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Pattern of expression of the *F8A24.12* gene from *Arabidopsis thaliana* which encodes a NAK ser/thr protein kinase and functional analysis of the suORF present in its 5'UTR

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ABSTRACT

Pattern of expression of the *F8A24.12* gene from *Arabidopsis thaliana*
which encodes a NAK ser/thr protein kinase
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Frédéric Piot

To overcome stresses such as osmotic shock and pathogen attack, internal signals activate plant defense responses. The *F8A24.12* gene from *Arabidopsis thaliana* encodes the serine/threonine protein kinase with the highest similarity at the structural and functional level to a salt-stress induced gene, *Esi47*, from the salt tolerant wheatgrass *Lophopyrum elongatum*. Both genes have a small upstream open reading frame (suORF) in their 5' untranslated region (5'UTR). This element was previously shown to have a regulatory function in the *Esi47* gene. Northern blot analysis revealed that under salt stress both genes have an increased level of transcription. Transgenic *Arabidopsis* plants were produced carrying reporter gene encoding the enzyme ß-glucuronidase (GUS) under the control of the *F8A24.12* promoter and 5'UTR. GUS histochemical assays revealed that *F8A24.12* gene expression occurs throughout the life of the plant, particularly in the root elongation zone, in the vascular tissues of roots and leaves, in the flower and in the petal and sepal abscission zone. GUS fluorometric assays demonstrated that the quantity of GUS protein is induced by methyl jasmonate (MeJA) treatment after 12, 24 and 36 hrs and by salt stress after 24 hrs treatment. These data suggest that the *F8A24.12* gene is part of the jasmonic acid signaling pathway. This phytohormone is related to the plant growth and plant defense responses. GUS fluorometric assays were also performed on transgenic *Arabidopsis* carrying an altered suORF promoter-GUS fusion. Mutation of the suORF obliterated the MeJA and salt responsiveness of the *F8A24.12* gene promoter plus 5'UTR transgene.

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LIST OF ABREVIATIONS

ABA: Abscisic acid

ABI: Abscisic acid insensitive

ABRC: Abscisic acid response complex

ABRC: Arabidopsis Biological Resource Center

ABRE: Abscisic acid responsive element

3-AT: 3-amino-1, 2, 4-triazole

Amp: Ampicillin

ANOVA: Analysis of variance

bZIP: basic leucine zipper

CaMV: Cauliflower mosaic virus

CE: Coupling element

cfu: colony forming unit

DRE: Dehydration-responsive element

EDTA: Ethylene diaminetetraacetic acid

EIN: Ethylene insensitive

Esi: Early salt-stress induced

EST: Expressed sequence tag

Gen: Gentamycin

GST: Glutathione-S-transferase

GUS: ß-glucuronidase

HOG: High osmolarity glycerol

HR: Hypersensitive response

HSP: Heat shock protein

JA: Jasmonic acid

JIPs: Jasmonic acid induced proteins

Kan: Kanamycin

LB: Luria-Bertani

LEA: Late embryogenesis abundant

MAP: Mitogen-activated protein

MAPK: Mitogen-activated protein kinase

MAPKK: Mitogen-activated protein kinase kinase

MAPKKK: Mitogen-activated protein kinase kinase kinase

MeJA: Methyl jasmonate

MEKK: MAPK/Erk kinase kinase

MIP: Major intrinsic protein

MS: Murashige and Skoog

MU: 4-methylumbelliferone

MUG: 4-methylumbelliferyl ß-D-glucuronide

NAK: Novel *Arabidopsis* protein kinase

OEE: Oxygen-evolving enhancer protein

ORF: Open reading frame

P: Probability score or p-value

PAL: Phenylalanine ammonia lyase

P5CS: ? [1]-pyrroline -5-carboxylate synthase

PCR: Polymerase chain reaction

pfu: plaque forming unit

PG: Polygalacturonase

PR: Patogenesis-related

SA:	Salicylic acid
SAR:	Systemic acquired resistance
SC:	Synthetic complete medium
SOS:	Salt overly sensitive
suORF:	small upstream open reading frame
uORF:	upstream open reading frame
UTR:	untranslated region
X-Gluc:	5-bromo-4-chloro-3-indolyl-ß-D-glucuronide
*	Interaction

CHAPTER 1. INTRODUCTION

1.1. The plant, a complex regulatory network

All stages of plant growth and development including seed germination, growth, flowering and reproduction are genetically programmed and also regulated by environmental stimuli. For example, the passage from vegetative growth to flowering can be regulated by the age of the plant, vernalization (*i.e.*, an extended period at low temperature), day length and osmotic stress. Throughout its life cycle a plant can be exposed to the biotic stress of pathogen attacks and abiotic stresses including extreme temperatures or light intensities, drought, lack of nutrients, or high concentrations of salts or heavy metals in the soil (Bonhert *et al.*, 1995). The plant has to overcome these stresses in order to follow its life cycle. The plant's response to environmental changes and the plant's development are tightly coordinated and an understanding of the plant at the genetic, biochemical and physiological level will reveal the complexity and the regulation of this finely tuned network. The challenge in the coming years will be to create plants tolerant to environmental stresses to enhance agricultural production to meet the nutritional demands of a growing world population. Moreover, growing populations and expanding industrial production will exacerbate environmental stresses by increasing greenhouse gas effects, water demands, and pressure to expand agriculture to soils of poorer quality (Epstein *et al.*, 1980).

Plants must manage a rapid adjustment of their metabolism in response to changes in temperature, water availability and exposure to toxic levels of salt (Bonhert *et al.*, 1995). Perception, signal transduction and gene induction are key components in the

chain of reactions of the signaling pathways that are activated by external stimuli. A classical model of perception involves receptors that may be transmembrane proteins transducing the new information from the external medium to the cytoplasm. This information passes through a transduction cascade often involving protein kinases and phosphatases and leads to the activation of specific transcription factors that bind promoter elements of genes involved in the response (Wang *et al.*, 2002). The plant's response is also under the control of growth regulators. The regulation of the biosynthesis of phytohormones is crucial because of the fine tuning between the different hormones which can determine the activation of specific signaling pathways. Many transduction pathways depend on several growth regulators in a concentration dependent manner. Moreover, a single gene can be implicated in different signaling pathways. This emphasizes the importance of cross talk and presents a challenge to understand the totality of the regulatory network of an organism (Moons *et al.*, 1997).

1.2. High salinity stress and osmotic shock

Cold, drought and high salinity all include an aspect of osmotic stress that gives rise to a change in the water status of the plant and causes the plant to dehydrate (Bonhert *et al.*, 1995). This "physiological drought" can lead to major cell damage including decreased photosynthesis, the accelerated death of plant cells and even plant death if the exposure to the osmotic stress is severe (Zhu *et al.*, 1998). While plants have some system of defense to counter salt stress and osmotic shock, most plants do not resist a high or long salt shock, although some species, referred to as halophytes, are able to grow normally in a high salinity environment. The halophytes have evolved adaptations such

as large vacuolar spaces for the sequestration of toxic sodium ions and show an effective response to salt stress. However, the vast majority of crop species including the major cereal crops (maize, rice and wheat) are not salt tolerant. Large areas of land have a salt concentration too high for the normal growth of these plants (Zhu, 2002).

The physiological drought that is generated by salt stress results from an ion imbalance across the plasma membrane. In order to survive this osmotic shock, the osmotic balance has to be reestablished. One way to recover the osmotic potential that is maintained during normal growth is through sodium partitioning (Niu *et al.*, 1995). In spite of the accumulation of high internal Na^+ concentrations, plant cells are able to keep low cytoplasmic Na^+ and high K^+ levels. Na^+ is transported from roots to shoots and accumulates in vacuoles especially in mature and old leaves (Hasegawa *et al.*, 2000; Munns, 1993). Elevated Na^+ and Cl^- concentration are toxic for the plant (Munns, 2002) but Na^+ uptake by the plant through cellular transmembrane proteins appears to be inevitable (Hasegawa *et al.*, 2000). Although the Na^+ uptake system is not understood, there is physiological evidence that Na^+ is a competitor for K^+ at the level of uptake (Niu *et al.*, 1995). K^+ is crucial for normal plant growth and Na^+ competes with K^+ through common carriers. Na^+ influx through the plasma membrane is possible via Na^+/H^+ and Na^+/K^+ symporters. When the sodium concentration increases in the cytoplasm, H^+ pumps including an H^+-ATPase on the plasma membrane (Niu *et al.*, 1996), a H^+-ATPase and an H^+-pyrophosphatase on the vacuolar membrane, are activated (Niu *et al.*, 1995). Induction of expression of the genes encoding these proteins leads to elevated levels of channel proteins and higher protein turnover rates (Hasegawa *et al.*, 2000).

Transmembrane proton pumps transport H^+ across the plasma membrane from the cytosol to the outside of the cell and across the vacuolar membrane into the vacuole. This leads to an increase of the proton concentration in the vacuole and outside the cell and creates an H^+-electrochemical gradient. This gradient drives Na^+/H^+ antiporters (Hasegawa *et al.*, 2000). Antiporters present on the plasma membrane export sodium ions and those on the tonoplast lead to the sequestration of Na^+ in the vacuole. In fact, overexpression of the gene *AtNHX1* which codes for a vacuolar Na^+/H^+ antiporter in the plant *Arabidopsis thaliana* gives salt tolerance to a transgenic plant (Apse *et al.*, 1999). Cl^- ions also accumulate in the vacuole but the transport mechanism for this phenomenon is unclear.

K^+ ions also play a crucial role in maintaining an appropriate osmotic balance across the plasma membrane. The plant cellular level of K^+ ions in the cytosol stays high even during a change in water potential; moreover, plant roots always keep a high cytoplasmic K^+ concentration (Binzel *et al.*, 1988). Some genes encoding proteins that are active in K^+ uptake have been characterized. While some K^+ and Na^+/K^+ transporters show high affinity for K^+, others show a low affinity depending on the external Na^+ concentration in the soil (Zhu, 2002). The gene *HKT1* encodes a Na^+/K^+ cotransporter with high affinity for K^+ that is expressed in wheat roots. This gene product is responsible for K^+ uptake at low K^+ concentration in the soil. During salt stress, K^+ uptake by HKT1 is blocked and a low affinity Na^+ uptake occurs through this cotransporter emphasizing HKT1 as part of a toxic pathway for the plant. This was confirmed by the analysis of mutations in the sixth putative transmembrane domain of HKT1 that confer salt tolerance to yeast cells (Rubio *et al.*, 1995).

Another response to protect against salt stress and other osmotic shocks is the elevated biosynthesis of small organic molecules referred to as osmolytes, which accumulate in the cytosol. These molecules lower the osmotic potential of the cytoplasm, balancing the osmotic potential of the vacuole, which accumulates Na^+. They also act as osmoprotectants without any damaging effect on cell metabolism and could be involved in the scavenging of reactive oxygen species (Hasegawa *et al.*, 2000). Osmolytes are classified in three categories: 1) sugars and sugar alcohols; 2) proline and the amino acid derivatives, glycine betaine, ectoine; and 3) dimethyl sulfonium propionate (Hasegawa *et al.*, 2000). Osmotic stress induces transcription of genes encoding enzymes of a secondary proline biosynthesis pathway but proline production is regulated by a negative feedback on one of the enzymes of the pathway, ? ^{l}-pyrroline-5-carboxylate synthetase (P5CS) (Yoshiba *et al.*, 1995). A comparison of transgenic tobacco producing either a wild type or an altered form of the *Vigna aconitifolia* P5CS protein lacking allosteric regulation showed that proline accumulated to higher levels under unstressed conditions and is even more elevated after salt treatment in the transgenic plant containing the mutated form of the enzyme (Hong *et al.*, 2000).

To alleviate a salt shock, the plant also can activate stomatal closure to avoid transpiration (Busk and Pagès, 1998), and also has water channel proteins such as aquaporins to reduce the water permeability and thus regulate water flux. Recent plant genomic projects have revealed the abundance of aquaporins, which are members of the Membrane Intrinsic Protein (MIP) family (Baiges *et al.*, 2002).

1.3. Phytohormones and the water deficit and defense response

Abscisic acid (ABA) is globally implicated in water-limiting stresses. The endogenous ABA concentration increases in shoot and root tissues in response to drought, salt stress and cold stress. Moreover, ABA exogenously applied to the plant is able to induce a plant dehydration response (Moons *et al.*, 1997; Hong *et al.*, 1997). The response to ABA at the physiological and gene regulation levels involves a complex controlled network. ABA is active in the seed and prevents germination during unfavorable growth conditions (Leung and Giraudat, 1998). It could enhance tolerance to drought by activating several groups of genes referred to as late embryogenesis abundant (LEA) genes, which encode proteins involved in membrane protection (Sales *et al.*, 2000).

Indirectly, ABA also controls a part of the plant pathogen defense response (Chao *et al.*, 1999). Defense and water limiting responses have similarities in that a pathogen attack triggers the desiccation and cell death of damaged plant cells and cells surrounding the damaged site (Sharp, 2002). ABA also acts locally and systemically after wounding. ABA levels were found to have a negative effect on the tomato plant defense response to the necrophitic fungul pathogen *Botrytis cinerea*. Tomato mutants with low levels of ABA are more resistant to *B. cinerea*. They show increased levels of phenylalanine ammonia lyase (PAL), an enzyme present during the plant defense response, a decreased level of expression of a pathogenesis related gene PR-1a and an increased sensitivity to a salicylic acid (SA) analog, a plant hormone involved in long distance signaling in the defense response. Exogenously applied ABA restored the sensitivity of the tomato mutants and even increased the sensitivity of wild type tomato. These data suggest that

ABA would be a negative regulator of the SA-dependent defense response of tomato to *B. cinerea.* In addition, nahG plants that cannot accumulate SA show higher sensitivity to *B. cinerea* than wild type plants (Audenaert *et al.*, 2002).

The induction of ABA biosynthesis by environmental stimuli is not well understood but the gene from maize, which encodes the enzyme 9-*cis*-epoxycarotenoid dioxygenase of the ABA biosynthesis pathway, was shown to be induced by water stress (Iuchi *et al.*, 2000). The analysis of *Arabidopsis* mutants has given insight into some aspects of ABA signaling. The production of mutants by *Agrobacterium tumefaciens*-mediated T-DNA insertion into the plant genome facilitated the identification of ABA response mutants (Leung and Giraudat, 1998). Abscisic acid insensitive (abi) mutants were identified as seeds whose germination was not inhibited by exogenously applied ABA. These mutations were found to be in genes encoding type 2C protein phosphatases, ABI1 and ABI2 (Leung *et al.*, 1997), and in genes encoding transcription factors ABI3, VP1, ABI4 and ABI5 (Brocard *et al.*, 2002). Both ABI1 and ABI2 inhibit the ABA activated pathway by dephosphorylation of ABA signaling components (Merlot *et al.*, 2001) and their action most probably takes place in the reaction cascade between the ABA receptor and other ABA-dependent downstream components such as transcription factors. Transcription factors ABI3, VP1, ABI4 and ABI5 were found to activate ABA-dependent genes and induce expression of genes encoding seed storage proteins and Group 1 LEA proteins in seeds (Brocard *et al.*, 2002). ABA related transcription factors are divided into two major groups: those that bind DNA directly and those that associate with proteins already bound to the DNA at specific promoter sites. For example, VP1 or ABI3 can first bind directly to the promoter region and allow

subsequent binding by ABI4 or ABI5. In *Arabidopsis*, ABI3 recruits ABI5 and also may recruit other factors of the transcription machinery to switch on the Group1 LEA *AtEm* gene (Finkelstein *et al.*, 1998; Finkelstein and Lynch, 2000). ABA activates several different signaling pathways and even though many genes are known to be ABA activated, the different steps of their signal transduction pathways and the interaction of the pathways are still largely not understood.

