overexpression lines than in WT (Figure 4.4, B). Importantly no difference in germination was observed when Rap2.4 overexpression lines and WT seeds were sown on MS plates without ABA (Figure 4.4, A and B).

4.3.1.4 Phenotypic characterization of Rap2.4b overexpression lines

None of the identified Rap2.4b overexpression lines had any discernible phenotypic characteristics compared to WT when grown on soil under normal growth conditions. Three independent Rap2.4b overexpression lines were further tested in stress conditions for enhanced/impaired tolerance phenotypes as described in the previous section.

As observed with the Rap2.4 overexpression lines, only exogenous ABA application led to distinguishable phenotypic alterations in the Rap2.4b overexpression lines. When sown on MS

A



B

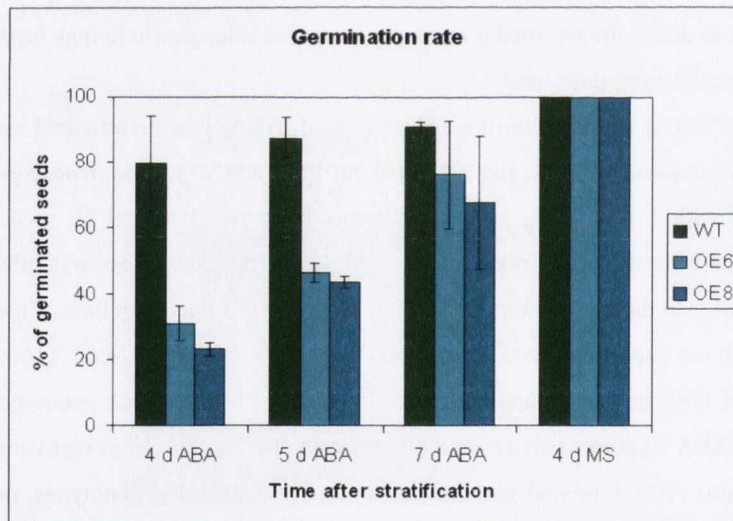


Figure 4.4. The effect of phytohormone ABA on early growth of wild-type plants (WT), Rap2.4 over-expression (OE6 and OE8) and Rap2.4 knockout lines (KO). A) ABA inhibited germination of Rap2.4 over-expression lines compared to WT whereas germination of WT and Rap2.4 knockout line were similar. B) On MS plates supplemented with 1 μ M ABA, Rap2.4 over-expression lines showed inhibited germination compared to wild-type. 160 seeds were used for WT, 311 for OE6 and 389 for OE8. The error bars correspond to double standard deviation, representing the 90% confidence range for data.

plates supplemented with 1 μ M ABA, the Rap2.4b overexpression lines germinated significantly later than the wild-type seeds, while on MS plates without ABA, all tested genotypes germinated uniformly (Figure 4.5 A and B). Germination tests for Rap2.4b were done in replicates of three using 25-40 seeds of each genotype per plate. The severest phenotype was observed for line number 8 where germination did not proceed further than *testa* rupture in the majority of the seeds tested (Figure 4.5, A).

ABA promotes maintenance of dormancy and inhibits wild-type seed germination (recently reviewed in Finkelstein et al., 2008). A high concentration of exogenous ABA causes mature *Arabidopsis* seeds to arrest germination with the seed coat split and the radicle held within the micropylar endosperm (Koornneef et al., 1982; Penfield et al., 2006). In our earlier experiments Rap2.4b was very strongly expressed in dry seeds but not seedlings and was strongly induced by exogenous ABA application. Overall, our results suggest that Rap2.4b is probably a positive mediator of ABA effects at germination and has a role in ABA-mediated processes maintaining dormancy in seeds.

The phenotype of Rap2.4 overexpression lines is more un-expected considering the lack of Rap2.4 expression in seeds and the low levels of induction by ABA observed in experiments described in Chapter 2. The similarity of Rap2.4 and Rap2.4b overexpression phenotypes supports the hypothesis of potentially shared functions for these closely-related transcription factors.

4.3.2 Knockout lines of Rap2.4

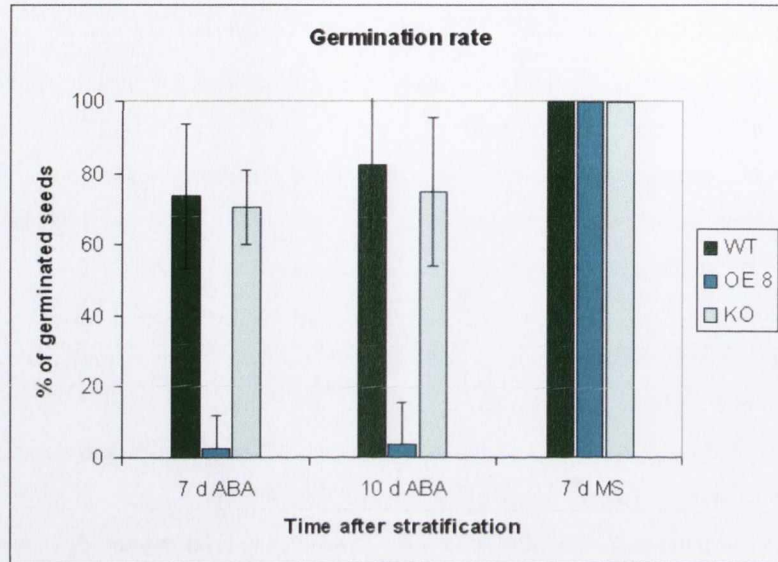
4.3.2.1 Identification of Rap2.4 T-DNA insertion lines

A BLASTN-based search using the Rap2.4 genomic sequence identified a number of T-DNA insertion lines in the NASC database. Two individual lines were obtained and subjected to PCR analysis in order to confirm the presence of T-DNA insertion in the plant genome. To determine the exact location of insertion, the PCR products were sequenced.

In the line SALK_020767 the Rap2.4 ORF was annotated as interrupted by a T-DNA insertion 169 bp downstream of ATG codon. Oligonucleotide primers TDNA2R and LB specific for the 3' junction between the T-DNA sequence and Rap2.4 amplified a fragment of approximately 500 bp instead of the expected 370 bp in five plants (Fig. 4.6, panel A, gel on the right, lanes 1, 4, 6, 8, 9) suggesting that the insertion might be located closer to the ATG codon of the Rap2.4 ORF than originally annotated. Amplification with Rap2.4-specific TDNA2F and TDNA2R primers detected a band of 323 bp in all but one tested plant indicating that 8 of the tested plants contained an intact copy of Rap2.4 while plant number 6 lacked an intact copy of Rap2.4 (Figure 4.6, panel A).

Sequencing the PCR products revealed that the T-DNA insertion in line SALK_020767 is located 30 bp downstream of the ATG codon instead of 169 bp annotated in NASC database. The TDNA2F primer is located at 23-52 bp down-stream of the ATG and is therefore not suitable for investigating whether the plants are homozygous for T-DNA insertion in Rap2.4 ORF. We carried out segregation analysis on MS plates supplemented with kanamycin in order to determine which lines of the 5 confirmed to carry a T-DNA were homozygous. The progeny of the five plants

A



B

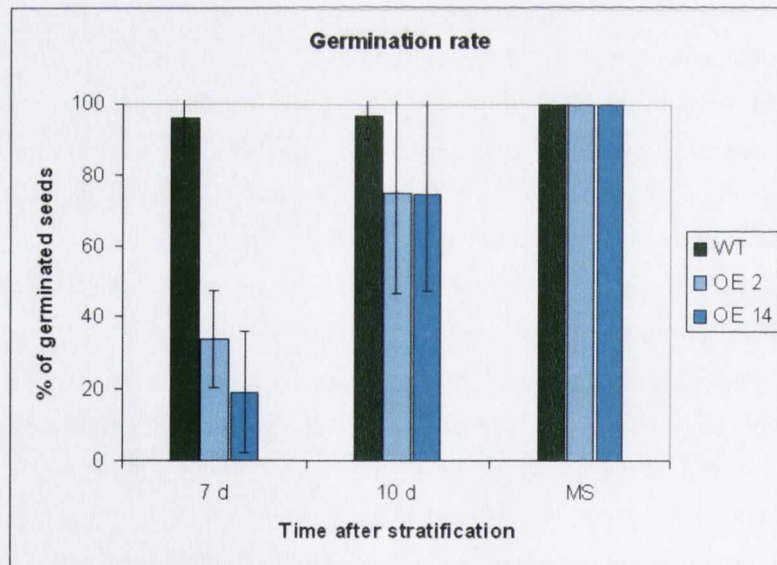


Figure 4.5. The effect of phytohormone ABA on early growth of wild-type plants (WT), Rap2.4b over-expression lines OE2, OE8 and OE14. A) ABA inhibited germination of Rap2.4b over-expression line OE8 compared to WT. B) On MS plates supplemented with 1 μ M ABA, Rap2.4b over-expression lines OE2 and OE14 showed inhibited germination compared to wild-type. A minimum of 100 seeds were used for each genotype for each condition. The error bars correspond to double standard deviation, representing the 90% confidence range for data.

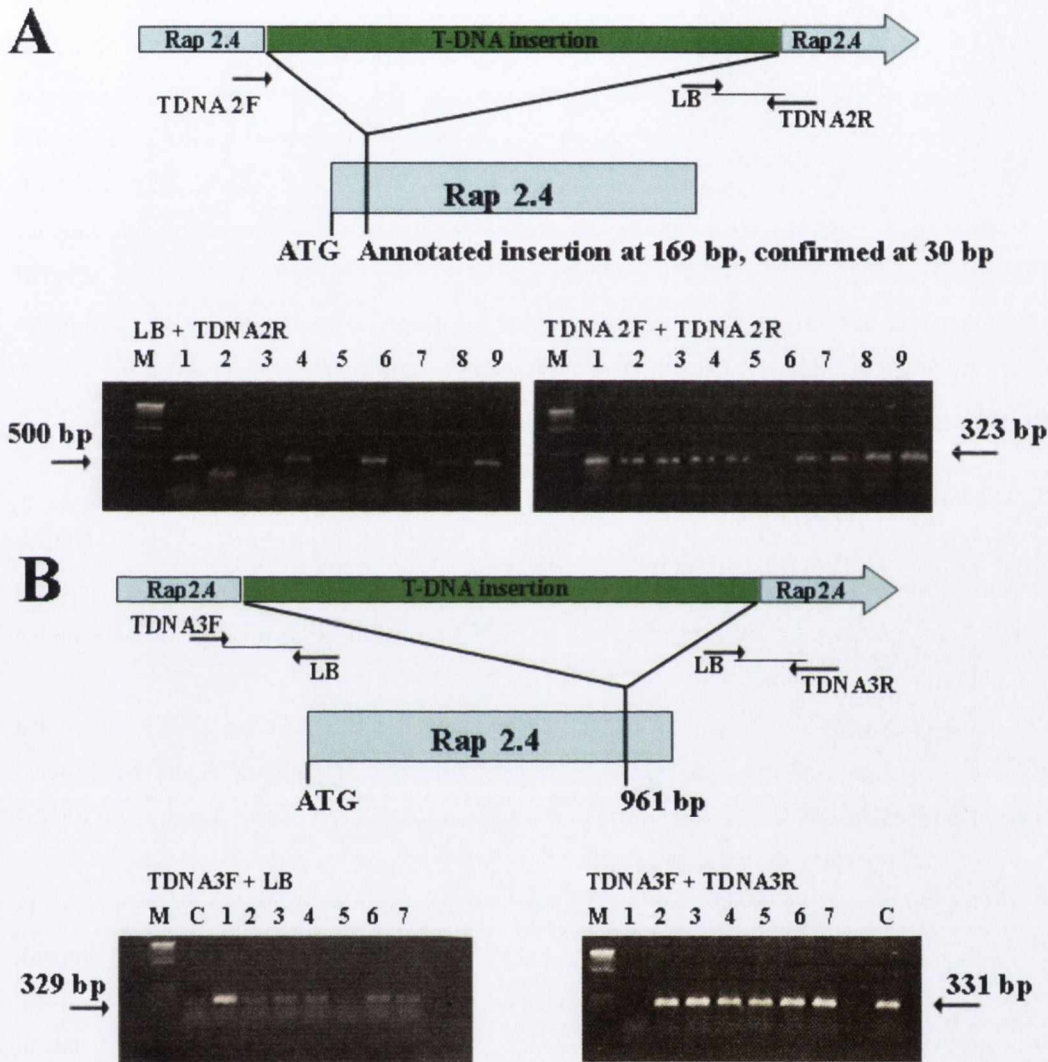


Figure 4.6. PCR analysis of Rap2.4 T-DNA insertion lines. A) PCR analysis of Rap2.4 T-DNA insertion line SALK_020767. Primers LB and TDNA2R specific to Rap2.4 with T-DNA insertion at 170 bp downstream of ATG codon, amplified a fragment of approximately 500 bp. Primers TDNA2F and TDNA2R amplified a fragment of 323 bp. B) PCR analysis of Rap2.4 T-DNA insertion line SALK_149156. Primers LB and TDNA3F specific to Rap2.4 with T-DNA insertion at 961 bp downstream of ATG codon, amplified a fragment of 329 bp. Primers TDNA3F and TDNA3R amplified a fragment of 331 bp. M- molecular weight markers. C- DNA from wild type plants. Numbers indicate independent lines of transgenic plants.

segregated as follows: #1 – 77 % kanamycin resistant (75 of 97 seedlings), #4 – 100 % kanamycin resistant (109 seedlings), #6 - 100 % kanamycin resistant (136 seedlings), #8 – 75 % kanamycin resistant (92 of 122 seedlings), #9 - 100 % kanamycin resistant (134 seedlings).

In summary, we identified three homozygous and two heterozygous lines in the progeny of SALK_020767. The segregation ratio of the heterozygous lines indicated that the original line carries only one T-DNA insertion.

In the line SALK_149156 the Rap2.4 ORF was annotated to be interrupted by a T-DNA insertion 961 bp. Out of 7 plants tested of line SALK_149156, only one was shown to have a T-DNA insertion. Plant line SALK_149156-1 gave an amplified fragment of 329 bp when the oligonucleotide primers TDNA3F and LB were used, but no fragment when the gene-specific primers TDNA3F and TDNA3F were used (Figure 4.6, panel B, lane 1 on both gels). The PCR results indicate that the line carries a T-DNA insertion in the Rap2.4 ORF and is homozygous for the insertion. Sequencing of the PCR product confirmed that the insertion is 961 bp downstream of the ATG codon of Rap2.4 as annotated in the NASC database. Segregation analysis on MS plates supplemented with kanamycin also indicated that the insertion line is homozygous for the T-DNA insertion with 100 % seedlings showing resistance.

4.3.2.2 Analysis of Rap2.4 SALK T-DNA insertion lines

The first step towards establishing that the plants identified as carrying a T-DNA insertion in the Rap2.4 locus are true knockouts was to confirm that no Rap2.4 mRNA is present in these lines. Northern blots were therefore used to study Rap2.4 expression in the putative knockout lines.

The expression analysis of wild-type plants showed earlier that Rap2.4 was expressed most abundantly in root tissue whereas expression in leaves of mature plants was almost undetectable (Chapter 2). Root tissue was therefore chosen as the RNA source for studying Rap2.4 expression in the putative knockout lines.

In the line SALK_020767 the progeny of 3 plants shown to be homozygous for the T-DNA insertion, were tested for Rap2.4 expression. After preliminary analysis (data not shown), the progeny of SALK_020767-9 (N520-9) was chosen for further investigation.

Northern analysis with a DIG-labelled Rap2.4-specific probe did not detect any bands in the line N520-9, suggesting that the line is a true homozygous knockout with no Rap2.4 mRNA present (Figure 4.7, A, lane marked 520-9). In wild-type plants the expected band of 1.8 kb, corresponding to the full-length Rap2.4 transcript, was observed. In the case of the line SALK_149156-1 (T-DNA insertion 30 bp upstream of stop codon of Rap2.4 ORF), Northern analysis with the Rap2.4-specific probe identified a strong band of approximately 2.4 kb (Figure 4.7, A, lane marked 649-1). This Rap2.4 transcript most probably carries extra sequences at the 3' end transcribed from the T-DNA insert.

In conclusion line N520-9 was found to be a true knockout and was subjected to Southern analysis in order to further investigate the T-DNA insertion at the Rap2.4 locus. Line SALK_149156-1 was excluded from further studies.

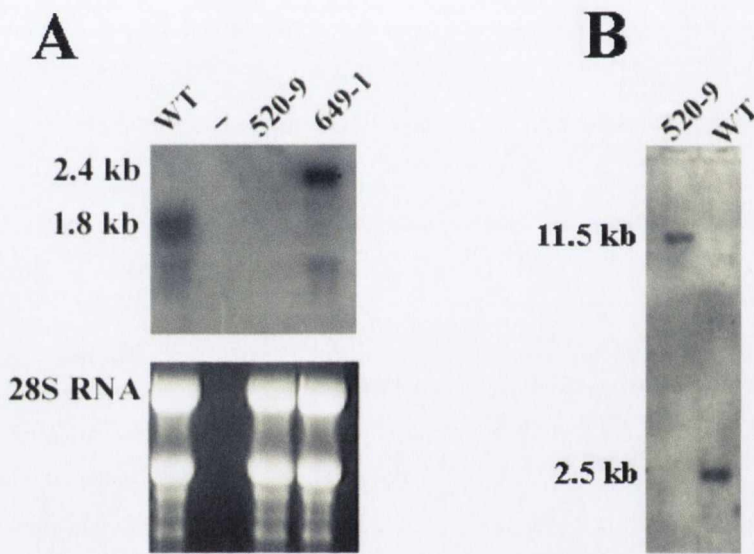


Figure 4.7 Analysis of the Rap2.4 knockout lines by Northern and Southern blotting techniques. A) Hybridization with Rap2.4 specific DIG-labelled probe revealed that the progeny of N520-9 is lacks Rap2.4 mRNA, while progeny of N649-1 showed a Rap2.4 transcript with increased size. Approximately 20 μ g of RNA was loaded into each lane. Bottom section shows RNA loading by ethidium bromide staining. B) Southern blotting utilizing a Rap2.4 specific probe showed that at least 9 kb of T-DNA has been inserted in the Rap2.4 locus.