As mentioned previously, salicylic acid is implicated in the stress response as are two other phytohormones jasmonic acid (JA) and ethylene. Plant growth regulators rarely act alone and growth processes may be under the influence of many factors that act in an additive or antagonist way (Ghassemian *et al.*, 2000; Sharp, 2002). Pathogen and insect attacks, physiological drought and wounding lead to the accumulation of jasmonates (Creelman and Mullet, 1995). Jasmonates, which include JA and its derivatives such as methyl jasmonate (MeJA) and hydroxy jasmonate, are known to play a major role in response to mechanical damage. In some instances their activity parallels that of ABA (Moons *et al.*, 1997). They activate some of the same genes and some wounding response genes are implicated in the osmotic response and *vice versa* (Cheong *et al.*, 2002). Some genes, such as the *Pin2* gene, which encodes a proteinase inhibitor, are ABA and JA activated during wounding but not during water deficit (Herde *et al.*, 1999). An extensive study carried out by Moons *et al.* (1997) compared the effects on the transcript and protein levels of several genes of ABA, JA and salt treatments on rice seedlings. These authors showed that during salt stress, the JA-induced proteins (JIPs) from roots such as the pathogenesis-related protein PR-10, the ABA-induced proteins oslea3 (Group3 LEA), and the salt-induced protein salT all accumulated. In fact, rapid

accumulation of the oslea3 mRNA and protein occurred in parallel with a rapid increase of the endogenous ABA concentration in response to high salinity. Prolonged salt stress or increasing salt stress gave rise to a delayed enhanced JA accumulation ultimately leading to a decrease of the oslea3 transcript. In contrast, the accumulation of salT transcript and protein was induced by JA, while ABA had an antagonist effect. Moreover, salt-stressed rice, pretreated with JA biosynthesis inhibitor followed with application of ABA, showed the disappearance of the salT transcript. Rice seedlings treated only with ABA showed increased levels of salT and PR-10 transcripts but no protein accumulation. Thus JA and ABA have antagonistic effects on the salt-induced proteins salT and oslea3. ABA and JA trigger, respectively, a rapid and delayed response in regulating genes. The regulation of ABA and JA-induced genes takes place at both the transcriptional and post-transcriptional level and is dependent on the ratio of ABA to JA (Moons *et al.*, 1997).

Ethylene, another important phytohormone, which is involved in plant development and in fruit ripening also interacts with ABA. The ethylene signaling pathway is well understood in *Arabidopsis*. In the absence of ethylene, ethylene receptors ETR1, ETR2, EIN4, ERS1, and ERS2 activate a Raf-like kinase, CTR1, which inhibits the downstream ethylene response pathway controlled by EIN2. The binding of ethylene inactivates the receptors resulting in the deactivation of CTR1, which allows EIN2 to function as a positive regulator of downstream responses (Wang *et al.*, 2002). Some mutants like the ethylene insensitive 2 (ein2) mutant deficient for the ethylene response show high endogenous ABA concentrations and altered ABA sensitivity. Analysis of this mutant allowed the conclusion that ABA inhibits root growth by signaling through

the ethylene signaling pathway and that ABA inhibition of germination is negatively regulated by ethylene (Ghassemian *et al.*, 2000). More recent additional studies on an ABA deficient mutant revealed that ABA prevents excess ethylene production during water stress, which leads to normal shoot and root growth. This finding supports the idea, contrary to the previous functions imputed to ABA as a growth inhibitor, that it is rather involved in maintaining normal growth during low water potential conditions (Sharp *et al.*, 2002). The interaction between the two phytohormones ethylene and ABA remains to be elucidated further and the putative role of ethylene in growth inhibition during water stress remains to be clarified.

1.4. Protein kinases, key enzymes in transduction pathways

The plant's initial perception of environmental stimuli leads to cellular responses through a cascade of reactions. The information travels within the plant cell by a series of chemical reactions typified by the phosphorylation cascades mediated by protein kinases. Protein kinases are enzymes that are able to regulate themselves or other proteins by phosphorylation of specific groups on histidine, serine, threonine or tyrosine residues. Protein kinases are classified by their target amino acid as serine/threonine, tyrosine or histidine protein kinases (Hardie, 1999). Changes in osmotic potential give rise to ATHK1 protein kinase autophosphorylation, which is hypothesized to subsequently activate, by a series of phosphorylation reactions, other downstream proteins including additional kinases to finally activate targeted proteins and gene expression in response to the original osmotic change (Urao *et al.*, 2001).

Signal transduction also is hypothesized to activate the biosynthesis of growth regulators such as ABA, which are crucial for the transmission of the information to the cells surrounding the site where the stress is perceived and ultimately to the whole plant. The hormones are thought to bind to specific plant plasma membrane or intracellular receptors, which subsequently activate intracellular secondary reactions (Leung and Giraudat, 1998). In the presence of high salinity in the soil, the first barriers to Na^+ toxicity are the K^+/Na^+ transporters and H^+/Na^+ antiporters that maintain a high K^+/Na^+ ratio inside the cytosol of plant root cells. During a salt stress, the Ca^{2+} dependent SOS3 Na^+/K^+ transporter protein recruits and activates the SOS2 protein kinase, which activates the SOS1 plasma membrane Na^+/H^+ exchanger by phosphorylation (Qiu *et al.*, 2002). This has been confirmed in yeast, where the reconstitution of the SOS pathway with the overexpression of the three *Arabidopsis* SOS genes is able to maintain a high K^+/Na^+ ratio in the cell and to enhance salt tolerance (Quintero *et al.*, 2002). In *Arabidopsis*, mutations in genes in the Salt Overly Sensitive (SOS) pathway that encode the three proteins SOS1, SOS2 and SOS3 decrease salt tolerance. Mutant plants display a deficiency in K^+ uptake at low concentration in the soil and are hypersensitive to salt stress. The SOS pathway is the best understood signaling pathway involved in plant salt tolerance and Na^+ extrusion. It reinforces the importance of protein kinases in the transduction of the information and the interaction of protein kinases and ion transporters (Zhu, 2002).

There are many plant protein kinase families but little is known about their role in the perception and the amplification of signals. More advanced studies in other organisms provide an excellent background and this has driven the interest in the

mitogen-activated protein kinase (MAPK) cascades, particularly in their role in environmental stress responses (Covic, 1999). MAPKK kinases (MAPKKK or MEKK) activate MAPK kinases that themselves activate MAP kinases (Hardie, 1999). An *Arabidopsis* gene, *AtMEKK1* is a member of the MAPKKK family and was shown to functionally complement a yeast mutant deficient for the gene *STE11* encoding a MEKK1, which is part of a MAP kinase cascade of the High Osmolarity Glycerol (HOG) response signaling pathway. This pathway induces glycerol biosynthesis during osmotic stress in yeast. *AtMEKK1* expression in yeast enhances tolerance to high salinity, probably by increasing the glycerol concentration, which acts as an osmoprotectant. The role of *AtMEKK1* in the salt stress response is suggested by the fact that its expression is induced in *Arabidopsis* after salt stress and the time of induction parallels that of osmolyte accumulation. This is the first plant MAP kinase implicated in the response to osmotic stress (Covic *et al.*, 1999). In addition, a yeast two-hybrid screen which used AtMEKK1 as bait revealed interactions with the MAPKK MEK1 and the MAPK AtMPK4 that may be additional components of the MAP kinase cascade in the plant response to osmotic stress (Ichimura *et al.*, 1998). Biotic and abiotic stresses lead to the accumulation of reactive oxygen species like hydrogen peroxide (H_2O_2), which also has been shown to initiate a MAP kinase cascade. An increase in H_2O_2 concentration activates the ANP1 MAPKKK, which activates by phosphorylation two MAPKs, AtMPK3 and AtMPK6, and induces oxidative stress activated genes encoding glutathione S-transferases (GSTs) and heat shock proteins (HSPs) (Kovtun *et al.*, 2000).

One of the best characterized plant signal transduction pathways in response to stress is the tomato defense response to the bacterium *Pseudomonas syringae* (Tang,

1996). AvrPto, the "avirulent" bacterial elicitor binds and activates the tomato protein kinase Pto that phosphorylates Pti1, another protein kinase that has been implicated in the hypersensitive response (HR) (Zhou *et al.*, 1995). Moreover, the Pto kinase activates the Pti4/5/6 transcription factors by phosphorylation, which induce pathogenesis related gene expression (Zhou *et al.*, 1997; Gu *et al.*; 2000). This demonstrates the critical role of the protein kinases, which facilitate plant resistance to bacterial attack.

As mentioned previously, the plant defense response is modulated by the three phytohormones JA, ethylene and SA but the signaling pathways controlled by these phytormones are also downstream regulated by proteins like protein kinases. During wounding, a tobacco plant overexpressing a MAP kinase gene increased SA accumulation and *PR-1* gene expression and demonstrated the strength of the protein kinases in modulating phytohormones effects (Seo *et al.*, 1995). A similar effect was observed for the Arabidopsis MAP kinase MPK4. A cDNA microarray experiment was performed on the *Arabidopsis* mutant mpk4 that exhibits a constitutive systemic acquired resistance (SAR), which represent a general defense response of the plant, not only in the cells surrounding the site of infection. The SAR is under the control of SA (Zhou *et al.*, 1995). This implicates MPK4 in negative control of the SAR through SA. Moreover, mpk4 mutants treated with JA revealed that the expression of two PR genes (PDF1.2 and THI2.1) that code for basic cysteine-rich peptides with antimicrobial properties (thionins) and which are usually induced by JA was blocked. This emphasizes that the MPK4 protein acts downstream of JA in activating defense and wound-induced genes (Petersen *et al.*, 2000). Kinases are key regulators and the F8A24.12 protein kinase could act downstream of one or several phytohormones. By investigating all of the characteristics

of this gene, including the localization and pattern of its expression, it will probably be possible to determine its role in the regulation of developmentally related genes or in the stress response.

Kinases are key enzymes because they are secondary messengers that determine the specificity for a precise stimulus of downstream reactions and activate specific transcription factors. In addition, kinase signaling cascades have additional negative control elements such as phosphatases or other protein kinases.

1.5. Transcriptional and posttranscriptional gene regulation

The induction of stress related genes is under the control of *trans*-acting factors that act at the transcriptional and post-transcriptional level. Transcription factors bind directly or indirectly to the DNA of the targeted genes, usually in their promoter region and induce or inhibit their transcription. Transcription factors bind specific domains in the promoter that have conserved sequences and recruit other factors for the initiation of transcription. The binding domains, *cis*-elements, are often conserved among genes that have common response patterns and are even conserved between different species (Yamaguchi-Shinozaki and Shinozaki, 1994).

Osmotic stress-induced genes are classified as being in ABA-dependent or ABA-independent pathways. The regulation of ABA-dependent genes is controlled by transcription factors that are members of basic leucine zipper (bZIP) (Hobo *et al.*, 1999b), MYB or MYC gene families (Abe *et al.*, 1997). Investigations in this area have focused on ABA-induced genes that are related to plant embryo dormancy where the chemical signal ABA plays a well known role (Finkelstein and Lynch, 2000). Many of these genes

have several abscisic acid responsive elements (ABREs) which are characterized by an ACGT core sequence recognized by the ABI5/TRAB1 class of bZIP transcription factors in their promoter regions (Hobo *et al.*, 1999b). Some genes, such as the barley gene *HVA1*, which is involved in seed dormancy, have only one copy of an ABRE. However, the binding of TRAB1 to the *HVA1* gene is not sufficient for ABA induction. TRAB1 also must bind to a Coupling Element 3 (CE3) to form, with ABRE, the abscisic acid responsive complex (ABRC) necessary for *HVA1* transcriptional activation (Hobo *et al.*, 1999a). Once the TRAB1 binds, it recruits ABI3/VP1, a member of the viviparous1 class of transcription factors, and then forms a new complex that recruits other factors of the transcriptional machinery and finally RNA polymerase. VP1 is able to bind a CATG *SphI* element (*SphI* restriction enzyme recognition sequence) but this alone is not sufficient to induce *HVA1* transcription. Some genes like the *HVA22* barley gene have several ABREs with additive effects on gene expression although each ABRE requires a Coupling Element 1 (CE1) for a maximal level of transcription (Busk and Pagès, 1998).

There is also an ABA-independent signaling pathway for osmotic stress responsive genes. Several genes are regulated by both the ABA-dependent and ABA-independent pathways. Such genes, which are exemplified by *rd29A* in *Arabidopsis*, are characterized by the presence of ABRE elements and a Drought Responsive Element (DRE). DREs have an A/GCCGAC conserved consensus sequence. In the case of an osmotic stress response the two transcription factors DREB1A and DREB2A bind the *rd29A* promoter. Cold stress induces the *rd29A* promoter through DREB1A and dehydration stress caused by drought or high salinity induces *rd29A* expression through DREB2A (Liu *et al.*, 1998).

1.6. The salt stress induced protein kinase ESI47

The tall wheatgrass *Lophopyrum elongatum* (Host) A. Löve (2n = 2x = 14) [synonym *Electrygia elongata* (Host) Nevski, *Agropyron elongatum Host*] is a wild species found in the littoral areas and salt marshes of the Mediterranean region. This highly salt tolerant diploid species has a close phylogenetic relationship to the bread wheat *Triticum aestivum* (McGuire and Dvorák, 1981). The genetic basis of the salt tolerance of *L. elongatum* was characterized by crossing it with the salt-sensitive wheat variety, Chinese Spring. The amphiploid derived from this cross is more tolerant to salt than the Chinese Spring (Omielan *et al.*, 1991). A comparison of gene expression in the roots of these genotypes noted the induction of a set of proteins in the roots of the amphiploid that were not induced in wheat (Gulick and Dvorák, 1987). Eleven different cDNAs from genes that were up-regulated in root tissues of *L. elongatum*, referred to as Early salt stress-induced (Esi) genes, have been cloned and identified (Gulick and Dvorák, 1990).