Genomic DNA from line N520-9 and wild type control plants was digested with NdeI (Figure 4.7, B, lanes marked N520-9 and WT, respectively). Hybridization with the Rap2.4-specific probe detected bands of 2.6 kb in the lane where DNA from the wild-type was loaded and >12 kb in the lane containing DNA from N520-9 (Figure 4.7, B). The absence of the wild-type 2.6 kb band in the N520-9 confirmed the line under investigation to be homozygous for the insertion. The size of the inserted fragment in the Rap2.4 locus in line N520-9 is at least 9 kb according to the Southern analysis which corresponds to two or more repeats of T-DNA. The integration of tandem repeats and duplication events are very frequent in case of T-DNA insertions into the plant genome (Grevelding et al., 1993; Tinland, 1996). Both direct and inverted repeats are reported to occur upon T-DNA transfer into plant cells (Zambryski et al., 1980).

The N520-9 and wild-type Columbia-0 seeds were grown on soil under greenhouse conditions to explore the phenotype of the knockout line. Regarding the germination speed, flowering time or morphology of plants, no discernible differences were observed between the knockout line and the wild-type control. Growing Rap2.4 knockout mutants on MS plates supplemented with varying concentration of NaCl (100, 200, 300 and 400 mM), mannitol (100, 200, 300 and 400 mM) and ABA (0.1, 0.25, 0.5, 1 and 1.25 μ M) did not reveal any phenotypic alterations compared to wild-type plants. Soil-grown knockout mutants also did not differ from the wild-type in their tolerance to dehydration.

4.3.2.4 Identification of Rap2.4b T-DNA insertion lines

A BLASTN-based search using the Rap2.4b genomic sequence identified a number of T-DNA insertion lines in the NASC database. Two individual lines were obtained and subjected to PCR analysis in order to confirm the presence of T-DNA insertion in the plant genome.

In the line SALK_139727 the ORF of Rap2.4b is annotated to be interrupted by a T-DNA insertion 226 bp downstream of the ATG codon. The oligonucleotide primers Rtdna639 and LB amplified a fragment of approximately 600 bp in three plants instead of an expected 394 bp fragment (Figure 4.8, A, gel on the left). Oligonucleotide primers Ftdna639 and Rtdna639 amplified a fragment of approximately 320 bp in all putative Rap2.4b knockouts tested and the wild-type control (Figure 4.8, A, gel on the left), suggesting at first glance that the lines confirmed to carry a T-DNA insertion by amplification with Rtdna639 and LB primers are heterozygous for the insertion locus. However the size of the PCR fragment amplified by Rtdna639 and LB primers indicates that the T-DNA insertion is located further upstream than annotated. The extra 200 bp would place the T-DNA insertion in the immediate vicinity of the ATG codon of Rap2.4b and would render the primer pair Ftdna639 and Rtdna639 inappropriate for detecting an interruption by T-DNA.

Segregation analysis utilizing MS plates supplemented with kanamycin showed that plants numbered 4 and 6 had 100 % kanamycin resistant progeny whereas the progeny of plant number 5 was 74 % resistant to applied antibiotic (84 of 113 seedlings). The approximate 3:1 ratio of kanamycin resistant seedlings to wild-type antibiotic sensitive seedlings suggests that the stock line carries a single T-DNA insertion.

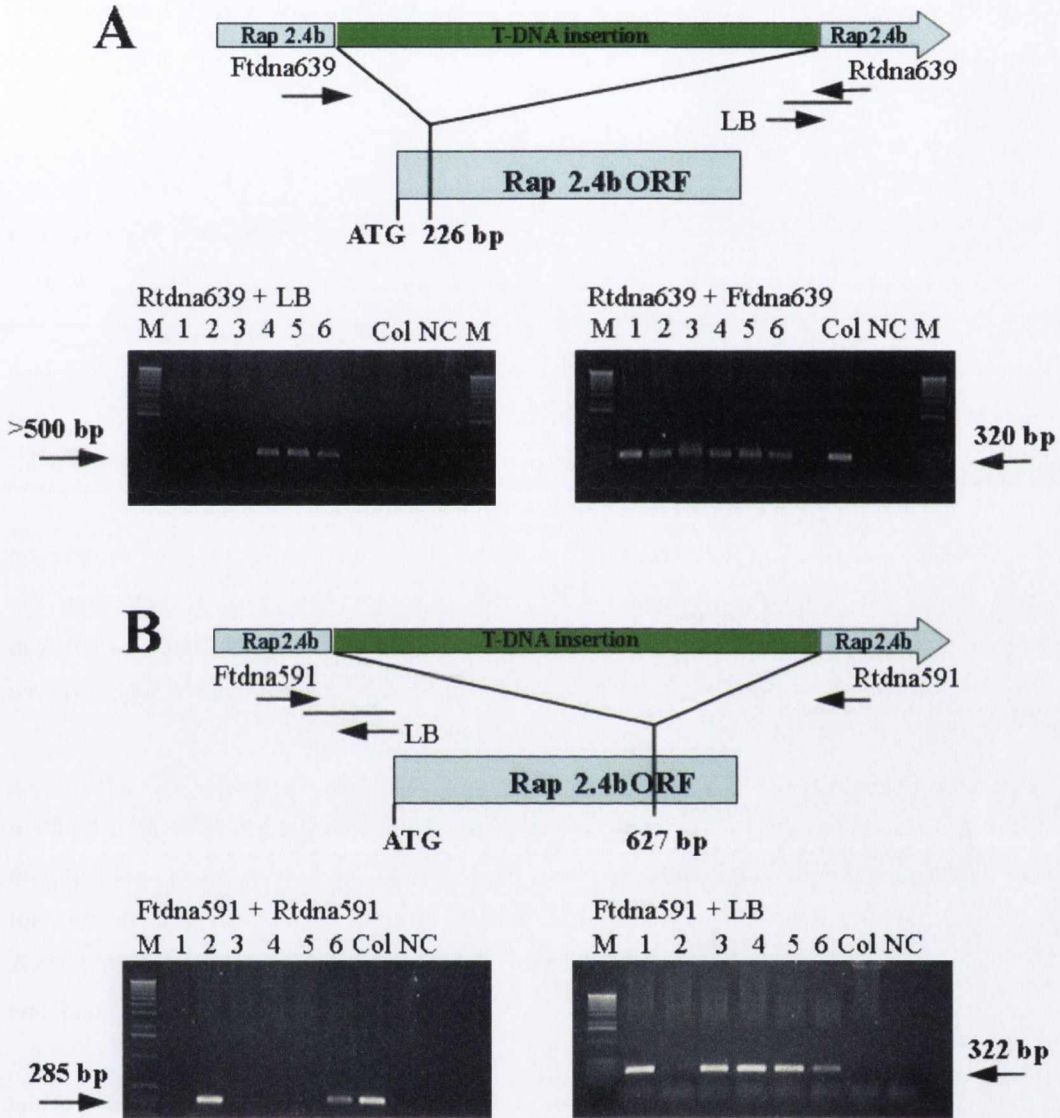


Figure 4.8. PCR analysis of the Rap2.4b T-DNA insertion lines. A) PCR analysis of Rap2.4b T-DNA insertion line SALK_139727. Primers Rtdna639 and LB, specific to the T-DNA insertion at 226 bp, amplified a fragment of approximately 500 bp, primers Rtdna639 and Ftdna639 amplified a fragment of 320 bp. B) PCR analysis of Rap2.4b T-DNA insertion line SALK_091654. Primer pair Ftdna591 and Rtdna591 amplified a fragment of 285 bp, primers Ftdna591 and LB, specific to the T-DNA insertion at 627 bp, amplified a fragment of 322 bp. NC- DNA from wild type plants. Numbers indicate independent lines of transgenic plants. M – molecular weight marker.

Out of six plants tested of line SALK_091654, five were shown to have a T-DNA insertion (Figure 4.8, B, gel on the right) by amplification with oligonucleotide primers Ftdna591 and LB of a fragment of approximately 300 bp indicating that the T-DNA insertion is only roughly in the position where it is annotated. Oligonucleotide primers Ftdna591 and Rtdna591 amplified a fragment of approximately 285 bp in only one putative Rap2.4b knockout tested (plant number 6) and the wild-type control (Figure 4.8, B, gel on the left), indicating that the other four T-DNA-carrying lines lack an intact copy of Rap2.4b and are therefore homozygous for the T-DNA insertion at the Rap2.4b locus.

4.3.2.5 Analysis of Rap2.4b SALK T-DNA insertion lines

Northern blots were used to study Rap2.4b expression in putative knockout lines in order to determine whether the plant lines confirmed by PCR to carry T-DNA insertions by PCR were true knockouts.

The expression analysis of wild-type plants showed earlier that Rap2.4b was expressed most abundantly following dehydration. With the ease of distinguishing between wild-type and Rap2.4b knockout plants in mind, the mRNA expression levels of Rap2.4b were studied in wild-type and putative knockout lines after 1 hour exposure to dehydration.

In the line SALK_139727 the progeny of two plants homozygous for T-DNA insertion based on segregation analysis (N139-4 and N139-6), were tested for Rap2.4b expression. The Rap2.4b-specific probe detected a band of 1.5 kb corresponding to full-length Rap2.4b mRNA in the drought-treated wild-type sample but not in RNA samples extracted from two drought-exposed individual Rap2.4b T-DNA insertion lines (Figure 4.9, A).

In the line SALK_091654 the progeny of four plants proven to carry T-DNA inserts by PCR, were tested for Rap2.4b expression. The Rap2.4b-specific probe detected a band of 1.5 kb in the dehydrated wild-type sample and a very faint band of higher molecular weight in at least two of the tested putative Rap2.4b knockout lines (Figure 4.9, B). This suggests the Rap2.4b transcript expressed at low level in SALK_091654 probably carries sequences transcribed from T-DNA insert in the 3' end, as was observed with the Rap2.4 SALK_149156 T-DNA insertion line discussed above.

In summary, Northern analysis confirmed the progeny of plant number 4 and 6 of the SALK_139727 to be true homozygous knockouts of Rap2.4b (N139-4, N139-6). Line SALK_091654 was excluded from further analysis due to the observed Rap2.4b residual expression.

Progeny of plants number 4 and 6 of the SALK_139727 line were grown on soil under standard greenhouse conditions but showed no discernible phenotypes when compared to wild-type plants. Growing Rap2.4b knockout mutants on MS plates supplemented with 200 mM NaCl or 1 μ M ABA also did not reveal any phenotypic alterations when compared with wild-type plants.

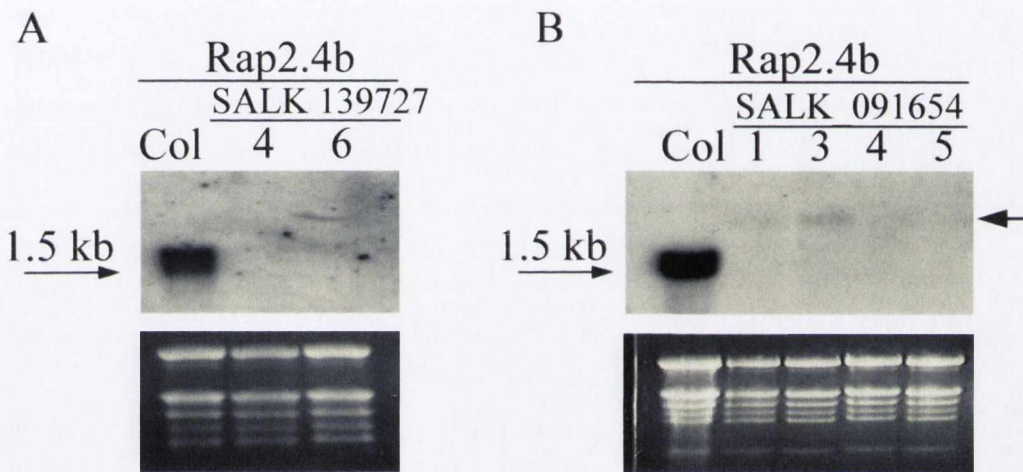


Figure 4.9. Rap2.4b expression in putative knockout lines. A) Rap2.4b specific DIG-labelled probe detected a strong band in lane containing RNA from drought-stressed wild-type plants (lane marked Col) and no band in lanes containing RNA from T-DNA insertion line SALK_139727. B) Rap2.4b specific DIG-labelled probe detected a strong band in lane containing RNA from drought-stressed wild-type plants (lane marked Col) and weak bands of higher molecular weight in lanes containing RNA from T-DNA insertion line SALK_091654. A and B) Approximately 10 μ g of RNA was loaded into each lane, bottom sections show RNA loading by ethidium bromide staining.

4.3.2.6 Rap2.4 expression in the Rap2.4b knockout line, and Rap2.4b expression in the Rap2.4 knockout line

Based on the 100 % conservation of amino acid sequences in their AP2 domains, it is probable that Rap2.4 and Rap2.4b regulate similar target genes. Knockout lines of either gene showed no immediately discernible phenotypes either under normal or stress conditions. We therefore set out to explore whether either gene might be transcriptionally up-regulated to compensate for the lack of the other under normal and stress conditions by studying the expression level of Rap2.4 in a Rap2.4b knockout and expression of Rap2.4b in a Rap2.4 knockout line. This might also reveal whether either gene was transcriptionally dependent on the other.

Two-week old *in vitro*-grown seedlings of the Rap2.4 knockout line N520-9, the Rap2.4b knockout line N139-6 and wild-type were subjected to drought, high salinity, heat and cold stress and sprayed with the stress-related plant hormone ABA. RNA was extracted after 3 hour treatments. Rap2.4 expression in the knockout lines did not differ from its expression in wild-type under normal growth conditions or following exposure to high salinity, ABA, heat or cold. However, after dehydration, Rap2.4b knockout seedlings accumulated Rap2.4 mRNA to higher levels than wild-type seedlings (Figures 4.10, panel A).

Similarly, Rap2.4b expression stayed the same in Rap2.4 knockout line as in wildtype during normal growth, following ABA application and under salt, heat and cold stress. Dehydration stress however induced Rap2.4b mRNA to higher levels in the Rap2.4 knockout line than in the wild-type control (Figures 4.10, panel B).

The DNA-binding domains of Rap2.4 and Rap2.4b are identical and the two transcription factors showed similar binding specificity towards the DRE-element in EMSA assays indicating that the targets of the two TFs could be shared at least to some extent. Both Rap2.4 and Rap2.4b transcripts accumulated to higher levels in reciprocal knockouts than in wild-type under drought conditions, suggesting a possible mechanism that compensates transcriptionally for the lack of the other gene to ensure appropriate regulation of down-stream targets. If Rap2.4 and Rap2.4b regulate an overlapping set of target genes, generation of double knockout lines could help shed light on the function of these transcription factors.

4.3.3 Generation of Rap2.4/Rap2.4b double knockout line

The confirmed knockout lines of Rap2.4 (N520-9) and Rap2.4b (N639-6) were crossed in order to obtain an *Arabidopsis* line lacking both transcription factors.

PCR analysis was utilized in order to find double knockouts in the T2 generation. Among 32 initially tested plants, the Rap2.4 specific primers Rap2.4orfFXba and TDNA2R amplified a fragment of 348 bp in wild-type, Rap2.4b knockout controls and in 26 out of the 32 tested putative double knockout plants. The primers failed to amplify a fragment in the Rap2.4 parental knockout line as expected and in 7 tested putative double knockout plants (Figure 4.11, A).

Six putative double knockouts lacking an intact copy of Rap2.4 were subjected to PCR analysis with Rap2.4b specific primers R24bcDNAFBamHI and Rtdna639. Rap2.4b specific primers amplified a fragment of 900 bp in three of the putative double knockout lines, wildtype

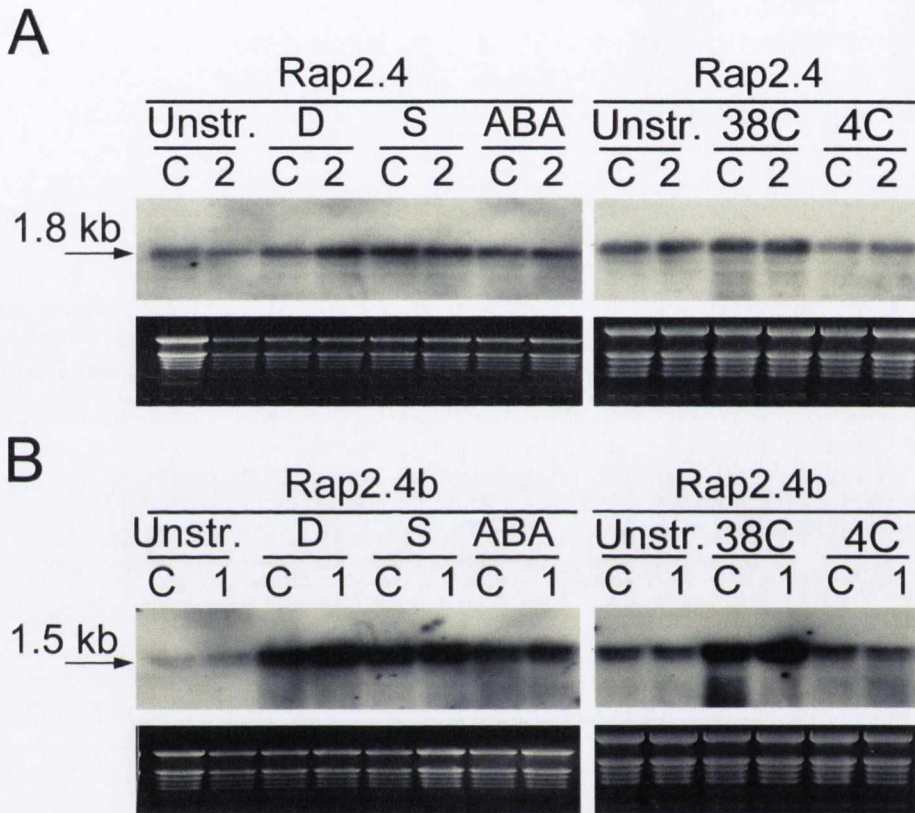


Figure 4.10. Rap2.4 expression in the Rap2.4b knockout and *vice versa*. Gene expression was explored in seedlings of wildtype (lanes marked C), Rap2.4 knockout line N520-9 (lanes marked 1) and Rap2.4b knockout line N139-6 (lanes marked 2) after exposure to drought (D), high salinity (S), heat (38C), cold (4C) and application of 100 μ m ABA (ABA). RNA from untreated seedlings was loaded to lanes marked Unstr. A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb in both seedlings of wild-type and Rap2.4b knockout line (lanes marked 2). B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb in both seedlings of wild-type and Rap2.4 knockout line (lanes marked 1). Approximately 10 μ g of RNA was loaded into each lane, bottom sections on both A) and B) show RNA loading by ethidium bromide staining.

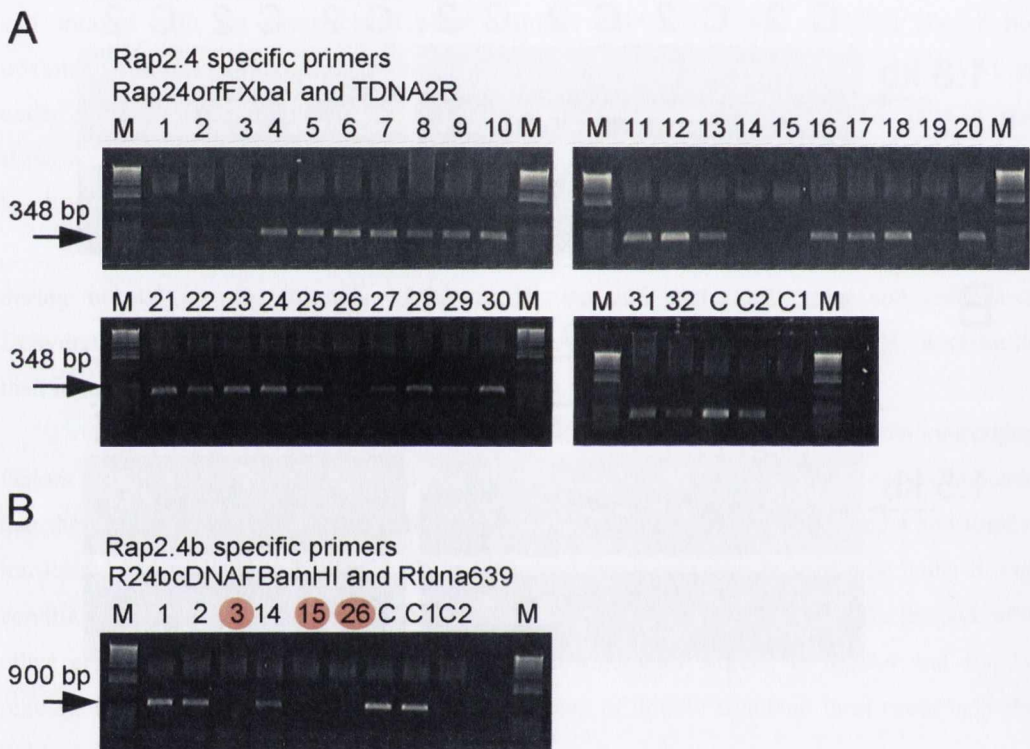


Figure 4.11. Identification of double knockout lines by PCR. A) Rap2.4 specific primers Rap24orfFXbaI and TDNA2R amplified a fragment of 348 bp in lines carrying at least one intact copy of Rap2.4 locus. B) Rap2.4b specific primers R24bcDNABamHI and Rtdna639 amplified a fragment of 900 bp in lines carrying at least one intact copy of Rap2.4b locus. Only lines confirmed to be homozygous for T-DNA insertion at the Rap2.4 locus were tested with Rap2.4b specific primers. Numbers denote individual putative double knockout plants tested. C- Columbia wild-type control, C1 – Rap2.4 knockout line (N520-9), C2- Rap2.4b knockout line (N139-6). M – molecular weight marker.

and Rap2.4 knockout controls. In three putative double knockout lines the Rap2.4b specific primers failed to amplify a fragment indicating the lack of an intact copy of Rap2.4b gene (Figure 4.11, B).

The two (of three) lines confirmed to lack intact copies of Rap2.4 and Rap2.4b by PCR were subjected to Northern analysis in order to verify the absence of both transcripts. Progeny of plants numbered 15 and 26 in the PCR analysis were grown *in vitro* and RNA was extracted from 2-week old plants.

The Rap2.4-specific probe detected a band of 1.8 kb in the wild-type control and Rap2.4b knockout line and did not hybridize to lanes where RNA from double knockout 26 (DKO26) or the parental Rap2.4 knockout line was loaded (Figure 4.12, A). In the lane where RNA extracted from double knockout 15 was loaded, the lack of a Rap2.4 specific band was not convincing.

The Rap2.4b-specific probe detected a band of 1.5 kb in the wild-type control and Rap2.4 knockout line and did not hybridize to lanes where RNA from either the double knockouts (DKO26 or DKO15) or parental Rap2.4b knockout line was loaded (Figure 4.12, B).

As a result of PCR and Northern analysis, double knockout line 26 (DKO26) was confirmed to lack intact copies, and more importantly, transcripts of both Rap2.4 and Rap2.4b.

4.3.4 Identification of Rap2.4/Rap2.4b targets by transcriptional profiling of a double knockout line

4.3.4.1 Preliminary analysis of RNA

Rap2.4 mRNA accumulated to higher levels in Rap2.4b knockout line than in wild-type and vice versa under drought conditions. If the genes are up-regulated in order to compensate for the lack of the other transcription factor, investigation of double knockout lines could help to clarify the functions of Rap2.4 and Rap2.4b. We carried out a global expression profiling experiment using the obtained double knockout line in order to identify the targets of Rap2.4 and Rap2.4b.

Seeds of the double knockout DKO26 and wild-type *Arabidopsis* were grown *in vitro* on MS medium for two weeks. Seedlings were then exposed to dehydration stress for 90 minutes and RNA was extracted from the whole seedlings. Four biological repeats were prepared for each genotype.

Preliminary testing of RNA for microarray analysis was carried out by Northern blotting to confirm the lack of Rap2.4 and Rap2.4b transcripts in DKO26 and to assess whether the drought treatment applied was appropriate. The DIG-labelled probes specific to Rap2.4 and Rap2.4b detected bands of 1.8 and 1.5 kb, respectively, in the samples extracted from wild-type (Figure 4.13, A and B, lanes marked C1-C4) but not in the samples extracted from DKO26 (Figure 4.13, A and B, lanes marked D1-D4).

The effectiveness of applied dehydration treatment was assessed by examining the levels of two well-characterized drought-responsive genes RD29A and COR15A. In addition to drought-treated RNA samples of wild-type and DKO26, samples extracted from untreated wild-type and DKO26 seedlings were included in the Northern blot. The RD29A-specific probe detected a very

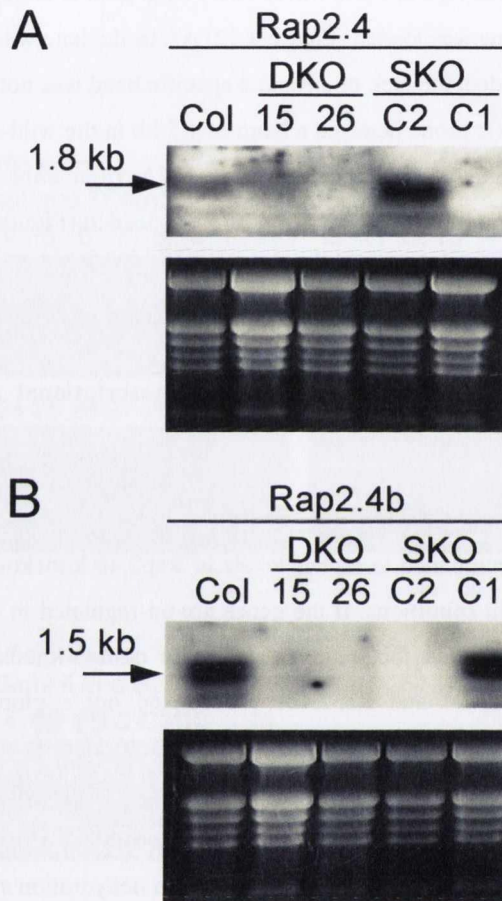


Figure 4.12. Confirmation of double knockout lines by Northern blotting. RNA was extracted from seedlings of wild-type (Col), putative double knockout plants (DKO, plants number 15 and 26 as in PCR analysis and Rap2.4 and Rap2.4b knockout lines (SKO, C1 – Rap2.4 knockout line N520-9, C2 – Rap2.4b knockout line N139-6). A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb in wild-type and Rap2.4b knockout lines. No band was detected in Rap2.4 knockout line or putative double knockout number 26. For putative knockout number 15 the result was not clear. B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb in wild-type and Rap2.4 knockout lines. No band was detected in Rap2.4b knockout line or either of the putative double knockout lines. Approximately 10 μ g of total RNA was loaded into each lane. Bottom sections on A and B show RNA loading by ethidium bromide staining.

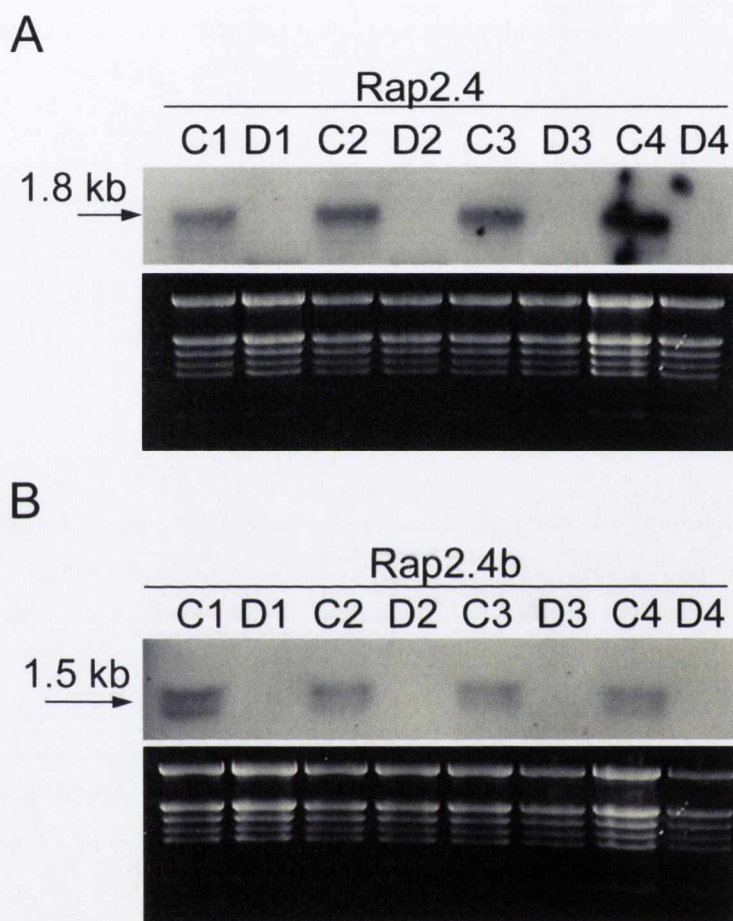


Figure 4.13. Preliminary testing of RNA samples for microarray. RNA was extracted from 4 samples of drought-treated seedlings of double knockout line 26 (D1 to D4) and 4 samples of drought-treated wild-type seedlings (C1 to C4). A) Rap2.4 specific DIG-labelled probe detected a band of 1.8 kb in lanes where RNA from wild-type samples was loaded but not in lanes where RNA from double knockout line was loaded. B) Rap2.4b specific DIG-labelled probe detected a band of 1.5 kb in lanes where RNA from wild-type samples was loaded but not in lanes where RNA from double knockout line was loaded. Approximately 10 μ g of RNA was loaded into each lane. Bottom section on both A and B show RNA loading by ethidium bromide staining.

strong band of 2.5 kb corresponding to the full-length transcript in drought-treated RNA samples and a much fainter band of same size in non-treated samples (Figure 4.14, A). The COR15A-specific probe detected a strong band of 0.75 kb in drought-treated RNA samples and a very faint band of same size in non-treated RNA preparations (Figure 4.14, B). RD29A and COR15A were expressed at similar levels in all tested wild-type and DKO26 samples under drought stress. In non-treated samples from wild-type and DKO26 both genes were also expressed at the same level, but transcripts accumulated at significantly lower levels than in drought-treated samples.

In summary, the preliminary results confirmed that the RNA samples for the microarray analysis were indeed of wild-type and Rap2.4/Rap2.4b double knockout genotype and that all the samples had been appropriately treated to induce a dehydration stress response.

4.3.4.2 Microarray analyses

Microarray hybridizations and analyses were carried out by VIB MicroArray Facility. The experiment consisted of 4 biological replicate RNA samples for wild-type and DKO26 each hybridized to an Agilent Technologies chip featuring 4 identical 44K *Arabidopsis* microarrays. Each microarray contains 43,803 *Arabidopsis* 60-mer oligonucleotide probes. Data and statistical analysis was performed by VIB using the Limma package from Bioconductor (<http://www.bioconductor.org>).

The microarray results showed up-regulation of 19 and down-regulation of 51 transcripts (p -value <0.001) when the cut-off for fold-change was set at 2. The MA plot showing fold-change between WT and mutant and signal intensities is shown in Figure 4.15. The volcano plot depicting the fold-change between WT and mutant, and confidence levels from replicates is shown in Figure 4.16.

In transactivation experiments described earlier (Chapter 3, section 3), Rap2.4 and Rap2.4b acted as transcriptional activators. For this reason, although the microarray analysis identified several genes that were up-regulated in DKO26 plants compared to wild-type, we concentrated our analysis on the down-regulated genes hoping to identify direct transcriptionally activated targets of Rap2.4 and Rap2.4b.

The 51 down-regulated transcripts included several *Arabidopsis* genes which were represented more than once. For example, Rap2.4 was represented five times and water channel protein TIP2;2 four times. After removing the repeated entries, pseudogenes and results for probes for which corresponding mRNAs could not be found, a list of 32 down-regulated genes was obtained (Table 4.17).

The microarray results were validated by checking the expression of a number of reported differentially expressed genes by Northern blotting. DIG-labelled probes designed against down-regulated genes At2g45960 (PIP1;2), At3g53420 (PIP2;1), At2g37180 (PIP2;3), At4g17340 (TIP2;2), At2g36830 (TIP1;1) and At2g20880 confirmed the microarray results by detecting stronger bands in lanes where samples from wild-type plants were loaded and weaker bands in lanes where RNA from double knockout lines was loaded (Figure 4.18). All the bands detected corresponded in size to the appropriate full-length mRNAs.

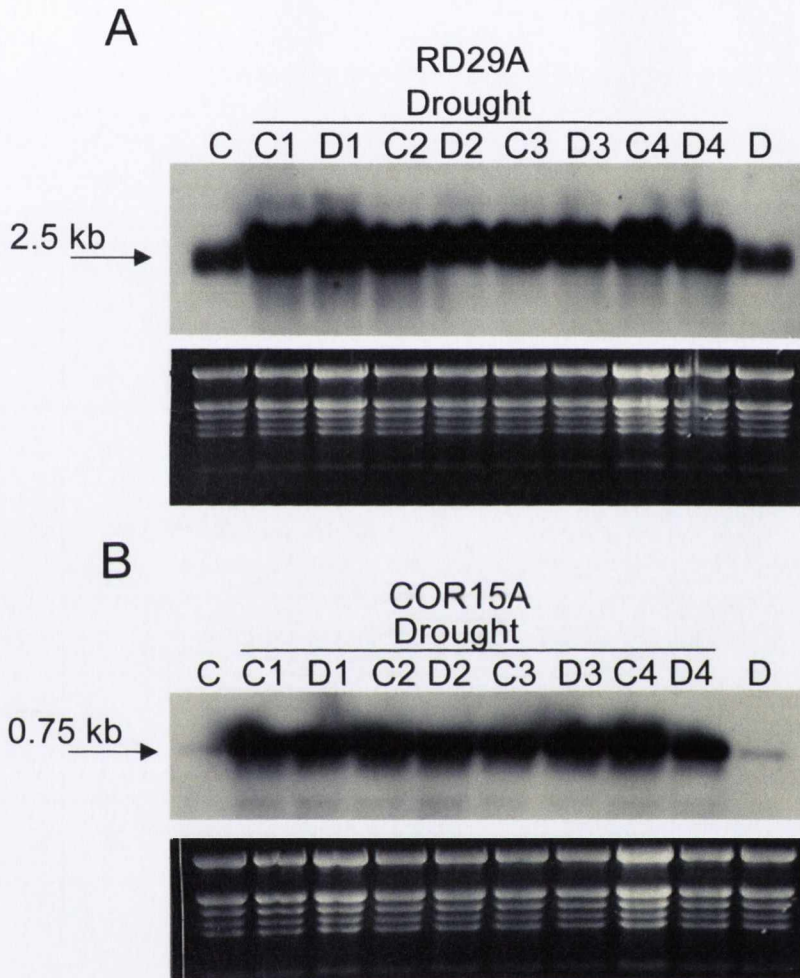


Figure 4.14. Preliminary testing of RNA samples for microarray. RNA was extracted from 4 samples of drought-treated seedlings of double knockout line 26 (D1 to D4) and 4 samples of drought-treated wild-type seedlings (C1 to C4). Untreated samples of wild-type (C) and double knockout (D) were used as controls for monitoring the effectiveness of drought treatment. A) RD29A specific DIG-labelled probe detected a band of 2.5 kb corresponding to the full-length mRNA in all samples but the expression was lower in untreated seedlings. B) COR15A specific DIG-labelled probe detected a band of 0.75 kb corresponding to the full-length mRNA in all samples but the expression was lower in untreated seedlings. Approximately 10 μ g of RNA was loaded into each lane. Bottom section on both A and B show RNA loading by ethidium bromide staining.