Sequence analysis indicated that the predicted *Esi47* gene product is similar to serine/threonine protein kinases. *Esi47* is responsive to ABA and its role in hormone signaling was confirmed with transient expression assays in barley aleurone where Esi47 suppressed the gibberelic acid induction of the a-amylase gene (Shen *et al.*, 2001). The precise function of the Esi47 gene product is still unknown. Transgenic *Arabidopsis* overexpressing *Esi47* or expressing its promoter upstream of a reporter gene did not give conclusive results (Shen, unpublished). The interaction of Esi47 with an *Arabidopsis* Oxygen-Evolving Enhancing protein 1 (OEE1) from photosystem II in a yeast two-hybrid system did not allow a function to be established (Elias, unpublished). Structural

analysis of the genomic clone of *Esi47* revealed the presence of one intron and one small upstream open reading frame (suORF) in the 5-untransleted region (5'UTR). Mutagenesis in this suORF enhanced the expression level in biolistic assays in corn callus of a reporter gene placed downstream of the *Esi47* promoter. The mutation eliminated the ABA responsiveness of this gene and raised its basal level of expression (Shen *et al.*, 2001).

1.7. Esi47 homologues in *Arabidopsis thaliana*

Among proteins predicted to be encoded in the *Arabidopsis* genome, Esi47 shows most similarity to the NAK protein kinase subfamily. Among the NAK protein kinases, *F8A24.12* is the gene with the highest similarity to *Esi47*. *F8A24.12* is salt stress-induced and also has one intron and one suORF in the 5'UTR-untranslated region of the gene. However, unlike *Esi47*, *F8A24.12* is not induced by ABA in the roots but is induced by salt stress in leaf tissue. Moreover, *F8A24.12* has a different timing of induction in roots and leaves (Shen *et al.*, 2001). The genes, *F12E4.50* and *T7F6.28*, are also members of the NAK protein kinase family and show high similarity to *Esi47*. *F12E4.50* is induced in roots by NaCl and ABA treatments while *T7F6.28* is induced in roots by ABA but not by NaCl. However, neither of these genes was induced in shoot tissue by these treatments (Shen *et al.*, 2001). The fact that the suORF in *Esi47* was shown to have regulatory properties and that the suORF is a feature that is conserved in the *Arabidopsis* homologue of *Esi47* led me to characterize *F8A24.12* in *Arabidopsis*.

In this work, the promoter of *F8A24.12* was characterized. Transgenic *Arabidopsis* plants were produced expressing the GUS reporter gene under the control of

the *F8A24.12* promoter to allow the visualization of the pattern of expression of the *F8A24.12* gene by GUS histochemical assays and to quantify GUS activity by fluorometric assays. A comparison of gene expression from constructs with and without the suORF demonstrated a role for this element in gene regulation that was analogous to that observed for *Esi47*.

CHAPTER 2. MATERIALS AND METHODS

2.1. Plant material

The *Arabidopsis* Colombia ecotype strain Col-0 from the Arabidopsis Biological Resource Center was used in this work. Plants used for *Agrobacterium tumefaciens*-mediated T-DNA transfer and transgenic plants were grown on soil containing equal amounts of black earth, peat moss, perlite and vermiculite and watered one time out of two with fertilizer Plant-Prod 20-20-20 at a concentration of 1g/l. Seeds were sown in soil and vernalized in the dark at 4°C for two days and then placed under fluorescent lighting or in growth chambers under 16 hours (hrs) light/8 hrs darkness. The controlled temperature in the growth chambers was 23°C and 21°C for the day and the night, respectively.

2.2. Plant nuclear DNA extraction

DNA was extracted from whole *Arabidopsis* plants in order to isolate and amplify the *Esi47* homologues, *F8A24.12*, *F12E4.50* and *T7F6.28*. Three grams of tissue were ground in a mortar with liquid nitrogen. The powder was vigorously resuspended in 15 ml of extraction buffer containing 100 mM Tris-HCl (pH 7.4), 50 mM EDTA (pH 8.0), 500 mM NaCl, 1.4% ß-mercaptoethanol (v/v), 1.33% SDS (w/v) and incubated for 10 minutes (min) at 65°C. Five ml of 5 M potassium acetate was added and mixed gently. The lysate was incubated on ice for 20 min to precipitate proteins and polysaccharides. The precipitate and insoluble tissue particles were removed by centrifugation for 20 min at 12 000 x g and the resulting supernatant was filtered through a Miracloth filter. Ten ml of isopropanol was added to the supernatant by gentle swirling

to precipitate the DNA. After centrifugation for 20 min at 18 000 x g, the supernatant was removed and the DNA pellet was rinsed with 70% ethanol, dried and resuspended in 4 ml sterile distilled water. Ten µl of 10 mg/ml RNaseA was added to the solution and incubated for 30 min at 37°C. One volume of phenol/chloroform (½ volume of phenol and ½ volume of chloroform/$^{1}/_{24}$ volume of isoamyl alcohol) was added and the mixture was agitated vigorously for 10 min and centrifuged for 5 min at 4 500 x g. The aqueous phase again was extracted with one volume of chloroform/isoamyl alcohol (24/1). One tenth volume of 3 M sodium acetate and two volumes of 95% ethanol (v/v) were added and the sample was kept at 4°C overnight to precipitate the DNA. After centrifugation at 28 000 x g for 30 min, the supernatant was removed and the pellet was rinsed with 70% ethanol, dried and resuspended in sterile distilled water until the DNA was completely dissolved.

2.3. Cloning and plasmid construction

2.3.1. Recombinant DNA methods

Recombinant DNA methods were based on Sambrook *et al.* (1989). Polymerase chain reaction (PCR) for DNA amplification and site-directed mutagenesis of cloned genomic DNA used the *Pfu* DNA polymerase (Stratagene). PCR products were purified by phenol/chloroform extraction followed by ethanol precipitation as described previously and gel purified using the QIAEX II gel extraction kit from Qiagen. The purified PCR product and the corresponding plasmid were digested with the appropriate restriction enzymes (in some cases partially digested) according to the manufacturer's specifications, ligated with T4 ligase and used to transform *E. coli* strain MC1061 competent cells. The plasmid containing the DNA of interest was extracted and checked

by restriction analysis or by DNA sequencing. See Table 1 for the primer sequences used in PCR.

Table 1. List of primers and a description of their purpose.

Name	Sequence (5'? 3')	End	RE site	Description
Esi47				
ESI47Xho1	CCG*CTCGAG*GCGCGATGCAGTGCTTC	5'end	*XhoI*	cDNA cloning into pta7001
ESI47Spe1	GG*ACTAGT*CAGCAAAAATACGAATTCC	3'end	*SpeI*	cDNA cloning into pta7001
ESI47Ala1	CTGTATGGATATACTCAGCAGCTGCATACCCTATA			Mutation of P(171) to A
ESI47Ala2	TATAGGGTATGCAGCTGCTGAGTATATCCATACAG			Mutation of P(171) to A
F8A24.12				
F8A5xho1	CCG*CTCGAG*TCGATAATGAAGTGTTTC	5'end	*XhoI*	cDNA cloning into pta7001
F8A3xho1	*CCGCTCGAG*TTAATATACATATCTTAC	3'end	*XhoI*	cDNA cloning into pta7001
F8Abam1	CG*GGATCC*TCGATAATGAAGTGTTTC	5'end	*BamHI*	cDNA cloning into pBI121
F8Asst1	TCGC*GAGCTC*TTAATATACATATCTTAC	3'end	*SstI*	cDNA cloning into pBI121
Antif8asst1	TCGC*GAGCTC*TCGATAATGAAGTGTTTC	3'end	*SstI*	cDNA cloning into pBI121
Antif8abam1	CG*GGATCC*TTAATATACATATCTTAC	5'end	*BamHI*	cDNA cloning into pBI121
F8AproBam	CG*GGATCC*TATCGAGAGGAGATTAAAATAC	3'end	*BamHI*	Promoter cloning into pBI101
F8AproSal	GC*GTCGAC*AGTAGGACCAGTATGACTCG	5'end	*SalI*	Promoter cloning into pBI101
F8Aorfm1	ATTCATTAACCACTTCTTGATAATTATAATCTTCC			Mutation of the suORF
F8Aorfm2	GGAAGATTATAATTATCAAGAAGTGGTTAATGAAT			Mutation of the suORF
Intronpfu1	CTTTGAATTTACTTTGTATA			intron deletion in the 5'UTR
Intronpfu2	TGTTTTATTGGTGTCCTGCA			intron deletion in the 5'UTR
YF8A1	CG*GGATCC*TAATGAAGTGTTTCTTG	5'end	*BamHI*	cDNA cloning into pAS2-1
YF8A2	GC*GTCGAC*TCAACAAGCTCTTATTG	3'end	*SalI*	cDNA cloning into pAS2-1
F12E4.50				
F12proPst1	AA*CTGCAG*AGTAGAATCACTTTAACGATG	5'end	*PstI*	Promoter cloning into pBI101
F12proXba1	GC*TCTAGA*CTTCGTAAAGATGTTAAAACAC	3'end	*XbaI*	Promoter cloning into pBI101
T7F6.28				
T7F6proPst1	AA*CTGCAG*TCAGAGTTTCAAGTAGTTACAG	5'end	*PstI*	Promoter cloning into pBI101
T7F6proXba1	GC*TCTAGA*TGCCTTAGATGAAACCAGCCT	3'end	*XbaI*	Promoter cloning into pBI101
ARSK1				
Arssal1	GC*GTCGAC*AAAATGGCAGTGTTCAAG	5'end	*SalI*	cDNA cloning into pta7001
Arsspe1	GG*ACTAGT*ACCAGAGAATCAGGATAC	3'end	*SpeI*	cDNA cloning into pta7001
Arshind1	ACG*AAGCTT*CTGCGATGTGAAGG		*HindIil*	To amplify Arsk1 cDNA
Arshind2	CAG*AAGCTT*CGTTTGTTCACGTTG		*HindIII*	To amplify Arsk1 cDNA
Arskin1	AATGGCTGGCGGAGATATTATTTTTC			First intron deletion in the EST
Arskin2	ATCTCCGCCAGCCATTCTCTATGCC			First intron deletion in the EST
Arsbam1	CG*GGATCC*AAAATGGCAGTGTTCAAG	5'end	*BamHI*	cDNA cloning into pBI121
Arsst1	TCGC*GAGCTC*AATATGAATGCTTAATAA	3'end	*SstI*	cDNA cloning into pBI121
Antiarsst1	TCGC*GAGCTC*AAAATGGCAGTGTTCAAG	3'end	*SstI*	cDNA cloning into pBI121
Antiarsbam1	CG*GGATCC*AATATGAATGCTTAATAA	5'end	*BamHI*	cDNA cloning into pBI121
Antiarssal1	GC*GTCGAC*AATATGAATGCTTAATAA	3'end	*SalI*	cDNA cloning into pta7001

2.3.2. *Esi47* and *F8A24.12* overexpression

The *Esi47* cDNA and its mutant form, K124Q, in which the lysine at position 124 was replaced by glutamine, were both cloned in the pBluescript SK- vector obtained from W. Shen. Mutants, P171A, were generated with proline at position 171 replaced with alanine, both in the *Esi47* cDNA and in the K124Q clone. For this purpose, two primers ESI47Ala1 and ESI47Ala2 were designed (Table 1). This created three different mutant forms of the *Esi47* cDNA: the single mutants K124Q and P171A and the double mutant K124Q/P171A. The P171A mutation was done using the Quick Change site-directed mutagenesis strategy of Stratagene. The vector containing the mutant version of the insert of interest was transformed into *E. coli* competent cells. The resulting constructs were verified by DNA sequencing. The entire coding region of the *Esi47* cDNA and the mutant forms of *Esi47* plus a part of the 3'UTR, from –5 to +2551 with respect to the start codon, were PCR amplified with the primers ESI47XhoI and ESI47SpeI (Table 1), digested with *XhoI* and *SpeI* and ligated into the similarly digested Pta7001 vector (Aoyama and Chua, 1997) downstream of the glucocorticoid (dexamethasone) inducible promoter. The four constructs were verified by DNA sequencing.

The *F8A24.12* cDNA was obtained from the Arabidopsis stock center (accession # F3A9T7). The cDNA region from –6 to +1484 with respect to the start codon was PCR amplified with primers F8A5XhoI and F8A3XhoI (Table 1), digested with *XhoI* and cloned into the Pta7001 vector in both sense and antisense orientations using the *XhoI* site for cloning. The *F8A24.12* cDNA from –7 to +1484 with respect to the start codon was also amplified using PCR primers F8ABamI and F8ASstI for sense expression (Table 1) and using PCR primers AntiF8ASst1 and AntiF8ABamI for antisense expression. The

inserts were subsequently cloned into the pBI121 vector (Clontech) using the two restriction sites *BamHI* and *SstI*. *F8A24.12* cDNA was cloned into the pBI121 vector for constitutive overexpression under the strong cauliflower mosaic virus CaMV *35S* promoter. Constructs were verified by restriction analysis.

2.3.3. ARSK1 overexpression

The *Arabidopsis* gene *ARSK1*, which encodes a protein kinase has been shown to be salt- and ABA-induced (Hwang and Goodman, 1995). The *ARSK1* cDNA was obtained from the Arabidopsis stock center (accession # 166N10T7) but the first intron of the major ORF from the EST clone was unspliced and a portion of the first exon was missing at the 5'end. The missing 5'end of the first exon was ligated to the partial length *ARSK1* clone taking advantage of the *Hind III* restriction site present in the first exon of the EST clone. To accomplish this, the missing 5'end of the first exon was amplified from genomic DNA with the two primers, ArsSalI and ArsHind1 (Table 1), which included the restriction sites *SalI* and *HindIII* at their 5'and 3'ends, respectively. The partial length *ARSK1* cDNA was PCR amplified with the two primers, ArsHind2 and ArsSpeI, which included the restriction sites *HindIII* and *SpeI* at their 5'and 3'ends, respectively. Both PCR products were digested with *Hind III*, gel purified, and ligated at the *Hind III* site to obtain the full length *ARSK1* cDNA which then was cloned in the pSport vector (Gibco BRL). The unspliced intron was deleted by PCR based cloning . One pair of primers ArsSalI and Arskin2 (Table 1) was used to amplify a fragment from the 5'end of the clone to the intron border and a second pair Arskin1 and ArsSpeI was used to amplify from the 3' border of the intron to the 3' end of the cDNA. Primers for

the extremities of *ARSK1* contained *SalI* and *SpeI* restriction sites. Two independent PCR fragments were amplified, one on each side of the intron. Both PCR products were ligated and cloned in the pSport vector. The *ARSK1* cDNA from –3 to +1411 with respect to the start codon was cloned in the Pta7001 vector (Aoyama and Chua, 1997) for sense and antisense expression using the *SalI* site for non-directional cloning. The *ARSK1* cDNA was also cloned in the pBI121 vector (Clontech) using the two restriction sites *BamHI* and *SstI*. The latter construct was designed for constitutive overexpression under the strong CaMV *35S* promoter. Constructs were verified by restriction analysis.