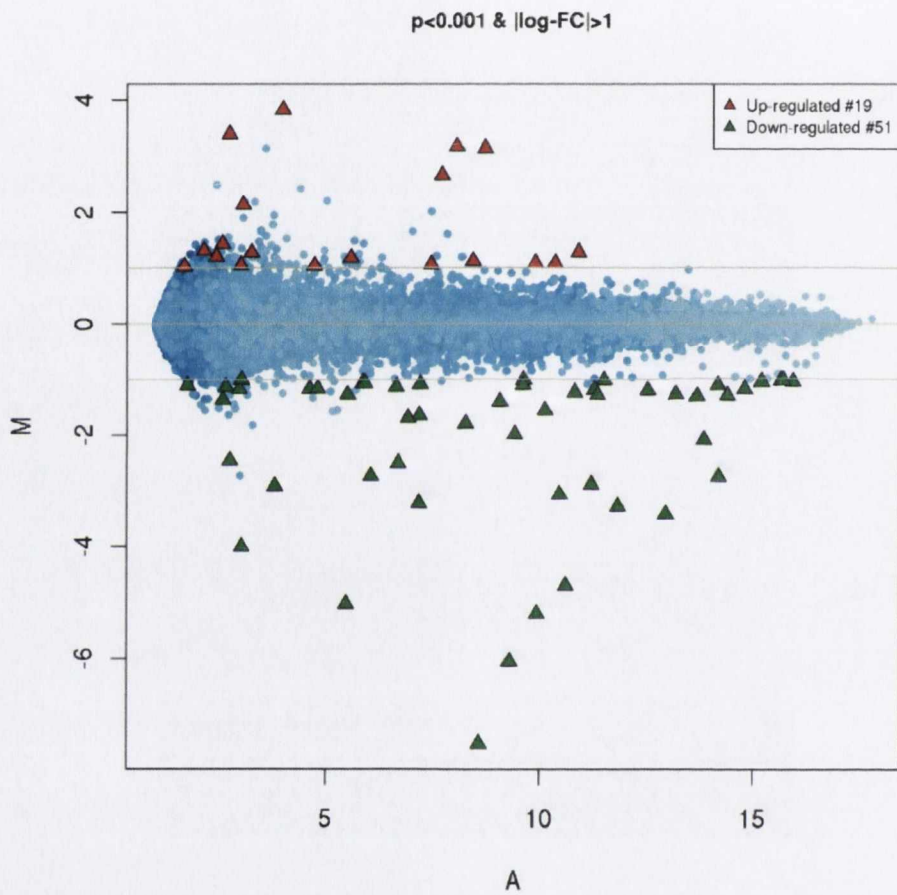


Figure 4.15. The MA plot showing fold-change between WT and mutant and signal intensities for significantly differentially expressed genes. The plot was generated by VIB.

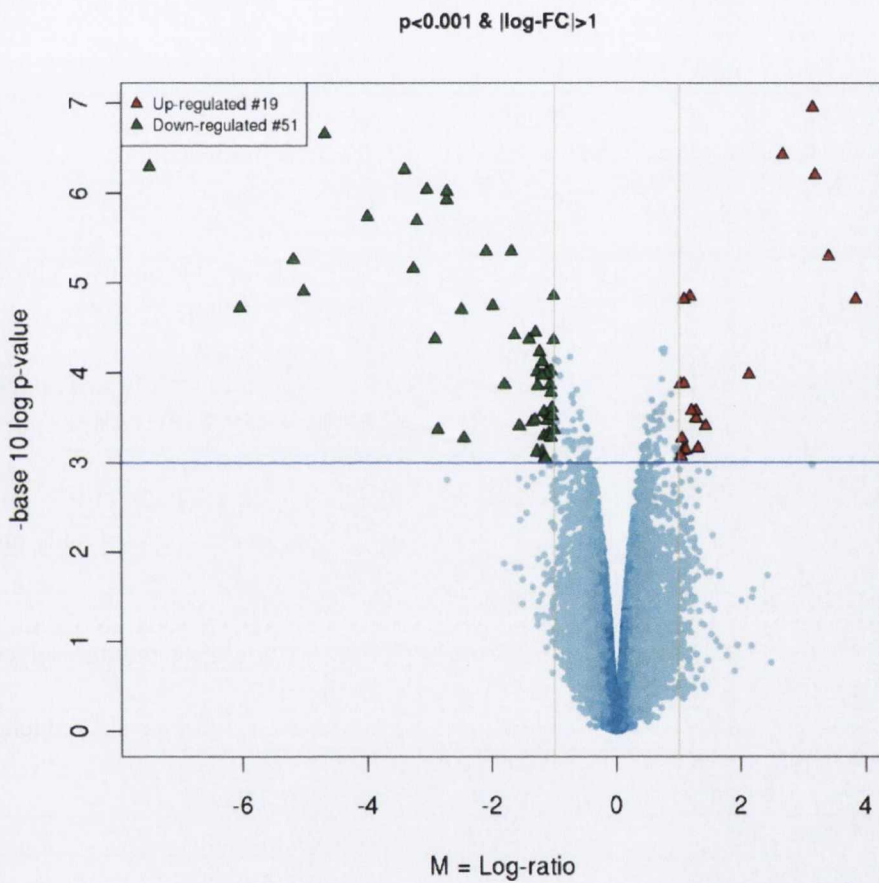


Figure 4.16. The volcano plot depicting the fold-change between WT and mutant and confidence from replicates for significantly differentially expressed genes. The plot was generated by VIB.

logFC	P.Value	Fold-change	Accession	Description
-7.516	5.06E-07	-183.07	AT1G53480.1	AtMRD1
-6.043	1.90E-05	-65.94	AT1G78080.1	Rap2.4
-5.190	5.49E-06	-36.51	AT2G20880.1	A-6 DREB TF
-5.024	1.24E-05	-32.55	AT5G54700.1	<i>Arabidopsis thaliana</i> ankyrin repeat protein
-4.687	2.19E-07	-25.75	AT1G22190.1	Rap2.4b
-3.997	1.83E-06	-15.96	AT1G53490.1	zinc-finger TF
-3.261	6.90E-06	-9.59	AT2G37180.1	RD28, PIP 2;3; aquaporin
-2.866	0.00044	-7.29	AT4G28140.1	A-6 DREB TF
-2.726	1.19E-06	-6.61	AT4G38410.1	putative dehydrin
-2.450	0.00055	-5.46	AT4G38140.1	zinc finger TF
-2.093	4.45E-06	-4.26	AT2G37170.1	PIP2;2; aquaporin
-1.988	1.80E-05	-3.97	AT1G20070.1	Unknown protein
-1.797	0.000138	-3.47	AT5G03890.1	Unknown protein
-1.639	3.80E-05	-3.12	AT1G02460.1	glycoside hydrolase family 28 protein
-1.562	0.0004	-2.95	AT5G24080.1	protein kinase family protein
-1.358	0.000363	-2.56	AT2G16830.1	PIP3 pseudogene
-1.311	0.000343	-2.48	AT4G17340.1	DELTA-TIP2/TIP2;2; aquaporin
-1.234	5.95E-05	-2.35	AT2G36830.1	GAMMA-TIP/TIP1;1; aquaporin
-1.192	0.000109	-2.28	AT2G45960.2	PIP1;2; aquaporin
-1.184	0.000774	-2.27	AT2G36590.1	PROLINE TRANSPORTER 3
-1.162	0.00028	-2.24	AT2G38600.1	acid phosphatase class B family protein
-1.161	0.000517	-2.24	AT1G24270.1	Unknown protein
-1.154	0.000905	-2.23	AT5G03090.1	similar to MRD1
-1.128	0.000902	-2.19	AT4G28850.1	XTH26, xyloglucan endotransglycosylase
-1.128	0.000105	-2.19	AT5G37210.1	zinc-finger TF
-1.101	0.000347	-2.14	AT3G26760.1	short-chain dehydrogenase/reductase family protein
-1.097	0.000296	-2.14	AT5G05280.1	zinc finger TF
-1.081	0.000489	-2.12	AT5G59760.1	Unknown protein
-1.052	0.00054	-2.07	AT2G38640.1	Unknown protein
-1.029	0.000308	-2.04	AT3G53420.2	PIP2;1; aquaporin
-1.009	4.36E-05	-2.01	AT5G06530.3	AtWBC23, ABC transporter family protein
-1.009	1.39E-05	-2.01	AT1G33700.1	catalytic; glucosylceramidase
-0.934	7.60E-05	-1.91	AT3G61430.1	PIP1;1; aquaporin
-0.921	0.018598	-1.89	AT5g47450.1	TIP2;3; aquaporin

Table 4.17. Genes down-regulated in drought-stressed Rap2.4/Rap2.4b double knockout lines compared to drought-stressed wild-type plants. Blue highlighting marks the genes also up-regulated in the Rap2.4 over-expression lines.

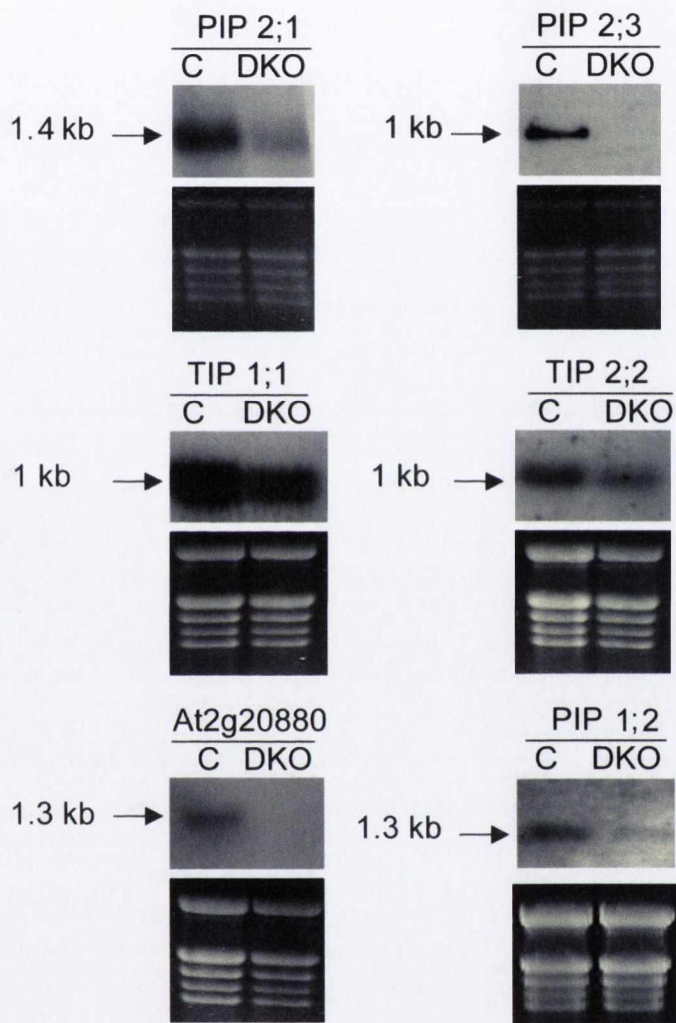


Figure 4.18. Microarray validation by Northern blotting. RNA of drought treated wild-type and double knockout was loaded into lanes marked C and DKO, respectively. Probes designed against PIP1;2, PIP2;1, PIP2;3, TIP2;2, TIP1;1 and At2g20880 detected bands corresponding to the appropriate full-length mRNAs. 5 μ g of RNA was loaded into each lane for the Northern blots with PIP2;1 and PIP2;3. 10 μ g of RNA was loaded into each lane for the rest of the Northern blots. Bottom section on each panel shows RNA loading by ethidium bromide staining.

4.3.4.3 Genes down-regulated in the double knockout line

As expected Rap2.4 and Rap2.4b were found to be down-regulated in double knockout lines compared to wild-type plants. Surprisingly two other DREB transcription factors (At2g20880 and At4g28140) from the same small A-6 subgroup that includes Rap2.4 and Rap2.4b were also down-regulated in double knockout lines compared to wild-type, suggesting that Rap2.4 and Rap2.4b could function as up-stream regulators for other closely related DREBs.

While a number of unknown genes were differentially regulated in the Rap2.4/Rap2.4b double knockout lines, several of the genes with altered expression were associated with drought stress response pathways in *Arabidopsis*. Based on Genevestigator data, most of the genes differentially regulated in the Rap2.4/Rap2.4b double knockout, are drought-responsive (Figure 4.19).

Most strikingly, six aquaporin genes and a pseudogene similar to water channel protein PIP3, were found among the down-regulated genes. Out of the six water channel protein genes down-regulated in double knockout lines, four (PIP1;2; PIP2;1, PIP2;2, PIP2;3) belong to the plasma-membrane intrinsic protein (PIP proteins) group which has 13 members in total in *Arabidopsis* and two (TIP1;1 and TIP2;2) to the tonoplast intrinsic protein group (TIP proteins) which has 10 members in total in *Arabidopsis* (Johanson et al., 2001). Furthermore, mRNA of PIP1;1 was down-regulated by 1.9 fold and mRNA of TIP2;3 down-regulated by 1.89 in the double knockouts compared to wild-type plants. Interestingly, a PIP3 pseudogene (At2g16830) was also down-regulated in the double knockout lines. Down-regulation of eight PIP- and TIP-type aquaporins out of a total of 23 TIP and PIP genes present in *Arabidopsis* suggests that Rap2.4 and/or Rap2.4b may play an important role in the regulation of water-channel activity during the plant life-cycle.

Other drought-response associated genes down-regulated in the double knockout were a root-specific putative dehydrin (Hundertmark and Hinch, 2008) encoded by At4g38410, PROLINE TRANSPORTER 3 (AtProT3) encoded by At2g36590 and an ABC (ATP-Binding Cassette) transporter AtWBC23 encoded by At5g06530. Dehydrins have been implicated in abiotic stress tolerance, especially desiccation tolerance in *Arabidopsis* and proline transporters have an important role in mediating the trafficking of compatible solutes as a part of the drought response (Bray et al., 2002). The ABC transporters traffic a broad range of molecules including hormones, sugars and cuticular waxes, and regulate stomatal opening/closure (Schulz and Kolukisaoglu, 2006).

Genes down-regulated in the double knockout line compared to wild-type also included genes coding for proteins involved in signal transduction such as an ankyrin repeat protein (At5g54700), a protein kinase (At5g24080) and an acid phosphatase class B family protein (At2g38600). Several zinc-finger DNA-binding proteins (At1g53490, At4g38140, At5g37210 and At5g05280) were down-regulated in the double knockout lines indicating that Rap2.4 and/or Rap2.4b-regulated stress-response pathways involve further layers of transcriptional regulation. A cell wall metabolism-associated putative glucosylceramidase (At1g33700), a xyloglucan endotransglycosylase-like protein XTH26 (At4g28850) and a pectin-degrading glycoside

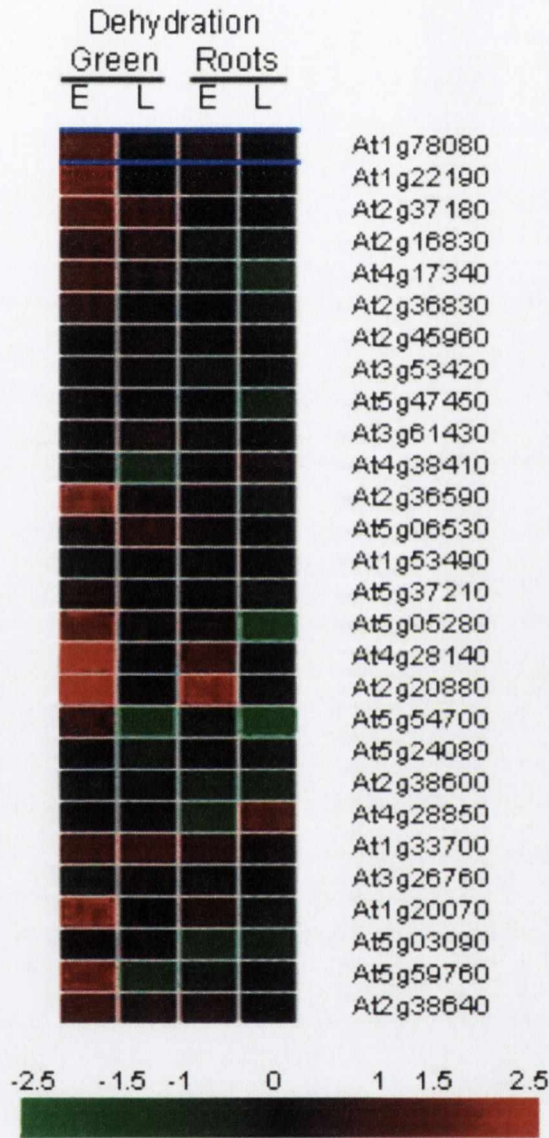


Figure 4.19. Expression patterns in dehydration for genes differentially regulated in double knockout lines. E – early drought response, L – late drought response. Green – RNA samples from aerial parts of seedlings, root – RNA samples from root tissues. Figure was adapted from Genevestigator (www.genevestigator.com).

hydrolase family 28 protein (At1g02460) were all found to be down-regulated in the double knockout line. AtMRD1 (At1g53480) which also accumulated less mRNA in the double knockout than in wild-type plants, encodes a protein of unknown function that has lower than normal expression in *mtol-1* mutant lines which over-accumulate soluble methionine (Goto and Naito, 2002).

Overall, the Rap2.4/Rap2.4b double knockout displayed an interesting molecular phenotype under drought conditions. The putative target genes of Rap2.4 and/or Rap2.4b include a number of water channels, transporter proteins, cell-wall associated enzymes and regulators of transcription and protein activity.

4.3.4.4 Promoter analysis of the differentially regulated genes

The genomic regions upstream of transcription start sites of the identified differentially regulated genes were scanned for DRE *cis*-elements using the PLACE signal scan tool. DRE-elements were found within the 1000 bp region upstream of the transcription start site for most genes down-regulated in the double knockout supporting a role for Rap2.4 and/or Rap2.4b in their transcriptional regulation. Two aquaporin genes identified as differentially expressed, TIP2;2 and PIP1;2, did not have DRE elements within the first 1 kb upstream of the transcription start site but exploring further upstream regions (up to 3 kb) revealed DRE *cis*-elements.

Earlier (Section 3.2), we explored the DNA-binding specificity of Rap2.4 and Rap2.4b and found that mutating the 4th A within the CCGAC consensus sequence of DRE-element containing oligonucleotides did not considerably affect the binding of Rap2.4 or Rap2.4b to the DNA *in vitro*. The 4th basepair in the DRE core has also been reported to be replaceable with any base in case of DREB1A and DREB2A (Sakuma et al., 2002). When 1 kb regions upstream of the transcription start sites of the differentially regulated genes were searched for DRE consensus elements allowing for substitutions in the 4th position (CCG*C, * denoting any of the four nucleotides), a number of DRE-like sequences were detected in the majority of the promoters surveyed, in addition to earlier identified DRE consensus *cis*-elements (Table 4.20).

4.3.5 Identification of Rap2.4 targets by transcriptional profiling of an overexpression line

4.3.5.1 Preliminary analysis and microarray experiment

In order to determine the targets of Rap2.4, a second microarray experiment was conducted which compared global gene expression in seedlings of a Rap2.4 overexpression line to that in wild-type.

Four biological repeat RNA samples were prepared for each genotype from two-week old *in vitro* grown seedlings. Preliminary testing of RNA for microarray analysis was carried out by Northern blotting to confirm the expression levels of Rap2.4 in overexpression and wild-type samples. The Rap2.4-specific probe detected a strong band of 1.8 kb in the samples extracted from seedlings overexpressing Rap2.4 cDNA and a very faint band of the same size in lanes loaded with

Accession	DRE elements present in the 1 kb region upstream of the transcription start site	CCG*C
AT1G53480.1	Shares several with AT1G53490	
AT1G78080.1 Rap2.4		
AT2G20880.1 A-6 DREB	ACCGAC -403, RCCGAC -207	
AT5G54700.1		
AT1G22190.1 Rap2.4b		1
AT1G53490.1	Shares several with AT1G53480	
AT2G37180.1 PIP2;3	Shared with AT2G37170 - PIP2;2	
AT4G28140.1 A-6 DREB	ACCGAC -589, ACCGAC -685, CCGAC -491	1
AT4G38410.1 Dehydrin	CCGAC -900, CCGAC -255, CCGAC -85, CCGAC -847, CCGAC -158	2
AT4G38140.1	CCGAC -922, CCGAC -596, CCGAC -393, CCGAC -81	3
AT2G37170.1 PIP2;2	CCGAC -720, CCGAC - 1678	1
AT1G20070.1	CCGAC -470, CCGAC -324	3
AT5G03890.1	CCGAC -235	1
AT1G02460.1	CCGAC -569,	
AT5G24080.1	CCGAC -778, CCGAC -634	1
AT2G16830.1 pseudo PIP3	ACCGAC -982	
AT4G17340.1 TIP2;2	CCGAC -1931	2
AT2G36830.1 TIP1;1	CCGAC -181	2
AT2g45960.1 PIP2;1	CCGAC -1734, CCGAC -2329	2
AT2G36590.1 AtProT3	CCGAC -947	2
AT2G38600.1	CCGAC -936	
AT1G24270.1		1
AT5G03090.1		2
AT4G28850.1		
AT5G37210.1	CCGAC -117,	3
AT3G26760.1		
AT5G05280.1	CCGAC -33, CCGAC -451	
AT5G59760.1	CCGAC -228	
AT2G38640.1		1
AT3G53420.2 PIP2;1	CCGAC -647	1
AT5G06530.3 AtWBC23		
AT1G33700.1		2
AT3G61430.1 PIP1;1	CCGAC -921, CCGAC -765	2
AT5G47450.1 TIP2;3	CCGAC -604	2

Table 4.20. DRE - elements found in the regions upstream of transcription start site of genes down-regulated in the Rap2.4/Rap2.4b double knockout line compared to wild-type. Upstream regions of 1000 bp were scanned for all genes but TIP2;2 (At4g17340) and PIP1;2 (At2g45960) for which regions of 3000 bp upstream of transcription start sites were scanned. The column titled CCG*C represents the number of DRE-like elements which differ from the core DRE sequence in the nucleotide in the fourth position present within the 1 kb region upstream of transcription start site.

samples from wild-type seedlings (Figure 4.21, lanes marked OE1-OE4 and lanes marked C1 to C4, respectively).

The microarray experiment was carried out in the VIB Microarray Facility as described earlier. As before, the lists of differentially-regulated transcripts contained several repeated entries and a number of pseudogenes which were excluded from further analysis. Overall, the microarray results showed up-regulation of 22 genes beside Rap2.4 and down-regulation of 5 genes (p -value <0.001) when cut-off on fold-change was set at 2. The MA plot showing fold-change between WT and mutant and signal intensities is shown on Figure 4.22. The volcano plot depicting the fold-change between WT and mutant and the confidence level from replicates is shown in Figure 4.23. The list of genes up-regulated in the Rap2.4 overexpression line compared to wild-type is shown in Figure 4.24.

Verification of the microarray data was carried out by checking the differential expression patterns by Northern blotting for a number of genes. DIG-labelled probes targeted against At3g53420 (PIP2;1), At2g36830 (TIP1;1), At4g17340 (TIP2;2), and At4g28140 confirmed the microarray results by detecting weaker bands in lanes where RNA from wild-type plants was loaded and stronger bands in lanes where samples extracted from the Rap2.4 overexpression line were loaded. All the bands detected corresponded in size to the appropriate full-length mRNAs (Figure 4.25).

4.3.5.2 Genes up-regulated by Rap2.4 overexpression

As expected the largest change observed in the microarray data was the 20-fold increase in Rap2.4 mRNA abundance in the Rap2.4 overexpression line compared to the wild-type.

Several of the genes up-regulated in Rap2.4 overexpression lines had been earlier identified as putative targets of Rap2.4 or Rap2.4b by the microarray comparing global gene expression in the DKO26 double knockout line and wild-type plants. The two A-6 subgroup DREB genes, two of the aquaporins (PIP2;3 and TIP2;2), a zinc-finger transcription factor (At4g38140), a protein kinase (At5g24080), a dehydrin (At4g38410) and an unknown protein (At1g20070) which had been down-regulated in the double knockout line, were up-regulated in the Rap2.4 overexpression line compared to wild-type. Furthermore, four more water channel proteins (TIP1;1, PIP2;1, PIP2;2, TIP2;3) and the ABC transporter (At5g06530) which were down-regulated in the double knockout line, showed higher expression levels in the Rap2.4 overexpression lines than in the wild-type plants. However, the difference in expression between the two genotypes for these five genes was less than two-fold.

Among genes up-regulated by two-fold or more in the Rap2.4 overexpression line that had not been previously identified as differentially expressed in the double knockout line, surprisingly many have putative functions in lipid metabolism in *Arabidopsis*. Lipid transfer proteins LTP3 and LTP4, palmitoyl protein thioesterase (At5g47330) and PQ-repeat gene (At4g36850) have all been associated with lipid synthesis or transport.

Genes with known functions up-regulated in the Rap2.4 overexpression line include abiotic stress responsive gene LEA76 and a defence-related gene GLIP1. LEA76 (*Late*

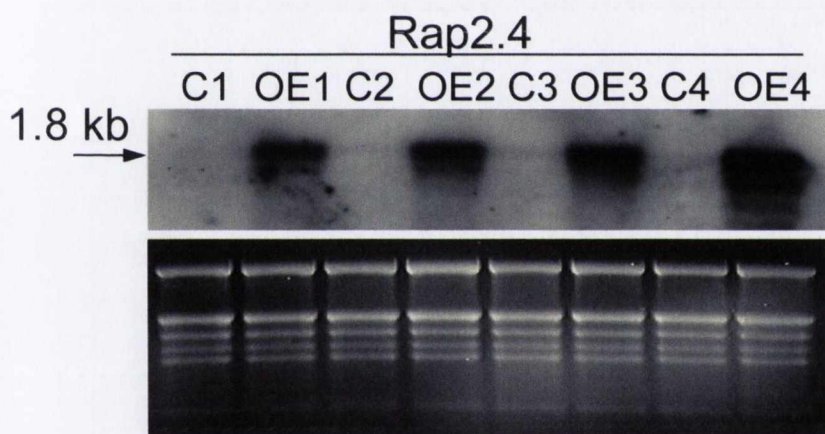


Figure 4.21. Preliminary testing of RNA samples for microarray. RNA was extracted from 4 samples of Rap2.4 over-expression line (OE1 to OE4) and 4 samples of wild-type seedlings (C1 to C4). Rap2.4 specific DIG-labelled probe detected a strong band of 1.8 kb in lanes where RNA from over-expression plants was loaded and faint bands of the same size where RNA from wild-type plants was loaded. Approximately 10 μ g of RNA was loaded into each lane. Bottom section shows RNA loading by ethidium bromide staining.

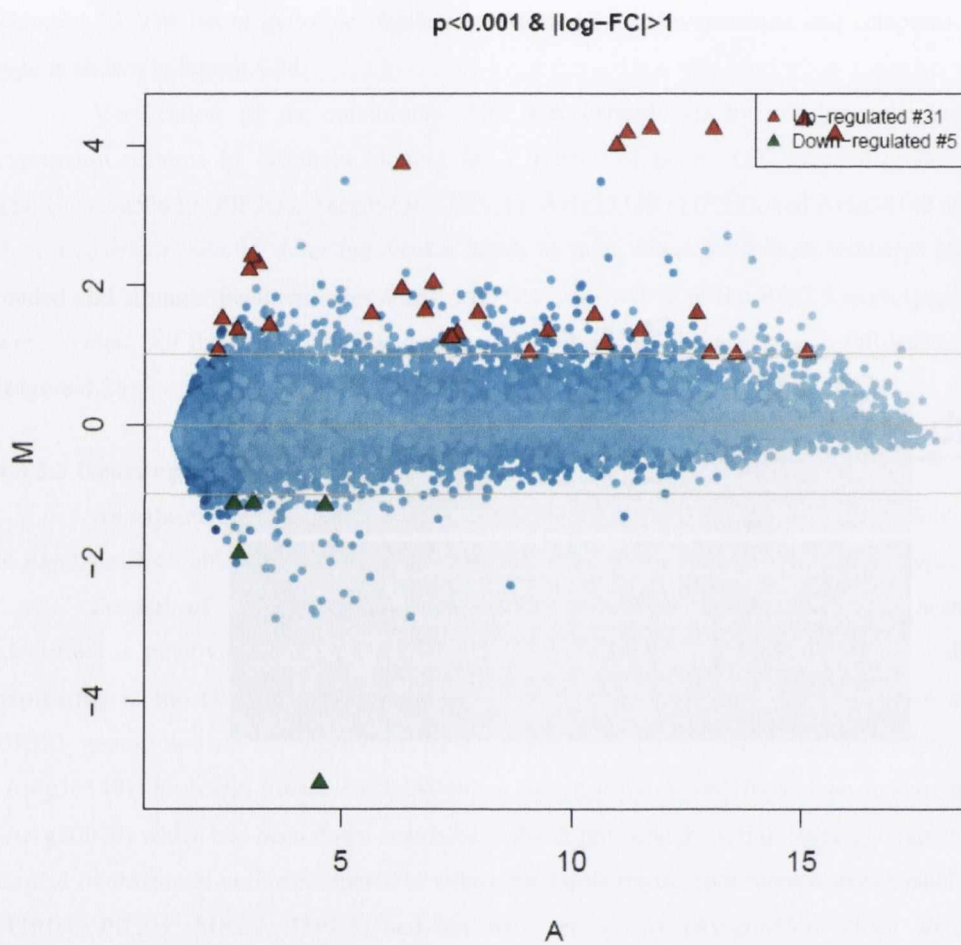


Figure 4.22. The MA plot showing fold-change between WT and mutant and signal intensities for significantly differentially expressed genes. The plot was generated by VIB.

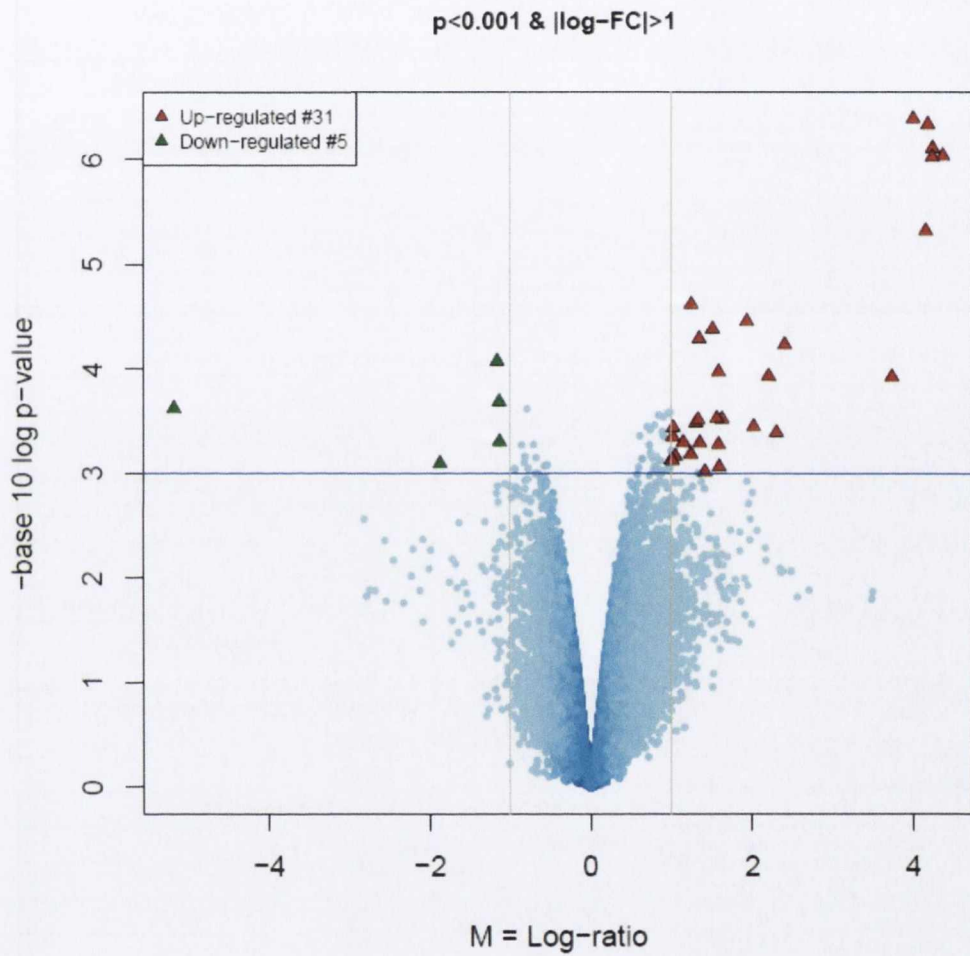


Figure 4.23. The volcano plot depicting the fold-change between WT and mutant and confidence from replicates for significantly differentially expressed genes. The plot was generated by VIB.

logFC	P.Value	Fold-change	Accession	Description
4.365	9.24E-07	20.61	AT1G78080.1	Rap2.4
3.733	0.000119	13.30	AT5G59310.1	LIPID TRANSFER PROTEIN 4
2.405	5.81E-05	5.29	AT2G20470.1	protein kinase
2.304	0.000406	4.94	AT4G38140.1	zinc finger TF
2.208	0.000117	4.62	AT1G66780.1	MATE efflux family protein
2.021	0.000357	4.06	AT4G38410.1	putative dehydrin
1.935	3.48E-05	3.82	AT5G47330.1	palmitoyl protein thioesterase family protein
1.627	0.000301	3.09	AT2G20880.1	A-6 DREB TF
1.592	0.000868	3.01	AT4G36850.1	PQ-loop repeat family protein
1.584	0.000529	3.00	AT5G24080.1	protein kinase
1.584	0.000106	3.00	AT4G28140.1	A-6 DREB TF
1.561	0.000297	2.95	AT1G52690.1	LEA protein
1.509	4.14E-05	2.85	AT5G40990.1	GDSL LIPASE1, carboxylesterase
1.416	0.00097	2.67	AT4G40020.1	unknown protein
1.352	0.00051	2.55	AT5G49600.1	unknown protein
1.342	5.12E-05	2.54	AT2G37180.1	RD28, PIP2;3; aquaporin
1.330	0.000306	2.51	AT5G59320.1	LIPID TRANSFER PROTEIN 3
1.302	0.000329	2.47	AT1G20070.1	unknown protein
1.245	2.40E-05	2.37	AT3G05640.2	putative protein phosphatase 2C
1.056	0.00069	2.08	AT1G23910.1	unknown protein
1.028	0.000365	2.04	AT4G17340.1	DELTA-TIP/TIP2;2, aquaporin
1.022	0.000449	2.03	AT5G57550.1	XTR3/XTH25 xyloglucan endotransglycosylase
1.004	0.000749	2.01	AT5G65730.1	XTH6, xyloglucan:xyloglucosyl transferase
0.906	0.000448	1.87	AT2G36830.1	GAMMA-TIP/TIP 1;1
0.817	0.000695	1.76	AT3G53420.2	PIP 2;1
0.860	0.001202	1.82	AT2G37170.1	PIP 2;2
0.876	0.001251	1.84	AT5G47450.1	TIP 2,3
0.785	0.000922	1.72	AT5G06530.1	AtWBC23

Table 4.24. Genes up-regulated in Rap2.4 over-expression lines compared to wild-type plants. Blue highlighting marks the genes down-regulated in the microarray experiments comparing the Rap2.4/Rap2.4b double knockout to wild-type plants.

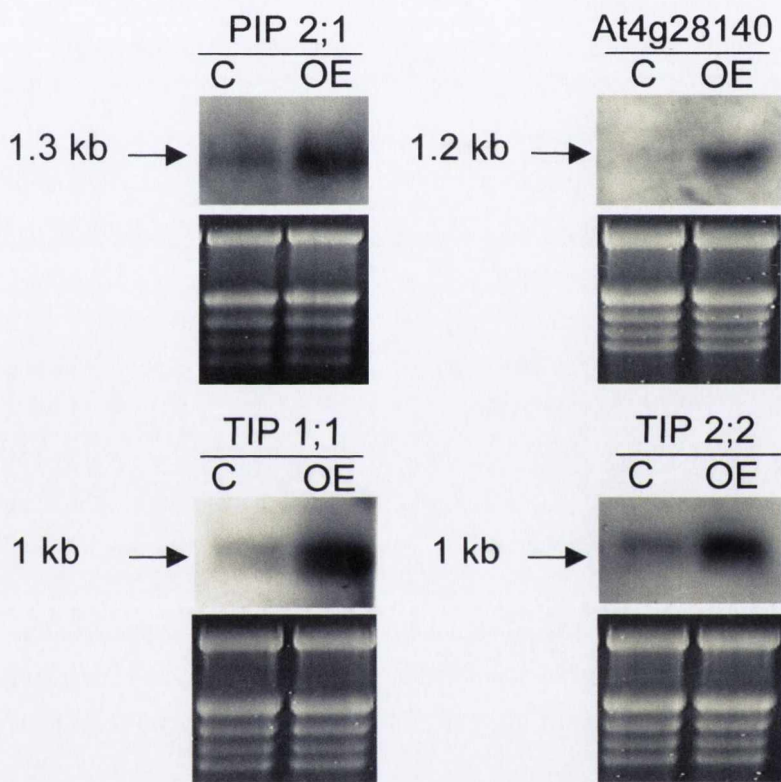


Figure 4.25. Microarray validation by Northern blotting. RNA of wild-type and Rap2.4 over-expression line was loaded into lanes marked C and OE, respectively. Probes designed against PIP2;1, TIP2;2, TIP1;1 and At4g28140 detected bands corresponding to the appropriate full-length mRNAs. Approximately 10 μ g of RNA was loaded into each lane. Bottom section on each panel shows RNA loading by ethidium bromide staining.