2.3.4. *F8A24.12*, *F12E4.50* and *T7F6.28* promoter analysis

Arabidopsis genes *F8A24.12*, *F12E4.50*, *T7F6.28* that encode protein kinases were chosen for characterization because of their high similarity to Esi47. The regions encompassing the upstream promoter region and the 5'UTR regions of the three *Arabidopsis* protein kinase genes *F8A24.12*, *F12E4.50* and *T7F6.28* were cloned into pBI101 upstream of the GUS reporter gene, which encodes ß-glucuronidase. The *F8A24.12* promoter and 5'UTR from –2056 to +3 with respect to the ATG translational start, was isolated from genomic DNA by PCR using primers F8AproBam and F8AproSal and cloned in the pBluescript SK- vector using the *SalI* and *BamHI* restriction enzymes at the 5' and 3' ends, respectively. suORF from –490 to –461 from the ATG in the 5'UTR of *F8A24.12* was removed by changing the start codon to TTG using the Quick Change (Stratagene) site-directed mutagenesis approach similar to the one used for the P171A mutation of *Esi47* described above. Two primers, F8Aorfm1 and F8Aorfm2, were designed for this purpose. The intron present in the 5'UTR from –394 to –57

upstream of the start codon was removed using two primers flanking the intron to amplify the entire plasmid minus the intron. The product of amplification was phosphorylated and ligated, and transformed into *E. coli*. Four versions of this promoter were made: (1) the intact promoter plus the 5'UTR (2) with the mutated suORF in the 5'UTR (3) with the first intron in the 5'UTR removed and (4) with both the mutated suORF and the intron removed. The constructs were built in the vector pBluescript SK- and verified by partial sequencing that covered the 5'UTR region. The promoter plus 5'UTR regions were transferred into pBI101 to make GUS reporter constructs using the *SalI* and *BamHI* restriction enzymes at this 5' and 3' ends respectively, and verified by PCR amplification.

The *F12E4.50* and *T7F6.28* promoters and 5'UTR regions extending from –2510 or –1418, respectively, to this translational start sites were isolated from genomic DNA by PCR (see Table 1 for the primers) and cloned in the pBluescript SK- and pBI101 vector using the *PstI* and *XbaI* restriction enzymes respectively at the 5' and 3' ends. The constructs were verified by restriction analysis.

2.3.5. F8A24.12 as bait in the yeast two-hybrid system

The *F8A24.12* cDNA from –2 from the start codon of the major ORF to the stop codon of this ORF was fused to the *GAL4* DNA binding domain in the pAS2-1 vector using the *BamHI* and *SalI* restriction enzymes at the 5' and 3' ends, respectively. The construct was verified by restriction analysis.

2.4. *Agrobacterium tumefaciens* plant transformation

To prepare *A. tumefaciens* competent cells, a colony was inoculated into 2 ml of Luria-Bertani (LB) medium plus 25 μg/ml gentamycin (Gen) and grown overnight at 28°C. Overnight culture (2 ml) was subcultured into 50 ml LB plus Gen (25 μg/ml) and grown until the OD_{600} was between 0.5 and 1.0. The culture was centrifuged at 1 000 x g for 5 min at 4°C. The bacterial pellet was resuspended in 1 ml of 20 mM $CaCl_2$ and divided into aliquots of 50 μl. One μg of each plasmid purified from *E. coli* was used to transform *A. tumefaciens* strain GV3101/pMP90 competent cells. A 50 μl aliquot of *A. tumefaciens* competent cells was mixed with the plasmid DNA and frozen in liquid nitrogen for one minute and immediately thawed and incubated at 37°C for 5 min. This was followed by the addition of 500 μl LB and incubation at 28°C for 4 hrs with gentle agitation. The cells were then plated on LB plates with 50 μg/ml kanamycin (Kan) and 25 μg/ml Gen to obtain *A. tumefaciens* transformants containing the plasmid of interest.

The protocol of Clough and Bent (1998) was followed for the plant transformation. The first flower stem of healthy plants was removed to induce secondary bolts and obtain a larger number of flower buds. Four days before the plant transformation, 2 ml of LB with 50 μg/ml Kan and 25 μg/ml Gen were inoculated with a single colony of *A. tumefaciens* harboring a plasmid of interest and grown at 28°C for two days. A 1 ml aliquot of this culture was subcultured to 25 ml LB Kan (50 μg/ml) and Gen (25 μg/ml) and grown overnight. The 25 ml culture was added to 400 ml LB with 50 μg/ml Kan and 25 μg/ml Gen for another overnight culture grown at 28°C until OD_{600} was between 0.8 and 1.2. The culture was centrifuged at 3 000 x g for 15 min, the supernatant was removed and the cell pellet was resuspended in 200 ml of a 5% sucrose

solution. Immediately before the plant dipping, Silwet L-77 was added with vigorous mixing to the bacterial suspension to a final concentration of 0.05%. This suspension was used to transform *Arabidopsis* plants whose flower stems had been cut four to six days prior to this treatment. By this time new flower stems had emerged. Any fully opened flowers were removed. The aerial part of the plants were gently agitated for a few seconds in the *A. tumefaciens* mixture, the pots were quickly placed in a large container with a few millimeters of standing water covered with saran wrap with and maintained under high humidity and low light intensity for 24 hr. Plants were subsequently grown normally under fluorescent lights in the laboratory at 6 000 lux intensity or in growth chambers at 7 500 lux intensity and T1 seeds were collected when the seeds were mature and dry.

To select transformed plants, T1 seed was surface sterilized with a mix of 12% (w/v) sodium hypochloride and 0.2% (v/v) Tween 80 for 10 to 15 min, rinsed several times with sterilized water and plated in Petri dishes containing a medium made of 1x Murashige and Skoog (MS) salts, 1% sucrose, 0.8% agar, 1x Gamborg's B-5 vitamins and 30 μg/ml Kan, (pH 5.7). The plated seeds were vernalized at 4°C in the refrigerator for two days and then placed under lights with an intensity of 6 000 lux with 16 hrs of light/8 hrs darkness at a temperature of 24°C. After 8 to10 days, Kan resistant transgenic lines were detected as rare green seedlings among white nonresistant seedlings. At approximately fifteen days, seedlings were transplanted to soil, maintained at high humidity for one day and then grown as described above (section 2.1) until seeds could be harvested. T2 seed was germinated under the same conditions as those for T1 described above and the frequency of Kan resistant seedlings was scored to determine the

number of transgenes inserted in each transgenic line. Transgenic lines with a single insertion have a 3:1 segregation of Kan resistance to Kan sensitivity. Transgenic lines with multiple insertions have a higher proportion of resistant plants in the T2 generation according to Mendelian segregation ratios, 15:1 and 63:1 for two or three unlinked insertions respectively. Only lines with single insertions were used for the analysis reported here. T2 seeds for *F8A24.12* promoter-GUS transgenic plants were sterilized and germinated as describe above and used for characterization of the transgenic promoter plus 5'UTR region.

2.5. MeJA and salt treatments of transgenic *F8A24.12* promoter reporter lines

After 8 days on the selective medium, the Kan resistant transgenic plants (showing a 3:1 segregation for Kan resistance) were transferred to sealed Petri dishes and grown on a modified Hoagland's solution (Gibeaut *et al.*, 1997) and 1% agar. Each Petri plate contained a 5mm hole in the lid blocked by sterile cotton to allow air exchange. The use of modified Hoagland's solution avoided the influence of sugar and salt present in the MS medium on expression. The modified Hoagland's solution used for *Arabidopsis* growth contains 1.25 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.5 mM KH_2PO_4, 50 μM KCl, 50 μM H_3BO_3, 10 μM $MnSO_4$, 2 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 0.1 μM $H_2Mo_2O_7$ 85% and 26.4 mg/l $[(O_2CCH_2)_2NCH_2CH_2N(CH_2CO_2)_2]FeNa?_2H_2O$ (Fe-EDTA, Aldrich), pH 6.0. Plants were grown for 7 days and then treated for 6, 12, 24 and 36 hrs either with modified Hoagland's medium with 250 mM NaCl or with 100 μM MeJA. The seedlings were either stained for GUS histochemical analysis or frozen for enzymatic quantification for GUS activity. Each experiment for the intact

F8A24.12 promoter plus 5'UTR assayed four treated whole plants and four control plants for each time point for each treatment. Six transgenic lines 63, 61, 111, 15, 16 and 202 were assayed.

For analysis of the transgenic plants with the suORF mutation, six transgenic lines (lines 3, 8, 9, 10, 13 and 20) were treated with 250 mM NaCl or 100 μM MeJA for 24 hrs. Four treated plants and four control plants were assayed for each line.

2.6. Histochemical and fluorometric assays

Transgenic plants were characterized by histochemical staining to detect GUS activity (Jefferson, 1987). *Arabidopsis* plants were submerged in small volumes of staining buffer (50 mM $NaHPO_4$ (pH 7.2), 0.5% (v/v) Triton X-100 and 2 mM of the substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-Gluc)) and incubated at 37°C with very gentle agitation until a blue coloration was visible. To stop the reaction, each plant was rinsed in distilled water and soaked in several changes of 70% ethanol over a 24 hr period until the green color disappeared from the tissue. Samples were stored in 70% ethanol for visual and photographic analysis.

For the fluorometric assays (Jefferson, 1987 and Gallagher, 1992), each *Arabidopsis* plant, including whole shoot and root tissue, was homogenized in 50 μl of GUS extraction buffer containing 50 mM $NaHPO_4$ (pH 7.0), 10 mM ß-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100. Each sample was cleared by centrifugation at 12 000 x g for 10 min at 4°C. The plant extract was diluted 10 fold with extraction buffer. An aliquot of 2.5 μl of the diluted extract was used to determine the protein concentration of the sample by Bradford assay and 10 μl was

used for the fluorometric assay of GUS activity. For the enzyme reaction, a solution of 1 mM 4-methylumbelliferyl ß-D-glucuronide (MUG) substrate in extraction buffer was prepared and aliquots of 690 µl were warmed to 37°C. A 10 µl aliquot of the diluted plant extract was mixed with each aliquot of the MUG solution which was incubated at 37°C. After 5 min, a 200 µl aliquot was removed and mixed with 1.8 ml of 0.2 M sodium carbonate stop buffer (Na_2CO_3). Additional 200 µl aliquots of the enzyme reaction were removed and neutralized in Na_2CO_3 at 15 and 25 min. To determine MU accumulation, 300 µl samples of the stopped reaction were checked by fluorometry at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Linear regression was done on each enzymatic reaction. Only the samples with an R squared above 0.975 were accepted for further analysis.

2.7. Statistical analysis

For fluorometric assays, plants were grown as those used for histochemical staining except that this was performed on the lines 63, 61, 111, 15, 16 and 202. Four replicate samples and controls were taken at four time points: after 6, 12, 24 and 36 hrs of treatment. Due to the expected variability of GUS expression between plants of the same line because of the use of the T2 generation containing a 2:1 ratio of heterozygotes over homozygotes for the transgene in the population and the difference between lines, the samples sets were randomized to allow an analysis of the variance (ANOVA). This analysis was performed on the 355 valid data taken from a total of 384 plant samples. For the F8A24.12 mutated promoter version, a similar procedure was followed except that the six independent transgenic lines 13, 9, 10, 8, 3 and 20 carrying the GUS reporter

driven by the F8A24.12 promoter with the mutated suORF were used and the treatments were done for 24 hrs. The 86 valid data from a total of 96 assays were used in this analysis.

GUS specific activity was used to determine the significance of the three factors, Treatment, Transgenic line and Time at a 95% confidence level (p-value of 0.05). P-values are probability scores (p). The ANOVA allows testing of the difference between two or more means. ANOVA examines the ratio of variability between two conditions and variability within each condition. The variability is measured as the sum of the difference of each score from the mean. ANOVA was used to identify factors with significant effects on GUS activity and the very conservative multiple comparison t-test of Bonferroni was used to test the significance of the difference of particular means for treatments, individual lines encompassing all times, times encompassing all the lines and also the significance of the difference between the intact 5'UTR and the mutated suORF during each treatment.

2.8. Yeast two-hybrid screening

In order to identify candidate proteins that interact with the protein kinase F8A24.12, the well-established yeast two-hybrid screen was used. The protocol used for this purpose was obtained from Bai and Elledge (1993). The pAS2-F8A24.12 plasmid was transformed into the yeast strain *Saccharomyces cerevisiae* Y190 and transformants were selected for their ability to grow without tryptophan since the pAS2-1 vector carries the *TRP1* gene and Y190 lacks this gene.

The cDNA library obtained from the Arabidopsis Biological Resource Center (ABRC) was constructed in the pACT vector which contains the leucine selectable marker and was hosted by *E. coli* strain BNN132. The cDNA library was made with mRNA isolated from mature *Arabidopsis* leaves and roots. cDNA had been generated by reverse transcription primed by random DNA oligomers and blunt ended cDNA was ligated to adaptors and inserted in the *XhoI* site of lambdaACT to be fused to the *GAL4* DNA activation domain. For the cDNA library, a titer of $6.7x10^{10}$ pfu/ml was previously measured by D. Elias using LE392 *E. coli* cells infected with the *Arabidopsis* ?ACT library, which produces plaques. I obtained a titer of $9.8x10^{8}$ cfu/ml for this library using the *E. coli* strain BNN132.

The library was amplified using BNN132 *E. coli* cells. They were grown to mid-log phase and centrifuged at 3 000 x g for 5 min, then resuspended in 10 mM $MgCl_2$ at an OD_{600} of 0.5. A 100 µl aliquot of the source library was added, the suspension was spread on 21 LB plates (150 mm) with 50 µg/ml ampicilin (Amp) and 0.2% glucose and incubated at 37°C overnight. Ten ml of LB were added to each plate to resuspend the bacteria colonies. The bacterial suspension was mixed in a 2 L solution of Terrific Broth and grown overnight at 37°C. Plasmid DNA representing the library was extracted following the Qiagen Maxi plasmid prep. protocol.

The plasmid from the library was transformed into the yeast Y190 strain containing the pAS2-F8A24.12 bait construct and plated on synthetic complete medium, SC-his/-leu/-trp supplemented with 3-amino-1,2,4-triazole (3-AT). Any positive interaction between the bait protein and the product of a cDNA clone from the library results in the association of the two chimeric subdomains to form an intact GAL4

transcription factor and induces the Lac Z reporter gene. Colonies maintaining both plasmids as evident from growth on the SC plates lacking histidine were tested by X-Gal filter assay, which causes a blue coloration due to the ß-galactosidase activity if an interaction takes place. Each positive blue colony was retested for ß-galactosidase activity.

CHAPTER 3. RESULTS

3.1. Three *Arabidopsis thaliana* homologues of Esi47, members of the NAK protein kinase family

The *Arabidopsis* ser/thr protein kinases F8A24.12, F12E4.50 and T7F6.28, are the three *Arabidopsis* proteins with the greatest amino acid sequence similarity to Esi47 (Figure 1) and they belong to the plant NAK group of protein kinases. They have 59%, 59% and 54% amino acid sequence identity respectively with Esi47. Moreover, their catalytic domains have highly conserved amino acids characteristic of different protein kinase families (Hanks and Quinn, 1991; Taylor *et al.*, 1993) and are 70%, 70% and 68% respectively identical to the Esi47 catalytic domain. *Arabidopsis* has eight other NAK protein kinase family members (Shen *et al.*, 2001) but these have lower amino acid sequence identity with Esi47 ranging from 51% to 34% overall and from 64% to 41% within the catalytic domain respectively for T9I4.2 to ARSK1.

3.2. Yeast two-hybrid screening for protein-protein interaction with the F8A24.12 protein kinase

A yeast two-hybrid library made from cDNA derived from *Arabidopsis* mature leaf and root was used to screen for proteins interacting with the F8A24.12 protein kinase. The pAS2-F8A24.12 construct was transformed in the yeast strain Y190 and $3.7x10^5$ yeast transformants were screened using the X-Gal filter assay. Interaction between the F8A24.12 protein kinase bait and a protein encoded by the cDNA library is

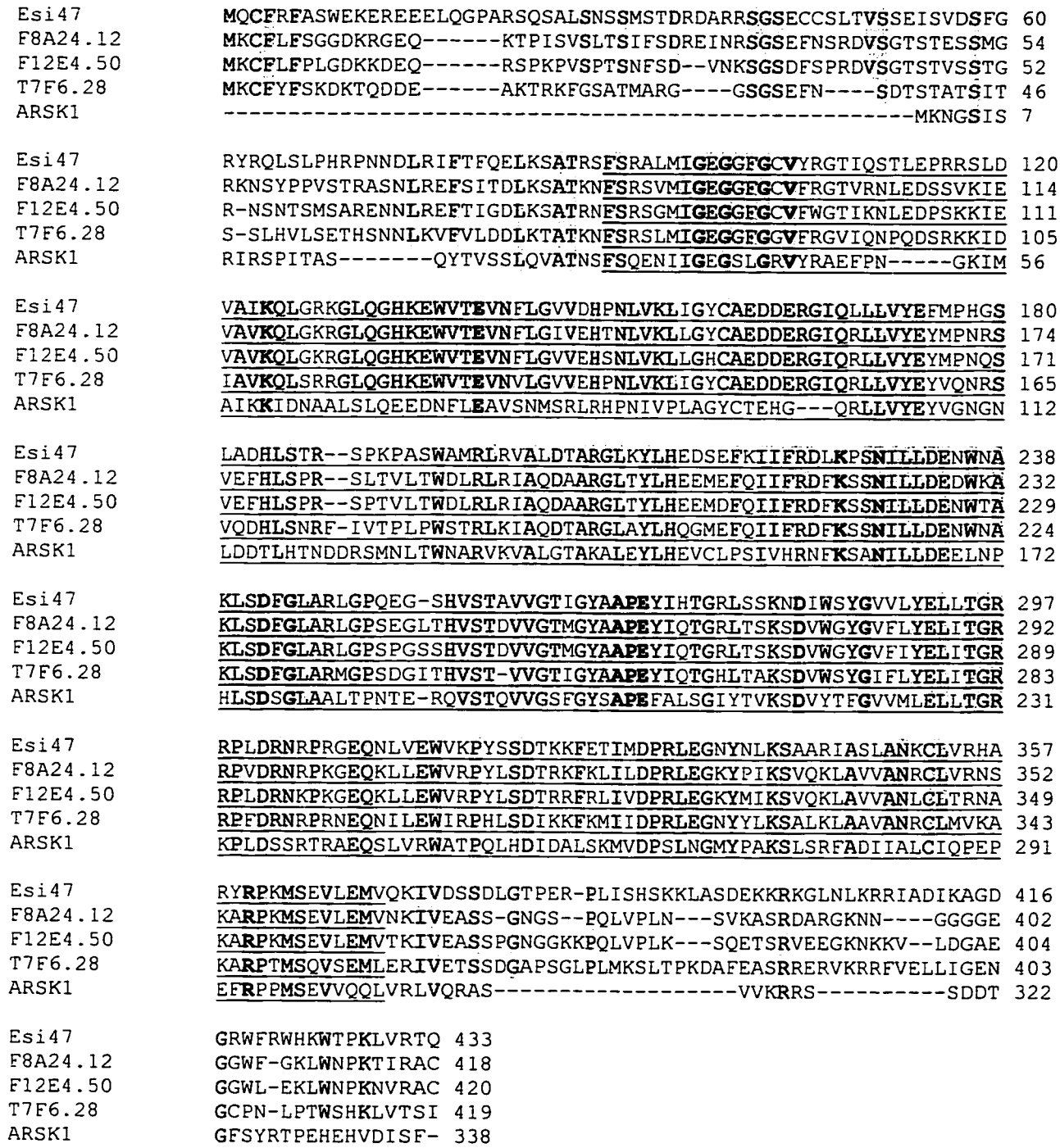


Figure 1. Multiple sequence alignment of Esi47 and similar protein kinases from *Arabidopsis*. CLUSTAL W (1.82) was used to align the ser/thr protein kinases Esi47, F8A24.12, F12E4.50, T7F6.28, and ARSK1 (a previously characterized member of the NAK group of plant protein kinases). Numbers represent amino acid position in each protein. The catalytic domain is underlined. The bold letters indicate the highly conserved amino acids of the protein kinase family and the conserved amino acids are highlighted.

expected to produce a blue colony. Three putative blue candidates were observed, picked and regrown. After a second X-Gal filter assay, none of the transformants gave positive blue coloration.

3.3. *F8A24.12* constructs

All of the *F8A24.12* promoter constructs were checked by partial DNA sequence analysis of the clones in the pBluescript SK- vector. Approximately 600 bp of sequence that included the vector/insert junction and all of the 5'UTR was sequenced. The intact *F8A24.12* promoter plus 5'UTR, the promoter with the mutated ATG of the suORF in the 5'UTR and the promoter with the deleted intron in the 5'UTR were as expected. However, a rearrangement was detected in the construct that contained both the mutated suORF and the deleted intron in the 5'UTR. The mutation of the suORF ATG was correct and the intron was removed but a 155 bp fragment from the –1643 to –1798 region was inverted and inserted at the site of the intron in the 5'UTR.

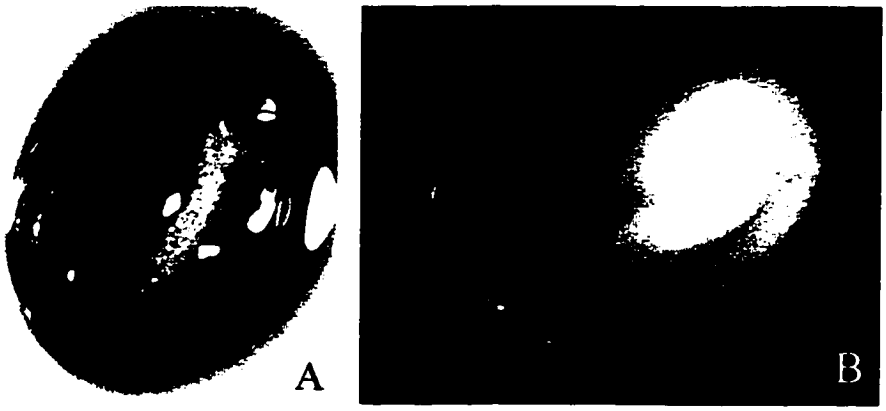
3.4. *F8A24.12* is expressed in roots, leaves, flower abscission zone and pistils

The intact *F8A24.12* promoter was fused to the GUS reporter gene and used for *A. tumefaciens*-mediated T-DNA transfer to *Arabidopsis*. Thirty healthy *Arabidopsis* plants referred to as the T0 generation were used for transformation with the intact *F8A24.12* promoter-GUS fusion. Seeds from each *A. tumefaciens* treated plant were germinated on Kan selective media and 21 resistant transgenic lines were obtained. Scoring for 3:1 segregation for Kan resistance indicated that 10 lines carried a single insertion of the

T-DNA. The ten independent transgenic lines with the intact *F8A24.12* promoter-GUS fusion were numbered: 3, 202, 204, 205, 15, 16,111, 61, 64 and 63.

To localize GUS expression *in vivo*, the GUS histochemical assay based on an enzymatic reaction in which the beta-glucuronidase cleaves a substrate (X-Gluc) that produces a blue coloration was used. The first GUS histochemical assay carried out on the ten lines revealed that GUS reporter gene expression had similar tissue specific localization but that the basal levels of expression were different in the different transgenic lines. The intensity of GUS staining was quantified by eye from the darkest (5) to the lightest (1) degree of blue coloration and grouped as follow; line 3 (5); lines 202, 204 and 205 (4); lines 15 and 16 (3); line 111 (2); and lines 61, 63 and 64 (1). Transgenic seedlings were grown on agar based medium in sealed Petri dishes then gently removed and immersed in GUS buffer. A blue coloration appeared in the root elongation and meristematic zone but not in the root cap respectively within a few seconds or within a few minutes for lines with a degree of coloration of (5) or (4) and (3) (Figure 2D). Subsequently staining in the vascular tissue of the entire root was visible within several minutes (Figure 2F) and also in first true leaves and at a lower level in the cotyledons, particularly in the vascular tissues (Figure 2C). One hour and 2 hrs were required for comparable staining respectively for the lines with a degree of coloration of (2) and (1). However for these lines, the blue coloration in root tip appeared within several minutes in the GUS buffer.

The *F8A24.12* promoter-GUS fusion is expressed at different stages of the plant development and GUS histochemical assays also allowed the localization of the GUS expression in mature embryo at the seed stage (Figure 2A) and in the newly germinated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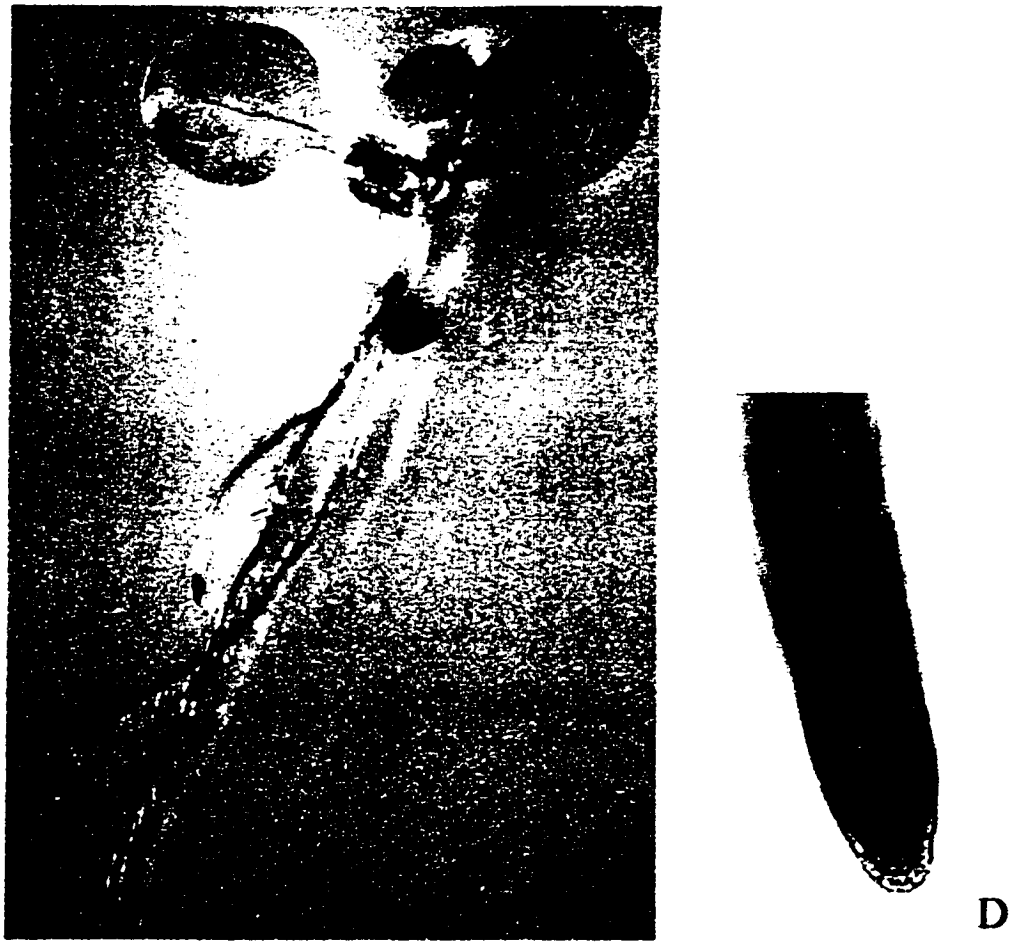
B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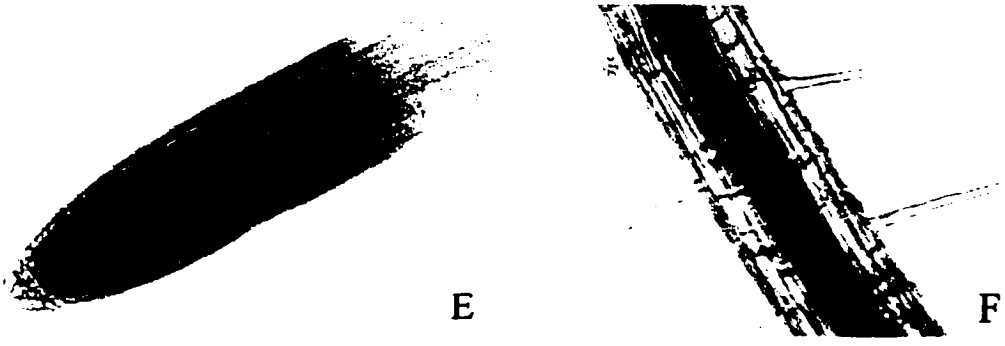
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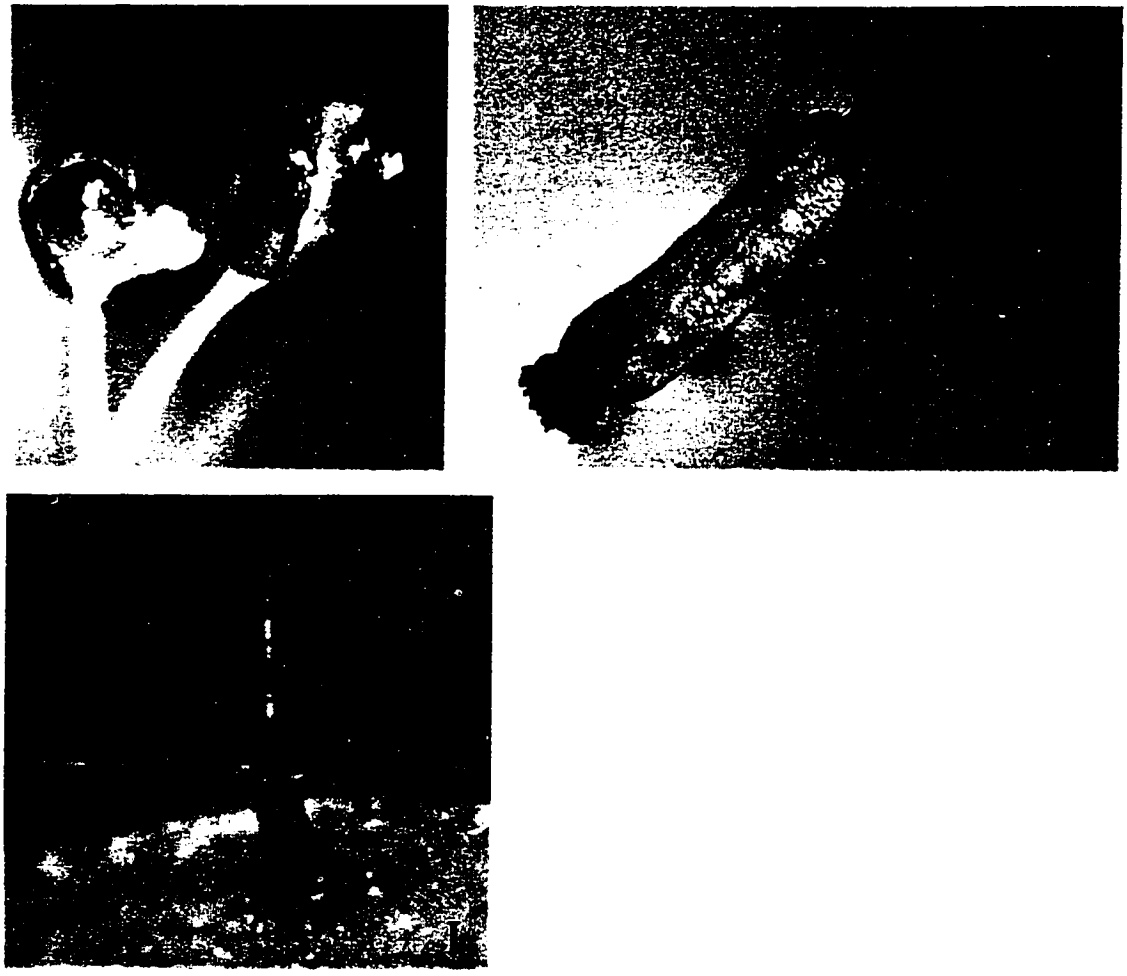