Embryogenesis Abundant 76, At1g52690) was previously identified as a direct target of DREB2A illustrating the overlapping functions within the DREB subfamily of proteins. GLIPI acts in ethylene-mediated defence signalling (Kwon et al., 2009).

Post-translational modifications of proteins have an established role in plant abiotic stress responses. In the Rap2.4/Rap2.4b double knockout protein kinase At5g24080 was down-regulated compared to wild-type. At5g24080 was up-regulated in the Rap2.4 overexpression line along with a protein kinase encoded by At2g20470 and a protein phosphatase 2C type gene At3g05640 suggesting that in addition to inducing the expression of target genes at the transcriptional level, Rap2.4 indirectly plays a part in modifying the activity of existing proteins post-translationally.

Two cell-wall metabolism associated genes XTH25 and XTH6 (At5g57550 and At5g65730, respectively) encoding xyloglucan endotransglucosylases/hydrolases (XTHs) (Yokoyama and Nishitani, 2001) were found to be up-regulated in the Rap2.4 overexpression line. Previously XTH26 (At4g28850) was found to be down-regulated in the Rap2.4/Rap2.4b double knockout line compared to wild-type. XTH genes have been associated with abiotic stress responses. For example, overexpression of CaXTH from hot pepper markedly improved tolerance to abiotic stresses including drought and high salinity in transgenic *Arabidopsis* (Cho et al., 2006).

In addition to the abovementioned genes, Rap2.4 up-regulation resulted in increased expression of a number of genes with unknown functions (At4g40020, At5g49600 and At1g23910) and a MATE efflux family protein encoded by At1g66780 which is thought to function in cellular detoxification processes.

To summarise, many of the genes up-regulated by Rap2.4 overexpression are potentially part of *Arabidopsis* abiotic stress response. LEA proteins, aquaporins, lipid and cell wall metabolism associated proteins can all be associated with drought or high salinity responses.

4.3.5.3 Promoter analysis of genes up-regulated in the Rap2.4 overexpression line

Sequences upstream of the transcription start site of genes up-regulated in the Rap2.4 overexpression line were examined in order to determine whether Rap2.4 could act as a direct transcriptional activator.

As observed earlier for genes down-regulated in the Rap2.4/ Rap2.4b double knockout lines, the promoter regions of up-regulated genes often but not always contained DRE consensus elements. When mutations were allowed at the 4th position of the DRE-consensus CCGAC, more putative Rap2.4 binding sites were revealed (Figure 4.26). Interestingly, no putative binding sites for Rap2.4 were identified in the promoters of the MATE efflux family protein encoding gene (At1g66780) or the PQ-loop repeat family protein encoding gene (At4g36850) indicating that the transcriptional regulation of the two genes probably includes an extra layer of complexity.

4.4 Discussion

In this chapter the functions of DREB transcription factors Rap2.4 and Rap2.4b were investigated utilizing overexpression and knockout lines. A double knockout line lacking both Rap2.4 and Rap2.4b expression was generated and studied. Although no discernible

Accession	DRE-elements present in the region upstream transcription start site	CCG*C
AT1G78080.1 Rap2.4	N/A	
AT5G59310.1	Shared with AT5G59320	
AT2G20470.1	CCGAC -749	
AT4G38140.1 Zinc-finger	CCGAC -922, CCGAC -596, CCGAC -393, CCGAC -81	3
AT1G66780.1		
AT4G38410.1 dehydrin	CCGAC -900, CCGAC -255, CCGAC -85, CCGAC -847, CCGAC -158	2
AT5G47330.1		1
AT2G20880.1 A-6 DREB	ACCGAC -403, CCGAC -207	
AT4G36850.1		
AT5G24080.1	CCGAC -778, CCGAC -634	1
AT4G28140.1 A-6 DREB	ACCGAC -589, ACCGAC -685, CCGAC -491	1
AT1G52690.1	CCGAC -4, CCGAC -41	1
AT5G40990.1	CCGAC -142	
AT4G40020.1		2
AT5G49600.1		2
AT2G37180.1 RD28/PIP2;3	CCGAC -720, CCGAC - 1678	1
AT5G59320.1		2
AT1G20070.1	CCGAC -470, CCGAC -324	3
AT3G05640.2	CCGAC -922	
AT1G23910.1		2
AT4G17340.1 TIP2;2	CCGAC -1931	2
AT5G57550.1		1
AT5G65730.1	CCGAC -915	2

Table 4.26. DRE - elements found in the regions upstream of transcription start site of genes up-regulated in the Rap2.4 over-expression line compared to wild-type. Upstream regions of 1000 bp were scanned. The column titled CCG*C represents the number of DRE-like elements which differ from the core DRE sequence in the nucleotide in the fourth position present within the 1 kb region upstream of transcription start site.

morphological phenotypes were detected for any of the obtained mutant lines under normal growth conditions, overexpression of either Rap2.4 or Rap2.4b significantly increased sensitivity to ABA at germination. In addition, double knockout and Rap2.4 overexpression lines displayed interesting molecular phenotypes. The results obtained will be discussed at length below.

4.4.1 Rap2.4 and Rap2.4b knockout lines

The knockout lines were obtained for Rap2.4 and Rap2.4b from the NASC seed stock centre and confirmed to express no Rap2.4 or Rap2.4b mRNA, respectively. The single knockout lines were crossed to generate a double knockout line lacking the expression of both transcription factors. However, neither the single or double knockout lines displayed any distinguishable gross phenotypic changes compared to wild-type plants under normal growth conditions.

In large-scale studies of *Arabidopsis* knockouts only a small fraction of mutant lines display directly informative phenotypes (Bouche and Bouchez, 2001). The lack of visible changes in vast majority of the knockout lines under standard growth or culture conditions can be either attributed to the prevalence of genetic redundancy revealed by the *Arabidopsis* genome sequence or to the difficulty of detecting the phenotypes (Bouche and Bouchez, 2001). The latter problem can sometimes be solved by subjecting the mutant lines to a variety of growth and stress conditions (Meissner et al., 1999). However, neither the single nor double knockout lines in our study displayed any gross phenotypes when exposed to salt or drought stress or exogenous ABA.

It has been reported that despite drastic changes at the molecular level, knockout lines might not display significant morphological alterations due to robustness in gene networks caused by structural or functional redundancy. In this case transcriptional profiling may be a suitable approach for detecting the alterations and obtaining information that can help to place the gene(s) under study in particular pathways (Cutler and McCourt, 2005).

Structural redundancy is a particularly relevant issue in our study because Rap2.4 and Rap2.4b belong to a family of 147 transcription factors all sharing at least the conserved DNA binding domain. Although structural similarities may indicate overlapping functions, they do not necessarily reflect complete functional redundancy as demonstrated by three CBF/DREB1 transcription factors. *CBF1*, *CBF2* and *CBF3* are almost 90 % identical in amino acid sequence and all function in the cold-response pathway (Gilmour et al., 1998). However, while ectopic expression of *CBF1* (Liu et al., 1998) and *CBF3* (Gilmour et al., 2000) enhances freezing tolerance in *Arabidopsis* through activation of cold-responsive genes, *CBF2* on the other hand is a negative regulator of freezing tolerance in *Arabidopsis* through repression of *CBF1* and *CBF3* (Novillo et al., 2004).

To address issues of redundancy between highly similar proteins, constructing and studying knockout lines that lack the products of multiple genes can prove to be a reasonable approach. For example, in the case of two MADS-box *Shatterproof* (*SHP1*, *SHP2*) genes which are 87 % identical in amino acid sequence, the combination of individual mutants each of which lacked a phenotype resulted in an informative combined phenotype (Liljegren et al., 2000).

Similarly, even though the knockouts of three individual *ANP* genes encoding MAPKKKs had no phenotypes, the triple knockout genotype was embryo lethal (Krysan et al., 2002).

When Rap2.4 expression was assessed in the Rap2.4b knockout and *vice versa* following dehydration stress in *Arabidopsis* seedlings, the mRNAs of both genes were up-regulated in the reciprocal knockouts compared to wild-type plants. The DNA-recognition sequences for Rap2.4 and Rap2.4b are very similar and we expect the two transcription factors to share target genes at least to some extent. The up-regulation of Rap2.4 in the Rap2.4b knockout line and *vice versa* might be the result of a mechanism that compensates for the lack of the other gene in order to maintain optimal levels of target (shared) gene expression. Other abiotic stresses such as heat, cold, high salinity or ABA application did not cause expression increases of either gene in the reciprocal knockout lines indicating that the function Rap2.4 and Rap2.4b could be more important in drought stress response than in other types of abiotic stress.

For further investigation of the function of Rap2.4 and Rap2.4b, double knockout lines lacking both transcripts were generated.

4.4.2 Genes down-regulated in Rap2.4/Rap2.4b double knockout lines

The generated double knockouts did not display any discernible phenotypic changes compared to wild-type plants when grown on soil or exposed to abiotic stresses such as high salinity or dehydration. To further the study, whole genome profiling experiment was undertaken in order to elucidate the function of the two transcription factors. Based on up-regulation of either gene in respective knockouts in drought conditions we hypothesised earlier that Rap2.4 and Rap2.4b are likely to be important for drought response in *Arabidopsis*. The microarray experiment was conducted using dehydrated tissues of wild-type and double knockout line in order to explore the role of the two DREBs in drought responses.

The microarray experiment carried out at the VIB Microarray Facility using the Agilent 44K *Arabidopsis* array revealed down-regulation of 32 genes by more than two-fold in the double knockout lines compared to wild-type. Several genes were found to be up-regulated but as Rap2.4 and Rap2.4b were observed to act as transcriptional activators earlier in this study, we concentrated on investigating the down-regulated genes. Most of the genes down-regulated in *Arabidopsis* seedlings lacking Rap2.4 and Rap2.4b expression, are responsive to drought treatments according to publicly available microarray data, a few genes have characterized functions in drought response and some can be associated with drought response through similarities with genes described in literature. The more interesting putative targets of Rap2.4 and Rap2.4b are examined in detail below.

Most intriguingly we found 6 aquaporin genes and an aquaporin PIP3-like pseudogene to be down-regulated by at least two-fold. Two more aquaporins were down-regulated by almost two-fold in the double knockout compared to wild-type. Aquaporins are ubiquitous and fulfil crucial roles in all life forms by providing a molecular mechanism for water transport and equilibration in cells (Agre, 2004). In plants, aquaporins contribute to water transport in both roots

and transpiring leaves; and are required during reproduction, seed germination and growth. Aquaporins can also transport carbon dioxide and other physiologically important molecules such as reactive oxygen species H_2O_2 and ammonia across membranes thereby participating in photosynthesis, nutrient acquisition and stress responses (Bienert et al., 2007; Loque et al., 2005; Uehlein et al., 2003).

The aquaporins down-regulated in the double knockout seedlings were either plasma membrane intrinsic proteins (PIPs) or tonoplast intrinsic proteins (TIPs). TIPs and PIPs along with Nodulin26-like intrinsic membrane proteins (NIPs) and the small basic intrinsic proteins (SIPs) belong to the superfamily of Membrane Intrinsic Proteins (MIPs) (Johanson et al., 2001). The TIP and PIP group aquaporins are the most abundantly expressed MIPs and are therefore thought to represent central pathways for water transport within and between cells (reviewed in Maurel, 2007).

Several studies have investigated the expression of the whole family of aquaporins in respect of tissue- and organ-specificity and stress-responsiveness. Alexandersson et al. (2005) found several aquaporin genes to be expressed in an organ-specific manner either in roots or flowers of *Arabidopsis* whereas no leaf-specific aquaporins were detected. In rice, however, 14 out of the 33 aquaporin genes in total were found to be leaf specific and eight were expressed in all tested organs (Sakurai et al., 2005).

Regarding drought stress responsiveness, the study by Alexandersson et al. (2005) reported down-regulation upon gradual drought stress for most PIP and TIP genes both at the mRNA and protein level. The authors hypothesized that the down-regulation of aquaporins could minimise the water flow through membranes and thereby assist in maintaining leaf turgor during times of water deprivation. Altered expression of *Arabidopsis* plasma-membrane aquaporins (PIPs) upon drought stress inflicted by mannitol application was also observed in RT-PCR expression profiling experiments conducted by Jang et al. (2004). The importance of PIP-type aquaporins in drought stress was further illustrated by experiments by Martre et al. (2002) which showed impaired recovery after dehydration for transgenic lines expressing reduced amounts of PIP1 and PIP2 type water channel proteins.

Aquaporin expression also responds at the transcriptional level to other abiotic stresses such as cold, high salinity, mineral starvation and diurnal changes (Bray, 2002; Jang et al., 2004; Maathuis et al., 2003; Sakurai et al., 2005; Ueda et al., 2004). While regulation of aquaporin activity (gating) by phosphorylation and by interactions with Ca^{2+} and pH is relatively well studied, an upstream transcriptional regulator has only been identified for *Arabidopsis* PIP2;1. Namely, repression of a basic leucine zipper transcription factor AtbZIP24 activated the expression of PIP2;1 among other genes and resulted in improved salt tolerance in transgenic plants (Yang et al., 2009a). A post-translational regulator has also been reported for PIP2;1. A drought stress-induced RING membrane-anchored E3 ubiquitin ligase homolog Rma1H1 interacts with PIP2;1 protein *in vitro* and is able to ubiquitinate it *in vivo*. Rma1H1 overexpression reduces PIP2;1 protein levels and inhibits the trafficking of PIP2;1 from the endoplasmic reticulum to the plasma membranes of protoplasts (Lee et al., 2009a).

The changes in mRNA abundance of eight TIP and PIP genes out of 23 TIP/PIP-type genes in *Arabidopsis* in the Rap2.4/Rap2.4b double knockout line suggests that either Rap2.4 or Rap2.4b or both genes have an important role in regulation of aquaporin expression under drought conditions.

Among the genes down-regulated in double knockout seedlings was a putative dehydrin encoded by At4g38410. The ten genes encoding dehydrins form the D-11 family of the LEA superfamily (Bray et al., 2002) and include well-characterized proteins with established roles in the response to drought e.g. COR47, ERD10, ERD14, XERO1, XERO2, and RAB18. The expression of dehydrins is often altered in transgenic lines which display enhanced tolerance to dehydration. For example, drought-tolerant *Arabidopsis* lines overexpressing DREB1A or DREB2A accumulate high levels of COR47 mRNA in unstressed plants (Sakuma et al., 2006a). Even though overexpression of single dehydrins does not correlate with enhanced drought tolerance, simultaneous overexpression of LTI29 and Xero2 or RAB18 and COR47 led to increased freezing tolerance in *Arabidopsis* (Puhakainen et al., 2004). The predicted amino acid sequence of At4g38410 contains a conserved domain known as the S-domain, which can be phosphorylated leading to calcium-binding activity (Hundertmark and Hinch, 2008), suggesting that post-translational modifications play a role in activity of the encoded protein. The promoter region of At4g38410 contains five DRE-consensus sequences and an additional two DRE-like DNA elements (CCG*C, * denoting any of the four nucleotides) which could act as recognition sites for Rap2.4 and/or Rap2.4b.

The proline transporter AtProT3, one of the three functional proline (Pro) transporters in *Arabidopsis*, was down-regulated in the double knockout line. In plants, exposure to high salinity, dehydration and cold can induce accumulation of compatible solutes such as Pro, glycine betaine, polyols, or sugars which act as cellular osmoprotectants that help maintain the correct conformation of macromolecules such as proteins and DNA (reviewed in Ingram and Bartels, 1996). *Arabidopsis* proline transporters (ProTs) mediate transport of Pro, glycine betaine, and the stress-induced compound γ -aminobutyric acid. Increased accumulation of Pro has been reported in a number of transgenic lines that exhibit enhanced drought tolerance (Chen et al., 2009; Ito et al., 2006; Xue et al., 2009). AtProT3 is expressed at very low levels in only the aboveground organs of *Arabidopsis*, has been shown to localize to the plasma membrane and is thought to mediate intracellular transport of compatible solutes (Grallath et al., 2005). The eFP browser shows up-regulation for AtProT3 following 1 hour exposure to drought conditions, supporting a hypothetical role for AtProT3 in drought response pathways.

At5g06530 encoding a protein named AtWBC23 of the large ABC (ATP-Binding Cassette) transporter family (Sanchez-Fernandez et al., 2001), was down-regulated in the double knockout line compared to wild-type plants. ABC transporter proteins are active in the transport of a broad range of substances including auxin, sucrose, mono- and divalent ions and heavy metals, across plasma membranes and regulate stomatal opening/closure (Schulz and Kolukisaoglu, 2006).

The *Arabidopsis* WBC subfamily members DSO (DESPERADO, AtWBC11) and CER5 (ECERIFERUM 5, AtWBC12) are associated with transport of plant cuticle components from the epidermis layer, where they are synthesised, to the surface of the plant. The plant cuticle layer has multiple functions – it prevents postgenital organ fusion and protects the plant against non-stomatal water loss, frost, UV and pathogens (Panikashvili et al., 2007; Pighin et al., 2004). *dso* mutant plants have abnormally functioning cuticles and in addition to developmental defects, display high susceptibility to salt stress.

AP2 transcription factors have previously been associated with enhanced drought tolerance through changes in the wax biosynthesis pathway. Overexpression of the Rap2.4 ortholog in *Medicago truncatula* WXP1 showed enhanced drought tolerance due to increased cuticular wax accumulation in transgenic alfalfa (Zhang et al., 2005). Overexpression of all three SHINE clade ERF genes not only changed the wax composition and increased cuticular wax levels, but led to alterations in leaf and petal epidermal cell structure, trichome numbers and stomatal index. Importantly, SHN overexpressing lines displayed significant drought tolerance and recovery which was attributed to changes in surface wax accumulation (Aharoni et al., 2004).

Other genes down-regulated in double knockout seedlings encoded proteins associated with signal transduction, transcriptional regulation, cell-wall metabolism and regulation of protein activity. Two DREB genes very closely related to Rap2.4 and Rap2.4b were also found to be down-regulated in the double knockout plants upon drought treatment.