Figure 2. GUS localization driven by the *F8A24.12* promoter plus 5'UTR. Figure 2A represents a seed of line 111 previously incised with a scalpel and incubated one hour in the GUS staining. Figure 2B, the embryo was taken out of the seed envelop. Figure 2C shows a 10 day old seedling from line 15 incubated for 20 min in the GUS staining buffer and Figure 2D, one of its root tips. Figure 2E and 2F show respectively the root tip and the vascular tissue of the root of a 10 day old seedling from line 63 and 15 incubated for one hour and 20 min in the staining buffer. Figure 2G reveals GUS localization in the unopened and newly opened flower; more precisely in the petals and sepals and strongly in the stigmatic tissue of the pistil. The staining was performed for one hour on a 24 day old plant from line 111. Figure 2H shows a silique of the same plant with a weaker staining in the stigmatic tissue but a strong GUS localization in the flower abscission zone. Figure 2I shows a trichome on one leaf of a seedling from line 15. The seedling was incubated for 20 min in the GUS staining buffer.

seed in the root tip, at the flowering stage in the sexual organs, more precisely in the stigmatic cells of the pistil and in the sepals (Figure 2G). At the silique stage, GUS expression was strong in the abscission zone of the petals and sepals at the base of the silique and in the stigmatic tissues but at a lower degree than observed at the mature and immature flower stage (Figure 2H). Staining also appears at the base of the trichome where it contacts the leaf surface (Figure 2I).

GUS histochemical assays were done on seedlings of the transgenic lines 63, 111, 15 and 202 treated with ABA. ABA treatment did not result in any visible increase of the GUS expression. The same GUS histochemical assays were done on seedlings of lines 202, 15, 16, 111, 61 and 63 treated with 250 mM NaCl or 100 μM MeJA for 6, 9, 12 and 24 hrs. There were three plants for each treatment and for each time point with the same number of untreated control plants. The salt treatment revealed a low induction of the GUS activity in the leaves at 6 hrs and 24 hrs but not at 9 and 12 hrs. MeJA increased the level of GUS expression in the leaves for lines 111 and 15 after 12, 24 and 36 hrs treatment. Results are not shown. The GUS histochemical staining is only a semi quantitative GUS activity assay, and the measurements were repeated in a more quantitative fashion below.

3.5. *F8A24.12* is MeJA-induced and is salt-induced specifically at 24 hrs treatment

To quantify the level of GUS expression and induction, the quantitative GUS fluorometric assay was performed. The GUS fluorometric assay is based on an enzymatic reaction in which beta-glucuronidase cleaves the substrate

4-methylumbelliferyl ß-D-glucuronide (MUG) to produce a fluorescent compound 7-hydroxy-4-methylcoumarin also known as 4-methylumbelliferone (MU).

3.5.1. MeJA treated plants

Transgenic plants carrying the intact promoter plus 5'UTR of *F8A24.12* fused to the GUS reporter gene showed increased expression of GUS in response to MeJA treatment (Table 2 and Figures 3, 4 and 5). The data for the replicated measurements for each line and time point had a large standard deviation, and the individual mean values for control and treated plants for each line and time point were not significantly different. Table 2 presents the summarized data for treatment with MeJA, Figure 3 shows data for each line, Figure 4 for each line averaged over all time points and Figure 5 for each time point averaged over all lines.

A three way global ANOVA test of the GUS activity in the MeJA treated transgenic plant is summarized in Table 3. ANOVA shows that when the data are analyzed globally there is a significant effect of MeJA on GUS expression (p-value <0.0001), there are significant differences in expression between transgenic lines (p <0.0001), and there is a significant MeJA*Transgenic Line interaction (p <0.0142). The latter indicates that MeJA has a significantly greater effect on some lines than others (Figures 3 and 4).

The Bonferroni t-test results give a level of confidence for concluding whether the means of MeJA treated plants and control plants are equal or not. In this case, the Bonferroni t-test that was used to compare the effect of MeJA on each line averaged over the 4 time points is presented in Figure 4. A significant increase in GUS expression is

seen in four of the six transgenic lines, shown in Table 4. Significant differences in the basal and induced levels of expression between lines were observed with histochemical staining and confirmed by the quantitative assay and statistical testing (Table 2 and 3). The lines with low levels of expression of GUS, 61 and 63, did not show significant effects of MeJA though the lines with higher expression levels did show significant effects (Table 4 and Figure 4). This is consistent with the ANOVA results which show significant MeJA*Line interaction (Table 3). Lines 111, 16 and 202 show a significant positive effect of MeJA at a 0.1 level of confidence (Table 4). The level of confidence of the results from these three lines is strong taking into account the use of the Bonferroni test, which is very conservative. Line 15 shows a significant MeJA effect at the 0.05 confidence level (Table 4).

The means for treated and control plants summarized over all six lines at each time point are shown in Figure 5. A Bonferroni t-test indicates that the increase in GUS expression in response to MeJA is particularly significant at 24 hrs and 36 hrs (Table 5) with an induction of 45.4% and 57.2% respectively (Table 2). The average increase in GUS expression was highest at 6 hr but was not significant because the response observed in the different lines is more variable at 6 hr than at 24 and 36 hr. The 44% MeJA induction at 12 hr (Table 2) is very close to the level of confidence ($p=0.0676$) taking into account the highly conservative Bonferroni t-test analysis (Table 5).

Table 2. The means of GUS specific activity for MeJA treated and untreated plants and the ratio between the two groups. GUS specific activity is calculated in nmole of MU/min/mg of protein for each line and for each time of treatment with exogenously applied MeJA at a concentration of 100 μM or with a mock treatment with water as control. The relative basal level of expression of GUS in the transgenic lines was initially judged by histochemical staining and graded from low to high as level 1 to 4 and marked in the table accordingly.

Line		6 hrs	12 hrs	24 hrs	36 hrs	Average
63 (Level 1)	Control	3.929	2.215	1.613	1.805	2.39
	MeJA	2.223	2.430	2.847	2.273	2.443
	Ratio	0.566	1.097	1.766	1.259	1.022
61 (Level 1)	Control	4.513	1.220	2.020	1.620	2.343
	MeJA	2.401	1.710	2.890	2.430	2.358
	Ratio	0.532	1.402	1.431	1.500	1.006
111 (Level 2)	Control	8.255	8.190	8.943	13.075	9.615
	MeJA	14.695	15.660	10.845	22.650	15.963
	Ratio	1.780	1.912	1.213	1.732	1.660
15 (Level 3)	Control	9.353	10.718	13.030	12.050	11.287
	MeJA	26.555	19.475	13.650	31.000	22.67
	Ratio	2.839	1.817	1.048	2.573	2.009
16 (Level 3)	Control	7.810	11.117	12.738	10.940	10.651
	MeJA	14.725	12.340	21.700	12.775	15.385
	Ratio	1.885	1.110	1.704	1.168	1.444
202 (Level 4)	Control	10.410	13.475	12.650	16.050	13.146
	MeJA	13.225	17.550	19.773	19.300	17.462
	Ratio	1.270	1.302	1.563	1.202	1.328
Average	Control	7.378	7.822	8.498	9.256	8.239
	MeJA	12.304	11.528	11.951	15.071	12.714
	Ratio	1.479	1.440	1.454	1.572	1.412

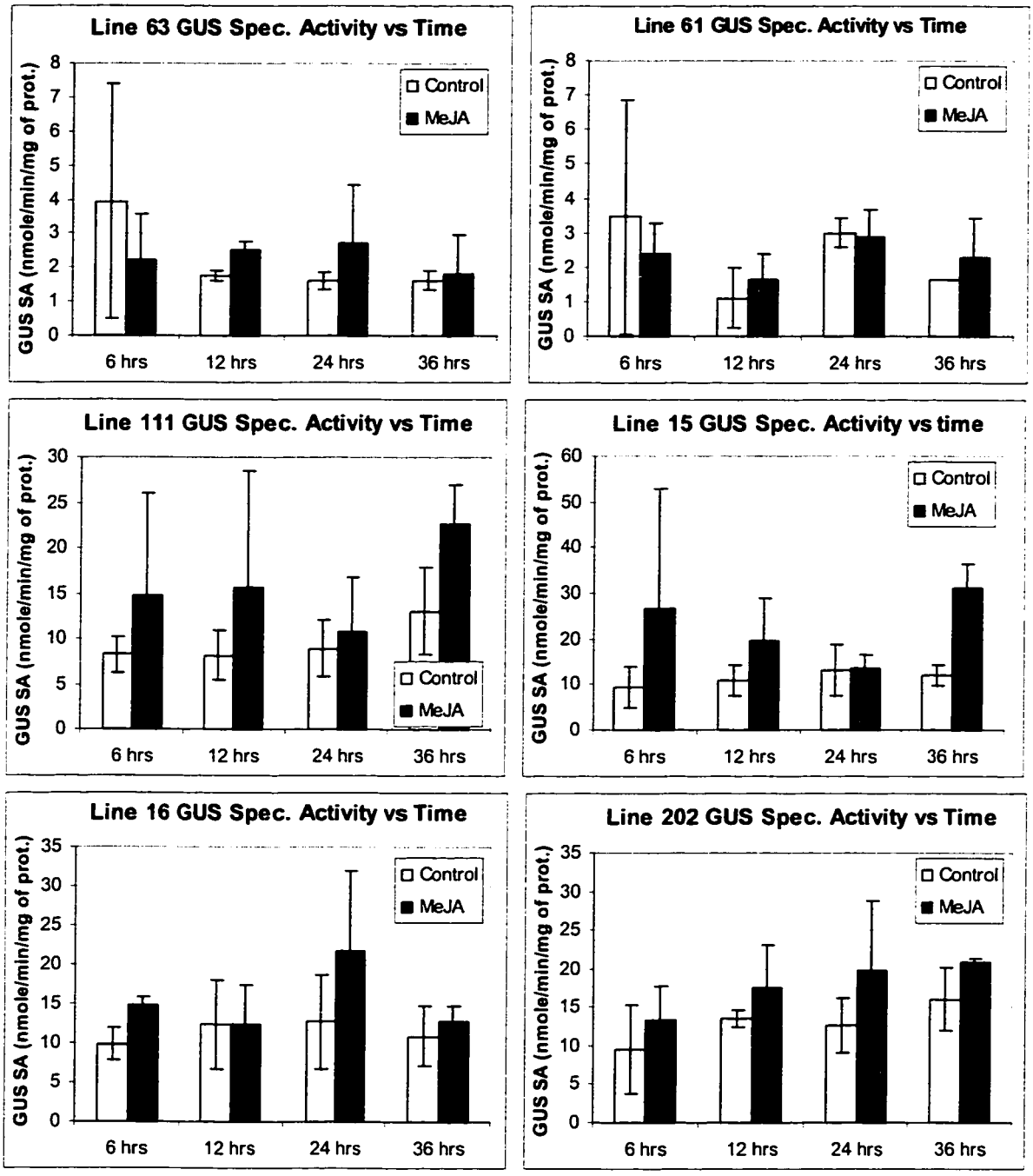

Figure 3. Time course for each line during MeJA treatment. GUS specific activities were measured in nmole of MU/min/mg of protein for each transgenic line after 6, 12, 24 and 36 hrs of exogenously applied MeJA at a concentration of 100 μM or with a mock treatment with water as control. Error bars indicate standard deviations of the means.

Analysis of variance for the MeJA treatment for 6 Lines and 4 Time points:

Table 3. ANOVA for MeJA treatment. ANOVA evaluation of the significance of the three factors, MeJA treatment, transgenic lines and duration of treatment and the interaction of these factors. P-values are probability scores; p-values less than 0.05 are significant at the 95% confidence level.

Factors and interactions	p-value
MeJA Treatment	**<0.0001**
Line	**<0.0001**
MeJA Treatment*Line	**0.0142**
Time	0.2899
MeJA Treatment*Time	0.829
Line*Time	0.3171
MeJA Treatment*Line*Time	0.577

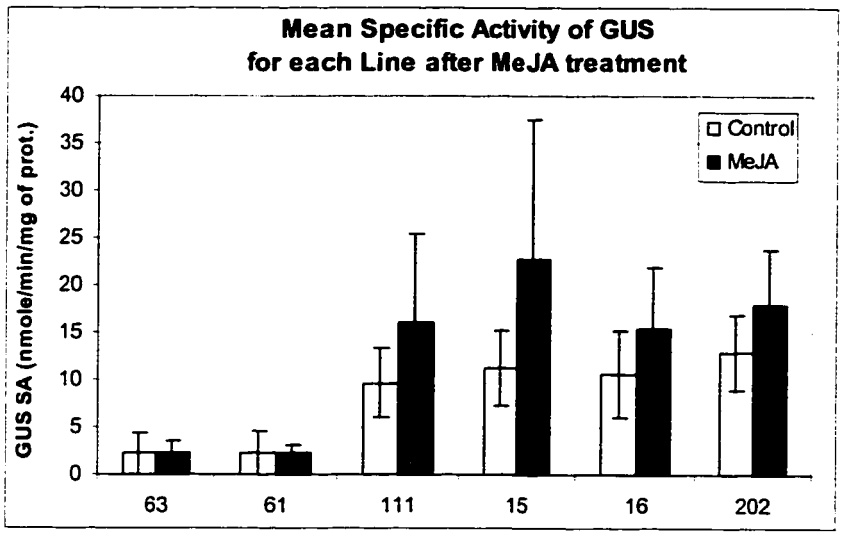

Figure 4. The means of GUS specific activity for MeJA effect on each line averaged over all time points. GUS specific activity is calculated in nmole of MU/min/mg of protein for each line and for each time of treatment with exogenously applied MeJA at a concentration of 100 μM or with a mock treatment with water as control. Error bars incidate standard deviations of the means

Table 4. Bonferroni t-test determined that the overall MeJA effect is not significant for all the lines. A p-value is given for a measure of significant difference between controls and MeJA treated plants for each line using the Bonferroni t-test. The mean values of GUS activity for MeJA treated and controls are significantly different for lines 111, 15, 16 and 202 considering the highly conservative Bonferroni test.

Line	63	61	111	15	16	202
p-value (MeJA)	0.9536	0.9832	**0.071**	**0.0064**	**0.0529**	**0.0291**

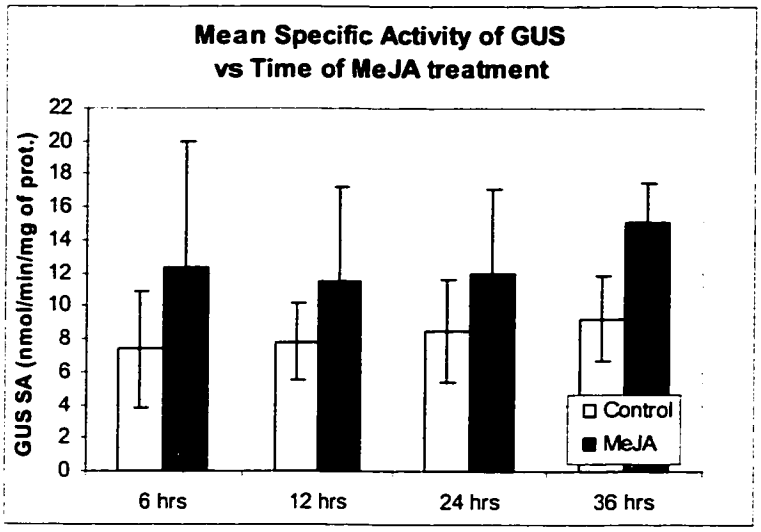

Figure 5. Time course of MeJA treatment. The mean GUS activity averaged for six transgenic lines carrying the intact promoter and 5'UTR of F8A24.12. GUS specific activity was measured in nmole of MU/min/mg of protein for each time of MeJA treatment encompassing all the Lines. Error bars indicate standard deviations of the means.

Table 5. Bonferroni t-test determined that the MeJA effect is not significant for all the time points. A p-value is given for a measure of significant difference between controls and MeJA treated plants for each Time using the Bonferroni t-test with a level of confidence of 95%. The mean values of GUS activity for MeJA treated and controls are significantly different at 24 and 36 hrs and close to the level of significance for 6 and 12 hrs treatment taking into account the highly conservative Bonferroni t-test.

	Time	6 hrs	12 hrs	24 hrs	36 hrs
p-value	MeJA	0.0898	0.0676	**0.044**	**<0.0001**
	MeJA*Line	0.3688	0.6846	0.5242	**<0.0001**

Though the randomized design for the quantification of GUS activity permitted ANOVA and the measurement of significant effects of MeJA treatment, the data were quite variable between lines and within a single line. This can be seen in the graphical data represented for each line in Figure 3.

3.5.2. Salt treatment

A 24 hr NaCl treatment appeared to increase the level of GUS expression under the control of the *F8A24.12* promoter (Figure 8). As it was observed for the previous MeJA treated plant analysis, the data for the replicated measurements for each line and time point had a large big standard deviation, and the individual mean values for control and treated plants for each line and time point were not significantly different. Table 6 summarizes the mean values for each transgenic line at each time of treatment with NaCl. Transgenic plants with the intact *F8A24.12* promoter plus 5'UTR showed little change in GUS expression in response to NaCl treatment (Figure 6 and 7).

ANOVA on the mean GUS specific activity showed that there was not a significant effect of salt on the *F8A24.12* promoter-GUS fusion when the six transgenic lines were considered globally (Table 7). However, the <0.0001 p-value for the Time factor and 0.0065 p-value for Salt*Time interaction are significant, and probably indicate a significant effect of salt at one or several specific time points (Table 7).

To confirm that there was a significant effect of salt treatment at one or more time of treatment, a Bonferroni t-test was performed to compare the means of GUS specific activity at the different time points averaged over all lines (Table 9). This revealed a significant p-value of 0.0005 for the time of 24 hr treatment even though the plants only showed a 61.2% increase in GUS activity (Table 6). Plants actually showed decreased GUS activity with NaCl treatment at 36 hr (Table 6), but this change was not significant (Table 9). Only the increase in expression at 24 hr was statistically significant (Table 9). Similarly to the MeJA data, the small p-values in Table 7 for line effect (<0.0001) and for the Line*Salt treatment interaction at 24 hr in Table 9 (p=0.0202) indicate that there were

significant differences in the level of expression between lines and there were significant differences in the level of response to NaCl treatment between transgenic lines (Table 8). This also was supported by the significant 0.0378 p-value for line 15 for Salt*Time interaction as compare to all other lines (Table 8) due to the strong salt effect at 24 hr (Figure 6).

Table 6. The means of GUS specific activity for salt treated and untreated plants and the ratio between the two groups. GUS specific activity is calculated in nmole of MU/min/mg of protein for each line and for each time of treatment with NaCl at a concentration of 250 mM or with a mock treatment with modified Hoagland's solution as control. The relative basal level of expression of GUS in the transgenic lines was initially judged by histochemical staining and graded from low to high as level 1 to 4 and marked in the table accordingly.

Line		6 hrs	12 hrs	24 hrs	36 hrs	Average
63 (Level 1)	Control	1.450	1.643	1.908	2.143	1.786
	Salt	1.281	2.643	3.018	3.463	2.601
	ratio	0.883	1.609	1.582	1.616	1.456
61 (Level 1)	Control	2.093	2.546	2.450	5.200	3.072
	Salt	2.277	1.131	2.957	2.420	2.196
	ratio	1.088	0.444	1.207	0.465	0.715
111 (Level 2)	Control	8.718	9.295	8.438	15.165	10.404
	Salt	7.255	11.723	16.798	13.610	12.347
	ratio	0.832	1.261	1.991	0.897	1.187
15 (Level 3)	Control	20.375	18.820	11.770	19.975	17.735
	Salt	11.250	17.975	28.075	18.025	18.831
	ratio	0.552	0.955	2.385	0.902	1.062
16 (Level 3)	Control	6.690	9.480	11.160	31.500	14.708
	Salt	11.165	7.660	13.775	17.320	12.480
	ratio	1.669	0.808	1.234	0.550	0.849
202 (Level 4)	Control	16.813	14.800	13.100	24.975	17.422
	Salt	10.588	14.975	16.700	22.125	16.097
	ratio	0.630	1.012	1.275	0.886	0.924
Average	Control	9.356	9.431	8.137	16.493	10.854
	Salt	7.303	9.351	13.554	12.827	10.759
	ratio	0.942	1.015	1.612	0.886	1.032

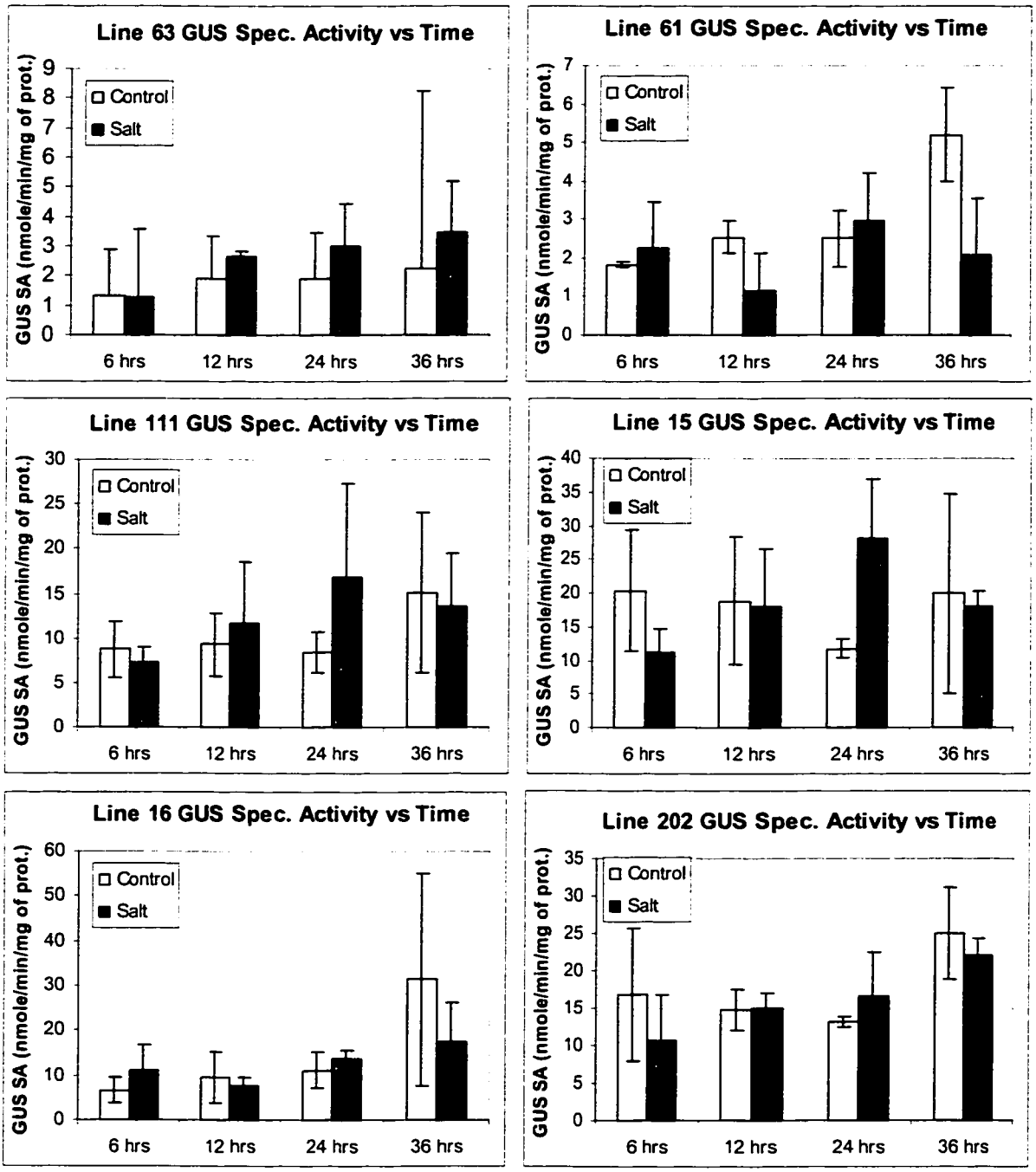

Figure 6. Time course for each line during salt treatment. GUS activities were measured in nmole of MU/min/mg of protein for each transgenic line after 6, 12, 24 and 36 hrs of NaCl treatment at a concentration of 250 mM. Error bars indicate standard deviations of the means.

Analysis of variance for the NaCl treatment for 6 lines, 4 time points:

Table 7. ANOVA for Salt treatment. ANOVA evaluation of the significance of salt treatment, transgenic lines, duration of treatment and the interaction of these factors. P-values are probability scores; p-values less than 0.05 indicate significant differences between means within the category listed at the 95% confidence level.

Factors and interactions	p-value
Salt Treatment	0.9207
Line	**<0.0001**
Salt Treatment*Line	0.7839
Time	**<0.0001**
Salt Treatment*Time	**0.0065**
Line*Time	0.0973
Salt Treatment*Line*Time	0.2526

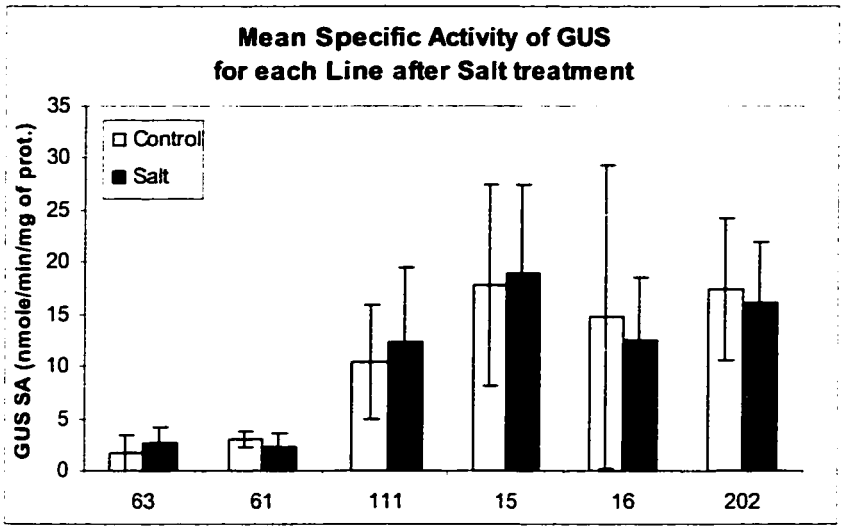

Figure 7. The means of GUS specific activity for overall Salt effect on each line. GUS activity is calculated in nmole of MU/min/mg of protein for each line and for each time of treatment with salt at a concentration of 250 mM or with a mock treatment with modified Hoagland's solution water as control. Error bars indicate standard deviations of the means.

Table 8. Bonferroni t-test determined that the overall NaCl effect is significant for one line. A p-value is given for a measure of significant difference between controls and salt treated plants for each line averaged over the time using the Bonferroni t-test. The mean values of GUS activity for salt treated and controls indicate there a significant salt*time interaction for line 15, indicating that there is likely a significant effect of salt at one or more time points.