In summary, the set of genes identified by microarray analysis utilising double knockouts of Rap2.4 and Rap2.4b mostly comprises of genes that either have established roles in drought response pathways or can be associated with drought responses through similarities with genes described in literature. In particular, Rap2.4 and/or Rap2.4b were found to regulate the expression of several genes belonging to the aquaporin family.

4.4.3 Rap2.4 and Rap2.4b recognition sequences in the promoters of putative target genes

Scanning the promoter regions of genes differentially-regulated in the double knockouts seedlings revealed that most of the putative targets of Rap2.4 and/or Rap2.4b have either consensus DRE (CCGAC) or DRE-like *cis*-elements present within the 1 kb region upstream of the transcription start site. The DRE-like element CCG*C, * denoting any of the four nucleotides, is a probable recognition sequence for Rap2.4 and Rap2.4b based on our earlier studies of the DNA-binding specificity of the two transcription factors and data published for DREB2A (Sakuma et al., 2002). The presence of DRE and DRE-like *cis*-elements within the promoters of differentially expressed genes suggests that Rap2.4 and/or Rap2.4b might directly regulate the expression of these genes.

Among the genes identified as down-regulated by the lack of Rap2.4 and Rap2.4b expression, three pairs of genes could potentially share promoter elements. The aquaporin genes PIP2;2 (At2g37170) and PIP2;3 (At2g37180) are neighbouring genes lying in a head-to-head conformation (i.e. divergently transcribed) on chromosome 2 with a 2.85 kb region separating the ATG codons. The expression patterns in the Genevestigator database for PIP2;2 and PIP2;3 are

similar, further supporting the hypothesis that the two genes might be controlled by the same regulatory elements. Both PIP2;2 and PIP2;3 are highly expressed root-specific genes according to publicly available microarray data.

AtMRD1 (At1g53480, (Goto and Naito, 2002)) and a DNA-binding zinc-finger protein (At1g53490) might also share regulatory elements. The coding regions of the two genes overlap on chromosome 1 with the AtMRD1 coding region located within the 5' UTR of the At1g53490. AtMRD1 is annotated to be transcribed from the negative and DNA-binding zinc-finger from the positive strand. It can not be determined whether AtMRD1 and At1g53490 show co-regulation based on publicly available microarray data as there is no probe for AtMRD1 on the widely used Affymetrix 22K assays.

The final pair of Rap2.4 and Rap2.4b regulated genes that could potentially share regulatory elements are the DREB transcription factors of the A-6 subgroup At2g20880 and At4g28140. At2g20880 and At4g28140 originated from large segmental duplications in the *Arabidopsis* genome (discussed in Chapter 2). Alignment of the 1 kb regions upstream of the transcription start site of the two genes revealed a 200 bp region with 66 % identity. Considering their common ancestry, similarities in promoter sequence and expression, and the shared upstream regulators, At2g20880 and At4g28140 might fulfil similar roles during the *Arabidopsis* life-cycle.

4.4.4 Overexpression of Rap2.4 and Rap2.4b

The overexpression lines of Rap2.4 and Rap2.4b were generated to complement the analysis of knockout lines in the investigation into the roles of the two transcription factors. Overexpression analysis is not affected by redundancy to the same extent as analysis of knockouts and provides informative phenotypes more often.

The Rap2.4 and Rap2.4b ORFs with the native 5' and 3' UTR sequences were expressed in *Arabidopsis* using the BASTA-selectable binary vector pFGC5941 (Figure 2.15). The upstream and downstream UTRs were included in order to achieve high-level stable expression in transgenic lines after several failed attempts to overexpress Rap2.4 using only the ORF.

Southern analysis revealed that the lines identified as Rap2.4 or Rap2.4b overexpressers contained multiple copies of inserted T-DNA sequences. One of the aims of plant transformation is to achieve simple integration patterns and low copy number of introduced genes, in order to minimize the probability of undesired gene disruption at the insertion site (Birch, 1997). To avoid incorrectly attributing phenotypic changes caused by disruption of unknown genes in the Rap2.4 or Rap2.4b overexpressers, the progeny of several independent overexpression lines were assessed.

4.4.5 Phenotypic characterization of Rap2.4 and Rap2.4b overexpression lines

Growing plant lines overexpressing Rap2.4 or Rap2.4b on soil under normal conditions did not reveal any morphological changes compared to wild-type plants. In addition, no phenotypic changes were detected in the overexpression lines upon exposure to dehydration or high salinity. However, Rap2.4 and Rap2.4b overexpressing lines were more sensitive to ABA

upon germination and during early growth stages under *in vitro* conditions. Importantly, no significant difference was detected between independent lines overexpressing Rap2.4 or Rap2.4b, respectively, confirming that the phenotypes observed were not due to random positional effects of the T-DNA insertions such as interruption of unknown genes.

ABA hypersensitivity has been documented for several mutant lines overexpressing genes associated with stress responses. For example, overexpression of AtMYB44 made plants more sensitive to ABA, while enhancing drought tolerance (Jung et al., 2008). Similarly, overexpression of either of the ABRE-ELEMENT BINDING basic leucine zipper transcription factors ABF3 and ABF4 resulted in ABA hyper-sensitivity during seedling growth and enhanced tolerance to abiotic stresses (Kang et al., 2002; Zhang et al., 2007c). Overexpression of rice NAC transcription factor SNAC1 also conferred higher sensitivity to exogenous ABA and improved drought tolerance (Hu et al., 2006). Overexpression of abovementioned transcription factors enhances drought tolerance through increased stomatal closure and reduced water loss. ABA hypersensitivity of Rap2.4 and Rap2.4b overexpresser plants observed here in combination with reported increased survival rates of Rap2.4 overexpresser plants in drought (Lin et al., 2008) could suggest a function for the two DREB transcription factors similar to that of AtMYB44, ABF3, ABF4 or SNAC1.

4.4.6 Genes up-regulated in Rap2.4 overexpression lines

The microarray experiment investigating global gene expression in the Rap2.4 overexpression lines was undertaken in order to identify targets of Rap2.4. Overall, mRNAs of 22 genes besides Rap2.4 accumulated to higher levels in the mutant line than in wild-type. A few transcripts were down-regulated compared to WT but as Rap2.4 acted as a transcriptional activator in our earlier experiments, we concentrated on the genes up-regulated by Rap2.4 overexpression.

Several of the genes previously identified as putative targets of Rap2.4 or Rap2.4b in the microarray experiment utilizing double knockout lines were up-regulated by overexpression of Rap2.4.

In particular, genes belonging to the aquaporin family had been previously down-regulated by lack of Rap2.4 and Rap2.4b. In the overexpression line of Rap2.4, PIP2;3 and TIP2;2 were up-regulated by more than 2-fold while four more aquaporins showed >1.7 fold up-regulation. PIP1;2 and the PIP3-like pseudogene which were down-regulated in the double knockout plants, did not display altered expression levels in Rap2.4 overexpression mutants suggesting that Rap2.4b is probably the upstream regulator for the two genes. The results obtained here support the earlier observation that the two DREBs probably have important roles in regulating the expression of water channel proteins.

The paralogous DREB transcription factors At4g28140 and At2g20880 were down-regulated in the double knockouts and up-regulated in the Rap2.4 overexpression lines. According to publicly available microarray data, At4g28140 and At2g20880, while expressed at much lower levels, are induced in a manner similar to Rap2.4 and Rap2.4b by drought, salt and general

osmotic stress and repressed by cold treatment. Overall, our results suggest that Rap2.4 controls expression of other closely related DREB genes.

The zinc-finger transcription factor (At4g38140), LEA dehydrin At4g38410, protein kinase At5g24080 and an unknown gene At1g20070 showed altered expression levels in both microarray experiments indicating that they could be targets of Rap2.4. A second LEA gene (LEA76-type 1 protein, At1g52690) was also up-regulated in the Rap2.4 overexpression line. The drought-responsive LEA76-type 1 protein is a known target of DREB2A (Sakuma et al., 2006a; Seki et al., 2001). Interestingly, a homolog of LEA 76 was found to be expressed in the desiccation tolerant bacterium *Deinococcus radiodurans* along with a homolog of PCC13-62 protein of the resurrection plant *Craterostigma plantagineum*. When the two genes were inactivated, bacteria became sensitized to desiccation (Battista et al., 2001). Involvement in the dehydration response of distantly-related species suggests an important role for LEA76-like genes in the acquisition of drought tolerance, while regulation by multiple similar up-stream regulators may reflect the robustness of the drought-signalling pathways in *Arabidopsis*.

Among the genes uniquely up-regulated in the Rap2.4 overexpression mutants, several surprisingly were associated with lipid metabolism. AP2 transcription factors have been associated with regulating lipid synthesis and transport before as discussed earlier in this chapter. Altered expression patterns in the Rap2.4 overexpression lines further support the hypothesis that Rap2.4-mediated stress responses include a component of lipid metabolism.

LTP3 and LTP4 are small (~9 kDa) basic proteins that belong to the type I subgroup of lipid transfer proteins of *Arabidopsis*. Lipid transfer proteins are abundant in plants (up to 4 % of soluble proteins) and are thought to play a role in several biological processes including pathogen defence, signalling and cuticle deposition (Kader, 1996; Yeats and Rose, 2008). In tree tobacco, Cameron et al. (2006) found that increased expression of LTPs correlated with increased cuticular wax accumulation further supporting the role of LTPs in transfer of lipids through the extracellular matrix for wax formation. LTP3 and LTP4 were strongly up-regulated by dehydration and ABA treatment in experiments conducted by Huang et al. (2007; 2008b) suggesting a role in abiotic stress response.

The palmitoyl thioesterase encoded by At5g47330 was up-regulated in Rap2.4 overexpression lines. Palmitoylation (S-acylation) which is the only reversible lipid modification of proteins, promotes protein membrane association and is known to play a role in protein-protein interactions, stress and Ca⁺ signalling and pathogenesis (Hemsley et al., 2008; Hemsley, 2009). At5g47330 was originally identified as a salt-responsive gene in a study by Gong et al. (2001) further supporting a stress-related role for this gene. At5g47330 was shown to be up-regulated in that part of the epidermis where cuticular lipids are actively deposited in *Arabidopsis* (Suh et al., 2005) providing a link between Rap2.4 and lipid metabolism.

The PQ-loop gene (At4g36850) up-regulated in the Rap2.4 overexpression line belongs to a family of 6 putative transmembrane proteins and was identified as a gene involved in recovery from dehydration stress by Oono et al. (2006). Altering the expression of some PQ genes has been

shown to result in differential regulation of a number of genes associated with defence and lipid biosynthesis (Pattison, 2008).

Up-regulation of LTP3, LTP4, At5g47330 and At4g36850 in the Rap2.4 overexpression lines further strengthens the links between AP2 transcription factors and lipid metabolism/transport.

An interesting gene up-regulated in the Rap2.4 overexpression line was the GDSL-motif lipase like-1 (GLIP1). GLIP1 is a well-characterized gene with an established function in plant immunity. *glip1* mutants are highly susceptible to infection by the necrotrophic pathogen *A. brassicicola*, fail to accumulate transcripts of the defence proteins PR1 and PDF1.1; and do not activate systemic resistance upon *A. brassicicola* infection. Dual roles have been proposed for GLIP1: direct interference with pathogen growth and activation of systemic resistance (Kwon et al., 2009). The transcripts of the defence proteins PR3, PR4 and PDF1.2 have been shown to over-accumulate in the Rap2.4 overexpression line upon treatment with ethylene precursor ACC (Lin et al., 2008). GLIP1-associated systemic resistance is dependent on ethylene signalling according to Kwon et al. (2009) suggesting that Rap2.4 might act as an upstream regulator of ethylene-dependent defence signalling pathways through GLIP1.

As observed before in the double knockout lines, expression levels of several cell wall metabolism associated genes were changed in the Rap2.4 overexpression line. We found up-regulation of two xyloglucan endotransglucosylase/hydrolase (XET/XTH) encoding transcripts in Rap2.4 overexpression lines, while down-regulation of XTH26 gene was observed in double knockout mutants. The genes up-regulated in the Rap2.4 overexpression lines were XTH6 and XTH25. XTH25 is also known as XTR3 and has been shown earlier to be up-regulated in *Arabidopsis acaulis* (*acl*) mutants which show a reduction in leaf expansion and stem elongation due to reductions in cell size (Akamatsu et al., 1999). Cell wall properties are known to change in response to cellular water stress (Wu and Cosgrove, 2000) and XTHs enzymes, which are involved in construction and modification of cellulose/xyloglucan framework of the cell wall, have been observed to respond to dehydration stress (Bray, 2002; Seki et al., 2001). Activity of XTH enzyme has been detected in expanding tissues but some members of the XTH family are also expressed in tissues which have ceased to expand (Yokoyama and Nishitani, 2001, and references therein). Perhaps changes in cell wall properties caused by differential regulation of XTH enzymes contribute to aspects of both drought tolerance and the dwarf phenotypes often observed in transgenic plants overexpressing DREB transcription factors.

Finally, a MATE (multidrug and toxic compound extrusion) protein was up-regulated in the Rap2.4 overexpression line. MATE efflux proteins are responsible for transportation of various substances in plants. For example, TRANSPARENT TESTA12 (TT12)-encoded MATE protein is required for proanthocyanin deposition in vacuoles of seed coat endothelial cells. Interestingly, a large proportion of MATE proteins in *Arabidopsis* are salt-stress inducible in root tissues (Jiang and Deyholos, 2006) and some MATE proteins are induced by high salinity in *Arabidopsis* shoots (Maathuis et al., 2003) suggesting a role in abiotic stress responses.

4.4.7 DRE and DRE-like elements in the promoters of putative Rap2.4 target genes

Scanning the promoters of genes up-regulated in the Rap2.4 revealed the presence of DRE consensus or DRE-like elements for all but two of the genes. The MATE efflux family protein encoding gene (At1g66780) or the PQ-loop repeat family protein encoding gene (At4g36850) did not have DRE or DRE-like sequences in the 1 kb region upstream of the transcription start site suggesting that they are probably regulated by transcription factors downstream of Rap2.4. Alternatively, Rap2.4 overexpression might have altered the expression of protein-modifying genes which in turn might alter the activity of transcription factors that control the expression of At1g66780 and At4g36850.

Some of Rap2.4 targets identified in the overexpression lines may share regulatory elements as a consequence of their location. In addition to the DREB transcription factors At2g20880 and At4g28140 and the aquaporins PIP2;2 and PIP2;3 which were discussed earlier, the lipid transfer proteins LTP3 and LTP4 are probably regulated through shared *cis*-elements. LTP3 and LTP4 are located on the 5th chromosome in head-to-head (divergently transcribed) manner. One DRE-consensus and several DRE-like elements are present in the 3 kb region spanning the distance between the respective transcription start sites.

4.4.8 Concluding remarks

The global changes in gene expression induced by drought stress have emerged from several microarray studies. Although the microarray data differ significantly, depending on the experimental conditions used, general tendencies can be deduced. The most commonly induced genes play roles in amino and fatty acid metabolism; encode proteases, protein phosphatases and chaperones; and function in transport or cellular organisation. Drought-induced genes include LEA proteins, transcription factors and defence-related genes (Bray, 2002; Seki et al., 2002b).

The changes in global gene expression observed upon Rap2.4 overexpression and in the Rap2.4/Rap2.4b double knockout line fit very well with the general changes in gene expression observed under drought stress conditions. Most remarkably, Rap2.4 and Rap2.4b control expression of aquaporins and Rap2.4 regulates the expression of a number of genes involved in lipid metabolism and transport. Overall, our results support the hypothesis that the two DREBs function in drought response pathways.

CHAPTER 5

GENERAL MATERIALS AND METHODS

Bacterial strains

Escherichia coli strain **DH5 α** – Genotype F- ϕ 80*lacZ* Δ M15 Δ (*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_k^- , m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ (Invitrogen).

Escherichia coli strain **BL21 (DE3)** – Genotype F *ompT hsdS_B($r_B^-m_B^-$) gal dcm met* (DE3) (Studier et al., 1990).

Agrobacterium tumefaciens strain **AGL-1** – Genotype C58, *RecA, Rif^R, Carb^R, Ti* plasmid type succinamyopine, pTiBo542 Δ T-DNA (Lazo et al., 1991).

Agrobacterium tumefaciens strain **GV3101** – Genotype C58, *Rif^R, Ti* plasmid type nopaline (Holsters et al., 1980).

Bacterial Growth Media

LB (Luria-Bertrani) media – 10 g/L bacto-tryptone, 5g/L bacto-yeast extract, 10 g/L NaCl; pH 7.0 (1.5 w/v agar was added for LB plates).

YM media - 0.04 % yeast extract, 10g/l mannitol, 1.7 mM NaCl, 0.8 mM MgSO₄, 2.2 mM K₂HPO₄, pH 7.0 (1.5 w/v agar was added for YM plates).

Plant Growth Media

MS (Murashige and Skoog) – 4.4 g/L MS salts with vitamins, pH 5.7, (0.8 w/v agar was added for MS plates) supplemented with 1.25 mg/L antifungal agent amphotericin.

MS for roots - 4.4 g/L MS salts with vitamin, 2 % sucrose, pH 5.7, supplemented with 1.25 mg/L antifungal agent amphotericin.

DNA manipulations

General DNA manipulations (DNA preparation, restriction digestion, ligation) were carried out using standard protocols (Sambrook et al., 1989). For separation of DNA fragments, electrophoresis in 1 x TBE buffer was used. 0.8 % agarose gels were stained with ethidium bromide (0.5 μ g/ml) and DNA was visualized by UV transillumination. Plasmid extractions were prepared using the JETquick Plasmid Miniprep Spin Kit and the accompanying protocol (Genomed).

Sequencing was carried out using the MWG Biotech AG services.

The restriction enzymes and buffers were obtained from New England Biolabs, Roche Molecular Biochemicals and Sigma-Aldrich.

Prior to ligation reactions, digested plasmid DNA was dephosphorylated by treatment with calf intestinal alkaline phosphatase (Roche Molecular Biochemicals).

Following modification by enzymes DNA was re-purified as follows: the DNA was extracted once with an equal volume of phenol, vortexed and centrifuged at 12 000 rpm for 4

minutes. The residual phenol was removed by spin chromatography on homemade Sepharose CL6B minicolumns.

General PCR conditions

PCR amplifications were performed in a volume of 50 μ l: the reaction mix contained 10-100 ng of plasmid template, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μ M forward and reverse primers and 2.5 units of proofreading *Pfu* DNA polymerase (Stratagene Cloning Systems). The cycling was carried out as follows: 94°C for one minute, 56°C for one minute, 72°C for two minutes; repeated 30 times and followed by a final extension step of 72°C for 10 minutes. Depending on specific primer combinations annealing temperatures were increased from 56°C up to 62°C in some instances. The PCR products were verified on 0.8 % agarose gels.

PCR conditions for generation of DIG-labelled probes

DNA fragments were digoxigenin-labelled (DIG) using a DIG Probe PCR synthesis kit (Roche Diagnostics GmbH, Germany) according to the manufacturers' instructions. Plasmid DNA was used as the DNA template where possible, other times probes were amplified from plant genomic DNA extracted with the REExtract-N-Amp Plant PCR kit (Sigma).

Transformation of *E. coli* by the heat shock method

The heat shock method was used for *E. coli* transformation. $CaCl_2$ -competent cells (Novagen, supplied with the pET system vectors) were thawed on ice and mixed with 10 ng of plasmid DNA for each transformation. The cells were incubated on ice for 20 minutes and transferred to a 42°C waterbath for 1 minute for the heat shock treatment. 500 μ l of SOC medium (Invitrogen) was added to each transformation and tubes were incubated for 1 hour at 37°C (shaken at 200 rpm). Cells were plated on selective medium (LB with appropriate antibiotics) and incubated overnight at 37°C. Four colonies were typically picked for each transformation and verified to contain the desired plasmids by plasmid isolation and restriction digestion.

Preparation of *Agrobacterium* competent cells for electroporation

Agrobacterium tumefaciens strains AGL-1 or GV3101 were streaked from glycerol stocks onto LB plates containing appropriate antibiotics (50 μ g/ μ l carbenicillin and 50 μ g/ μ l kanamycin for AGL-1; 100 μ g/ μ l rifampicin and 50 μ g/ μ l kanamycin for GV3101). A single colony was picked and grown in liquid YM medium overnight at 28°C shaking at 250 rpm. This culture was used to inoculate fresh YM medium and grown to an OD_{600} =0.5-0.7. Cells were collected by centrifugation at 7000 rpm at 4°C for 10 minutes, washed with and resuspended in cold 1 mM HEPES, 10 % glycerol pH 7.0. Aliquots of cells were stored at -70°C.

Agrobacterium transformation

The binary vector containing the gene of interest was transformed into *Agrobacterium tumefaciens* strain AGL-1 using the GIBCO Cell-Porator electroporator and the accompanying standard protocol (Life Technologies). Following electroporation cells were plated on YM

medium containing appropriate antibiotics for selection of transformed cells. Plates were incubated for 48 hours at 28°C. Four single colonies were picked from each transformation plate and grown in liquid LB medium. The presence of desired binary vectors in *Agrobacterium tumefaciens* was confirmed by restriction analysis of isolated plasmids.

Plant transformation via 'Floral Dip'

Plant transformation was achieved via the 'Floral Dip' method (Clough and Bent, 1998). *Arabidopsis* plants were grown in the greenhouse under normal growth conditions (22-24°C, 16 hours of light, 8 hours of dark) in pots covered with tulle cloth for approximately one month. The first flower bolt was clipped to encourage proliferation of secondary bolts. *Agrobacterium tumefaciens* strains containing the desired plasmid constructs was grown in large volume liquid culture until OD₆₀₀=0.8-1.0. Cells were collected by centrifugation at 10 000 rpm and resuspended in an autoclaved 5 % (w/v) sucrose solution at a cell density equivalent to OD₆₀₀=0.8. The surfactant Silwet L-77 was added to the resuspended cells (to a final concentration 0.05 % v/v) to mediate the entry of *Agrobacterium* into floral tissues. All aboveground organs of *Arabidopsis* plants were dipped into the solution containing *Agrobacterium* for 3 seconds with gentle agitation. Following "Floral Dip" plants were kept overnight in the cold room to improve recovery from treatment and returned to the greenhouse where they were kept under normal growth conditions.

Seeds were harvested approximately one month later. Seeds were sown on soil and selected by spraying twice or thrice, with two day intervals, with a 0.01 % solution of the herbicide glufosinate (ammonium salt) (BASTA by AgrEvo UK Ltd) at an early growth stage (typically 2 – 3 days post-germination).

Plant genomic DNA extraction for direct PCR screening

Plant genomic DNA was extracted using the REDExtract-N-Amp Plant PCR kit (Sigma) following the manufacturers' instructions. The PCR was carried out according to the kit manual. The reaction mix contained 50 %REDExtract-N-Amp Readymix, 20 % leaf extract and 0.4 µM of each primer. Cycling was carried out as follows: 94°C 3 minutes; 30 cycles of 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 1 minute; final extension of 10 minutes at 72°C.

RNA extraction

Total RNA was extracted from leaves, flowers, stems and siliques of *Arabidopsis* ecotype Columbia plants grown in the greenhouse under normal conditions (22-24°C, 16 hours light). Root RNA was extracted from roots of dark-grown *in vitro* seedlings (grown in liquid MS medium with added vitamins and 2 % sucrose, 2.5 µg/ml amphotericin, pH 5.7, shaken at 100 rpm at 23°C). Following stress treatments RNA was extracted from whole seedlings.

The RNA extraction protocol was based on procedure described by Eggermont et al. (1996). All solutions were treated with 0.1 % DEPC (diethylpyrocarbonate) and autoclaved. All equipment was soaked in 0.1 N NaOH and rinsed with DEPC-treated water. Approximately 500 mg of tissue was collected for each sample, ground to a fine powder in liquid nitrogen and

homogenised further in a 1:1 solution of extraction buffer (0.1 M Tris-HCl pH=8.0, 50 mM EDTA, 50 mM NaCl, 2 % SDS, 0.75 % β -mercaptoethanol) and phenol. 4M LiCl was used to specifically precipitate RNA at -20°C. RNA was recovered by centrifugation (12 000 rpm, 15 min), washed with 75 % ethanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

The integrity of RNA was assessed on a 0.8 % agarose gel and the concentration of RNA in samples was measured using a ND-1000 spectrophotometer (NanoDrop Technologies). Samples were stored at -70°C.

Northern blotting

The Northern blotting protocol was adapted from Eggermont et al. (1996). RNA was separated in 1 % agarose, 1 % formaldehyde gels in NBC buffer (50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH, pH 7.5). The gel was equilibrated in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 30 minutes and blotted by capillary transfer onto a positively charged nylon membrane (Roche) using 20X SSC overnight. The transferred RNA was UV cross-linked to the membrane before proceeding to the hybridisation steps. Pre-hybridisation and hybridisation were carried out in high SDS buffer (7 % SDS, 50 % deionised formamide, 5X SSC, 2 % blocking reagent, 50 mM sodium phosphate pH=7.0, 0.1 % N-lauroylsarcosine) at 50°C for 4 hours and overnight, respectively. Probes were labelled with digoxigenin (DIG) using the PCR DIG Probe synthesis kit and the accompanying protocol by Roche. For hybridisation 15 ml buffer containing the DIG-labelled probe at 20 ng/ml concentration was used per 100 cm² of membrane.

After hybridisation, washing and detection was carried out as follows: membranes were washed twice in 2X SSC, 0.1 % SDS and twice in 0.3X SSC, 0.1 % SDS at 68°C for 10 minutes and 20 minutes, respectively. Membranes were blocked in 2 % blocking solution (0.1 M maleic acid, 3 M NaCl, 2 % blocking reagent, pH 8.0) for 1 hour and incubated with an anti-DIG alkaline phosphatase conjugated antibody (Roche) for 30 minutes (diluted 1:10000 in blocking solution). Nonhybridised antibody was removed by washing the membrane 4 times in high salt buffer (0.1 M maleic acid, 3M NaCl, 0.3 % Tween 20, pH 8.0). A suitable pH for alkaline phosphatase-mediated revelation of hybridised bands was achieved by incubating the blot twice in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 3 minutes. The light-emitting substrate CSPD (Disodium 3-(4-methoxy spiro (1, 2-dioxetane-3, 2'-5'-chloro)tricyclo[3.3.1.1.3,7]decan)4-yl)phenyl phosphate) (Roche) was added for detection. Membranes were incubated at 37°C for 10 minutes and exposed to X-ray films for 8 to 16 hours.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In this study we set out to characterize the function of two closely-related genes, Rap2.4 and Rap2.4b, encoding AP2/ERF transcription factors. The two genes belong to the DREB subgroup, members of which have been demonstrated to function in plant responses to drought, cold and high salinity. Identifying the transcription factors that regulate responses to abiotic stresses is a topic of great interest in plant biology because regulatory genes that control whole cascades of stress signalling can offer significant potential for crop improvement. However, in order to realize this potential an in-depth knowledge of the individual transcription factors is essential.

Rap2.4 and Rap2.4b show 100 % conservation in their DNA-binding domains. According to the TIGR database, the two genes originated from a common ancestor via duplication of a large chromosomal DNA segment. They belong to the A-6 subgroup of DREB genes and share elements of genomic context with four of the A-6 group genes, suggesting a common origin. Expression profiling utilizing Northern blotting and promoter-GUS gene fusions revealed that the expression patterns of the two genes are only partially overlapping. Rap2.4 and Rap2.4b are both strongly induced at germination; are expressed in roots of seedlings and respond to drought and to application of exogenous ABA or the ethylene precursor ACC. However, the expression kinetics differ for the two genes with Rap2.4b showing stronger but more transient induction. Rap2.4 is also expressed in inflorescences; responds to high salinity stress and during drought is up-regulated to higher levels in ABA-deficient mutants than wild type plants. Rap2.4b on the other hand is strongly expressed in dry seeds, responds to heat stress and is not affected by mutations affecting the ABA biosynthetic pathway. Under stress conditions, Rap2.4 induction is negatively-controlled by FRY1 whereas the elevated IP₃ levels in *fryl* do not alter Rap2.4b mRNA accumulation.

Investigation of the molecular properties of the encoded proteins revealed further similarities. The amino acid region we determined to function as the nuclear localization signal for Rap2.4 is fully conserved in Rap2.4b. The DNA-binding preferences are also very similar – both proteins recognise the DRE-element with the 1st C and 5th C of the CCGAC core being crucial and the A at position 4 dispensable for DNA-protein interactions. The amino acid sequences of Rap2.4 and Rap2.4b differ considerably outside the conserved DNA binding domain suggesting that although these transcription factors probably recognise DRE or DRE-like elements in overlapping sets of promoters, the effect they have on the expression of target genes is not necessarily identical. However, in the experiments in which we tested their transactivation ability, both proteins behaved as activators. The dissimilar regions of Rap2.4 and Rap2.4b might therefore act as determinants for differential interactions with other proteins. However, this remains to be explored.

Overall, expression profiling and investigations of DNA-binding preferences support the idea that Rap2.4 and Rap2.4b functions overlap under some stress conditions and in certain

tissues, but are distinct in other circumstances. Both genes probably activate the same set of genes during early root growth and during the response to drought. Rap2.4b likely also plays a role in desiccated tissues of dry seeds and upon exposure to heat, whereas Rap2.4 up-regulation in ABA-deficient lines could compensate for faulty ABA-dependent drought signalling.

The knockout lines of either Rap2.4 or Rap2.4b had no discernible phenotypes when grown under normal conditions or when exposed to abiotic stresses. The lack of phenotypes in *Arabidopsis* mutants lacking individual genes is common and usually attributed to either genetic redundancy or to the functional robustness of plant physiological processes. Since Rap2.4 and Rap2.4b are indeed very closely related genes, we proceeded to generate double knockout lines in order to attempt to establish functions for both genes. While double knockouts also lacked a distinguishable physical phenotype, global expression profiling using dehydrated tissues revealed an interesting molecular phenotype. Simultaneous elimination of Rap2.4 and Rap2.4b resulted in down-regulation of a number of genes normally associated with the response to drought. Namely, the expression of several genes encoding aquaporins belonging to the TIP and PIP subclass was reduced, as was expression of a dehydrin encoded by At4g38410, the proline transporter AtProT3 and the ABC (ATP-Binding Cassette) family transporter AtWBC23. Other down-regulated genes encoded proteins associated with signal transduction, transcriptional regulation, cell-wall metabolism and regulation of protein activity. Interestingly, two DREB genes of the A-6 subgroup were also found to be down-regulated in the double knockout plants. In future, knocking out additional members of the A-6 subgroup, especially those genes that share genomic context with Rap2.4 and Rap2.4b, could possibly lead to more pronounced phenotypes.

To complement the knockout approach, overexpression lines were generated for Rap2.4 and Rap2.4b. Most noticeably, overexpression of either of the genes resulted in ABA hypersensitivity upon germination. ABA hypersensitivity has been associated with increased tolerance to abiotic stresses in the published literature. However, although enhanced drought resistance was previously described for Rap2.4 overexpression lines (Lin et al., 2008), our mutants failed to display such a trait. Perhaps the differences in the published Rap2.4 phenotype and that observed here might be attributed to differences in the sequences used to achieve overexpression. In the present study the overexpression construct included the non-translated regions of Rap2.4, while Lin et al. (2008) used only the open reading frame. Our situation could be similar to that of DREB2A where only overexpression of a certain truncated version of the open reading frame confers enhanced drought tolerance (Liu et al., 1998; Sakuma et al., 2006a). In future it would be interesting to investigate whether overexpression of different versions of the Rap2.4 cDNA/open reading frame might lead to additional phenotypes.

A DREB2A-like overexpression phenotype was also achieved by eliminating expression of the ubiquitin E3 ligases DRIP1 and DRIP2 which act as negative regulators of DREB2A (Qin et al., 2008). The dramatic increase in Rap2.4 and Rap2.4b mRNA accumulation that we observed upon application of the protein synthesis inhibitor cycloheximide may also indicate the existence of a negative regulatory mechanism for each gene and explain the lack of phenotypes in overexpression lines. The experimental procedures required to establish whether Rap2.4 and

Rap2.4b are regulated in a similar manner to DREB2A at the protein level might include overexpression of a tagged version in order to follow the expression at protein level and yeast two-hybrid experiments to find interaction partners.

The final experiment described in the present study – the expression profiling of the Rap2.4 overexpression line – confirmed our earlier observations. Rap2.4 overexpression enhanced the expression of several genes associated with drought responses in *Arabidopsis*. As seen before in the double knockout, several water channel genes, two dehydrins, genes associated with lipid transport and metabolism and several cell-wall metabolism related genes were up-regulated under normal conditions in the overexpression lines. Moreover, the two DREB factors that were down-regulated in the double knockout were up-regulated in the Rap2.4 overexpression line indicating that Rap2.4 could be an upstream regulator within the A-6 subgroup. Further microarray investigations comparing global gene expression patterns in the Rap2.4b overexpression line would provide interesting data concerning the extent to which the target gene sets overlap for our two DREBs. A schematic summary of Rap2.4 and Rap2.4b functions is given in Figure 6.1.

In summary, Rap2.4 and Rap2.4b are closely related DREBs with very similar molecular properties but expression patterns that differ to a significant extent. Both genes are involved in responses to abiotic stress – they are induced by dehydration and regulate genes with known or potential functions in the drought stress response. Our study shows how two DRE-element binding transcription factors are induced, how they carry out their functions and which genes are their targets, thereby increasing our understanding of stress-response pathways in *Arabidopsis*.

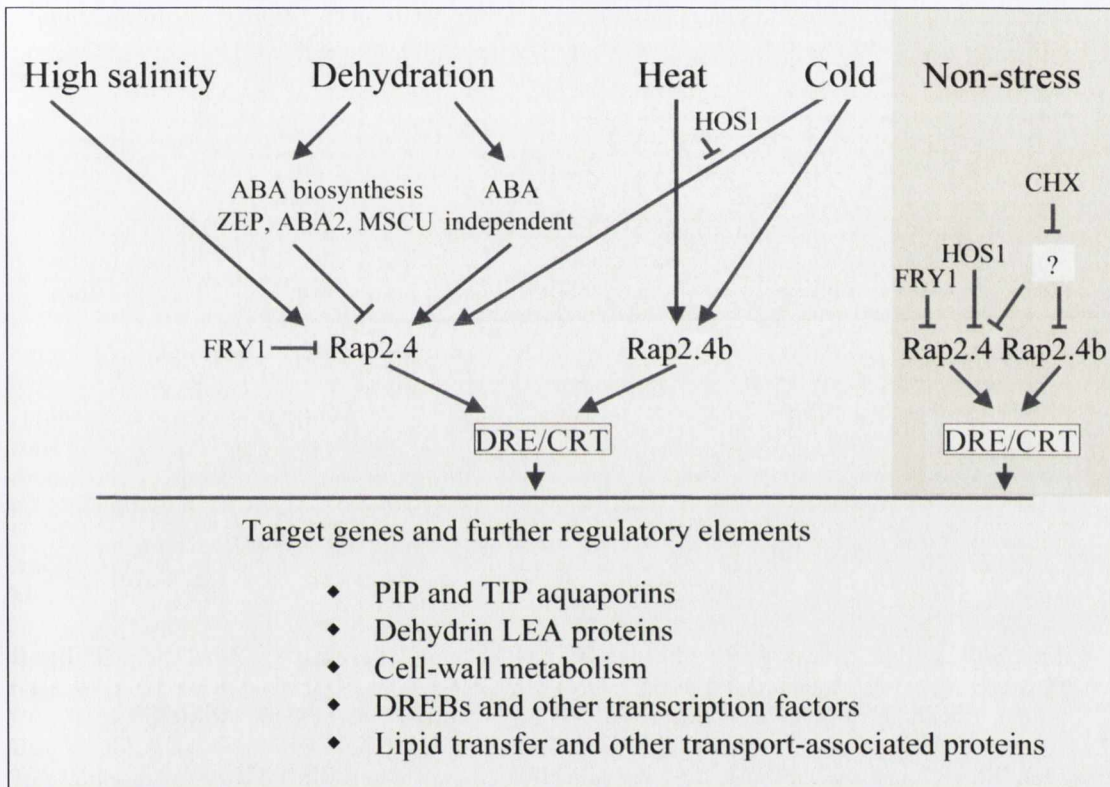


Figure 6.1. Rap2.4 and Rap2.4b position in *Arabidopsis* abiotic stress-signalling cascades.

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