Line	63	61	111	15	16	202
p-value (Salt*Time)	0.6476	0.664	0.3491	**0.0378**	0.2615	0.2728

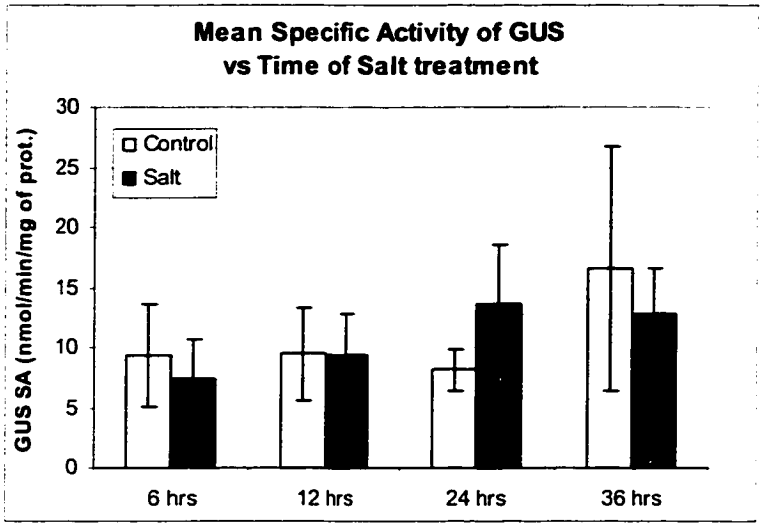

Figure 8. GUS expression for Time course of salt treatment. The mean GUS activity averaged for six transgenic lines carrying the intact promoter and 5'UTR of F8A24.12. GUS specific activity was measured in nmole of MU/min/mg of protein for each time of salt treatment encompassing all the lines. Error bars indicate standard deviations of the means.

Table 9. Bonferroni t-test determined that the Salt effect is significant for 24 hr treatment. A p-value is given for a measure of significant difference between controls and MeJA treated plants for each time using the Bonferroni t-test with a level of confidence of 95%. The mean values of GUS activity for salt treated and controls are significantly different at 24 hr when averaged over all lines.

	Time	6 hrs	12 hrs	24 hrs	36 hrs
p-value	Salt	0.1905	0.9554	**0.0005**	0.2121
	Salt*Line	0.1461	0.9524	**0.0204**	0.704

3.6. The *F8A24.12* promoter with the mutation in the suORF does not show a response to MeJA or salt treatment

To test the effect on gene expression of the suORF in the 5'UTR of *F8A24.12*, the start codon (ATG) of the suORF was replaced by TTG. Thirty healthy *Arabidopsis* plants of the T0 generation were used for transformation with the mutated suORF *F8A24.12* promoter plus 5'UTR-GUS fusion. Seeds from each *A. tumefaciens* treated plants were selected on Kan selective medium and 45 resistant transgenic lines were obtained. Scoring for 3:1 segregation for Kan resistant indicated that 24 lines carried a single insertion of the T-DNA. Histochemical staining of these transgenic lines revealed no change in the tissue specific pattern of GUS expression as compared to the transgenic lines carrying the intact promoter-GUS fusion. Variable levels of GUS expression were observed between independent transformed lines. The six independent lines 13, 9, 10, 8, 3 and 20, were classified by eye for their basal level of GUS expression with one being the lowest and five being the highest level. Lines 13 and 9 had low level (1), lines 10, 8 and 3 had intermediate levels respectively of 2, 3 and 4 and line 20 had a high level (5).

As in the previous analysis of transgenic lines with the full promoter of *F8A24.12* with the intact 5'UTR, fluorometric assays were performed, however, plants were analyzed only after 24 hr of treatment with MeJA or NaCl. The *F8A24.12* promoter with the mutation in the suORF does not show a response to MeJA or salt treatment. The averaged ratio of the mean specific activity of ß-glucuronidase for MeJA (0.904) or NaCl (0.972) treated over control plants on all the lines reveal no change in expression of GUS in response to MeJA or NaCl treatment (Table 10 and 11).

To confirm this, an ANOVA was performed on the means of the GUS activity to determine the significant effect of treatment and line. Also the ratios of GUS specific activity of the mutated and non-mutated lines were compared to see if the mutation had an effect on GUS reporter gene expression. The ANOVA on the means of the GUS specific activity determined that there are significant differences between the mutated lines and non-mutated lines in both the MeJA treatment and the NaCl treatment with p-values respectively of 0.0176 and 0.0221 (Table 12). The GUS activity means for the mutated lines are globally higher than for the non-mutated lines due to the strong level of GUS expression in the lines 3 and 20 (Table 10 and 11). This may not be due to the mutation but may due to variation between transgenic lines caused by an effect of the DNA surrounding the site of the T-DNA insertion. The p-value of 0.4159 for the MeJA treatment and 0.6902 for NaCl treatment indicates that there is no significant effect of MeJA or NaCl after 24 hr treatment on the suORF mutated *F8A24.12* promoter plus 5'UTR-GUS fusion when the six transgenic lines 13, 9, 10, 8, 3 and 20 are considered globally (Table 12). There was an non-significant decrease in expression in response to MeJA treatment (Table 10) and there was no significant line*treatment effect (p=0.2938) (Table 12).

This result is different from that found for the intact *F8A24.12* promoter plus 5'UTR (Table 13 and Figure 9). The mutation of the ATG of the suORF in the 5'UTR nullifies the MeJA and NaCl responsiveness of the GUS reporter gene driven by the *F8A24.12* promoter plus 5'UTR. To determine if the mutation effect is significant in the response of GUS expression to MeJA and salt treatments, the ratios of the GUS specific activity of the intact and mutated promoter at the time of 24 hr were compared. An

ANOVA on these ratios showed that the response to salt treatment of the transgenic lines with the intact promoter plus 5'UTR is significantly different from that of the transgenic lines with the mutated suORF and the difference between the intact and mutated 5'UTR in response to MeJA is slightly significant (at a cut-off of p=0.1) (Table 14 and Figure 10).

Table 10. The mean of GUS activity for transgenic plants with the mutated suORF in the 5'UTR of *F8A24.12* for MeJA treated and untreated plants and the ratio between the two groups. Activity was measured in plants treated with 100 μM MeJA for 24 hr and mock treated controls. GUS specific activity is reported as nmole of MU/min/mg of protein.

Transgenic Line	Control	MeJA	Ratio
13	3.514	3.232	0.920
9	4.015	2.04	0.508
10	4.97	6.155	1.238
8	9.73	12.967	1.333
3	45.2	44.5	0.985
20	214.025	94.67	0.442
Average	46.909	27.261	0.904

Table 11. The mean of GUS activity for transgenic plants with the mutated suORF in the 5'UTR of *F8A24.12* for salt treated and untreated plants and the ratio between the two groups. Activity was measured in plants treated with 250 mM NaCl for 24 hrs and mock treated controls. GUS specific activity is reported as nmole of MU/min/mg of protein.

Transgenic Line	Control	Salt	Ratio
13	1.865	1.783	0.956
9	2.44	2.01	0.824
10	5.782	5.27	0.911
8	8.735	11.045	1.264
3	35.6	28.575	0.803
20	118.85	127.825	1.076
Average	28.878	29.418	0.972

Table 12. ANOVA for the mutated lines. Means of the GUS specific activity comparison of the mutated and non-mutated promoter. Calculation of p-values determines the significance of MeJA or NaCl treatment, difference between mutated and non-mutated lines and the difference between the mutated lines. P-values less than .05 indicate significant differences between means within the factors or between them at the 95% confidence level.

	Factors interactions	p-value
MeJA	MeJA Treatment	0.4159
	Mutant vs intact line	**0.0176**
	MeJA Treatment*Mutant line	0.2938
Salt	Salt Treatment	0.6902
	Mutant vs intact line	**0.0221**
	Salt Treatment*Mutant line	0.7418

Table 13. Comparison of the mean GUS activity between transgenic lines with intact 5'UTR and mutated suORF constructs at 24 hrs. GUS specific activity is given in nmole of MU/min/mg of protein after 24 hrs treatment with MeJA and NaCl. Values are averaged over six transgenic lines for each genotype.

Treatment	Promoter version	Control	Treated
MeJA	intact 5'UTR	8.498	11.951
	Mutated suORF	46.909	27.261
Salt	intact 5'UTR	8.137	13.554
	Mutated suORF	28.878	29.418

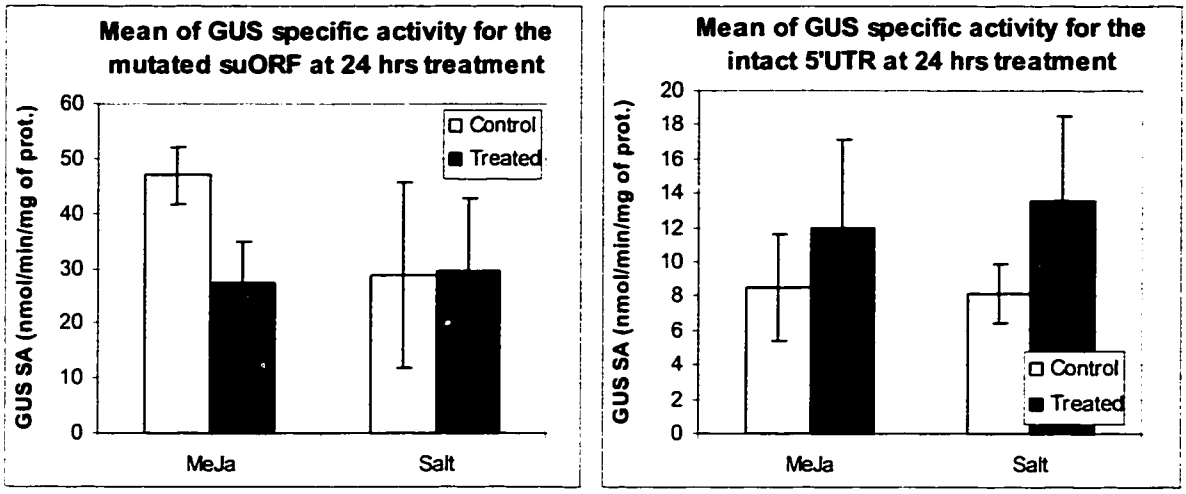

Figure 9. Comparison of the mean GUS activity between transgenic lines with intact 5'UTR and mutated suORF constructs at 24 hrs. Error bars indicate standard deviations of the means.

Table 14. Comparison of the ratios of the GUS specific activity between the mutated and intact promoter lines. The significance of the effect of the mutation is given by the p-value based on the Bonferroni t-test with a level of confidence of 95% for each treatment. The mutation has a significant effect in NaCl responsiveness and near significant effect in MeJA responsiveness.

Treatment	Promoter version	ratio	p-value
MeJA	intact 5'UTR	1.454	**0.083**
	Mutated suORF	0.904	
Salt	intact 5'UTR	1.612	**0.0091**
	Mutated suORF	0.972	

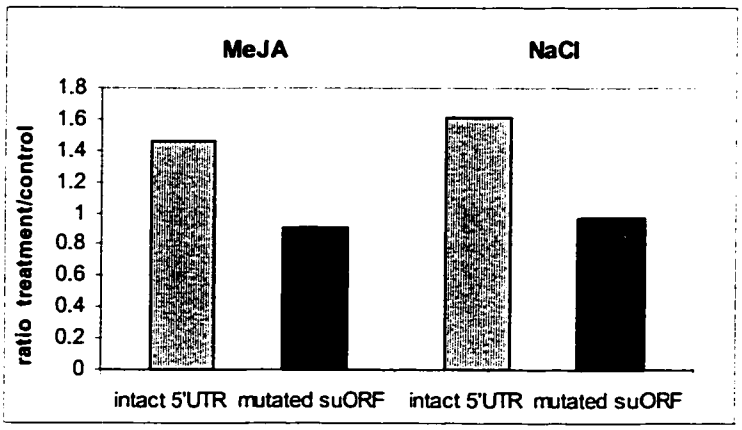

Figure 10. The ratio of GUS activity between treated and control plants comparing transgenic lines with intact 5'UTR and mutated suORF constructs at 24 hrs. The ratios are a measure of induction of activity in response to the two treatments in transgenic plants carrying the intact promoter plus 5'UTR and plants with a mutated suORF. Both ratios for the mutated suORF are close to one, the ratios of the intact 5'UTR show an increase of 45% and 61% for the MeJA and NaCl treatment respectively.

Esi47 5'UTR sequence:

AGAGAGCTTCGTGTCTTTCAACTTCTCTTGGGTTGGTTTTGGAGAACAAGGTTGAGCAGTTGGTTGGTCGG
TTGTTGGCTTGAAAGATCTTGCGGTTTCTGTCTGGCTGATCGTCTCCATGGGTGTCTGTTCTGGTCCTGGT
GGCTTCCTTTCGTCGACCGTCCTTGTTTAGATTGGTGCGTCTTTGCATTGGTAATTGATTGATCCAAAGTA
GGATCTCATCTTTATTTGAAAAGTGCCAATCTCTTTTGCCTCTTCTGAAACCCCTTTAGTTTTCTTCTTTT
GATGCCTTCAAGGTTTCTCTCTCTCTCTCTCTCTCTCTCTCTCCTTCTGGTTGTAGAATAATGGCTCTTTT
CCGCTGCTTTCTGCGCCTAGTCTTCTTCACAATCAGTGCTTCCAAGATCCGAAAGCTTGCCTTTACCGCCG
TCTGAAACTGATTACTATAGTAGCTGAAAGGCTGCGCCTTTGATGCTGTCATGTTATTGCAG**AGCAGGAGG**
GTCAAAGCAGAAGCGCGATG

F8A24.12 5'UTR sequence:

TAGCTTCTCAAATCCTCGATCTCTTTTGTATCGTGATTCATTAACCACTTCATGATAATTATAATCTTCCT
TCTCTTCTGACGATTGAATTACATCTTCTTCTGATAGCTGTTCTGATTTTTTTCTGTATACAAAGTAAATT
CAAAGGTGAGAGTTGTGTCATCTTTTGCTTTTTATATACTTGTTCAATAAGTCAAAGTTCTTTATGTTGCT
GTTGGGTTTTAGTTTATTGGAATCAACTTGCGAAATTTGATTACTTTTAGCTTCGAGATCCCTTCTATTAC
TCACTGGAACTTAGGGTTTGTGTTACTTGGAAAAGAATTTGGATCTGTTGGGTCTAAAGGCCTTCCTCTCT
GTTAAGTGGTTTTGTACTTGGGGGAAGCCTTAACTTTGGAAATGCTTGTACTTTGCAGATTAGTTGTTCGA
TAATATGCTTCAATGGTTTTGATTTTGTGGTTCGGTATATGTTATATGATCATTATCAG**TGTTTTATTGGT**
GTCCTGCAGTGGACAAACATCGGTATTTTAATCTCCTCTCGATAATG

Figure 11. The 5'UTR of the *Esi47* and *F8A24.12* genes contain one uORF and one intron. The bold letters represent the exonic portion of the region and the light letters are the intronic region. The suORFs are boxed and highlighted. The untranslated sequences end with the boxed ATG start codon of the major ORFs.

3.7. Non-analyzed transgenic lines

Other mutated versions of the *F8A24.12* promoter fused to the GUS reporter gene were used for *A. tumefaciens*-mediated T-DNA transfer. Twelve T0 plants were transformed with the *F8A24.12* promoter with the intron deleted from the 5'UTR. Three T0 independent transgenic lines were obtained and the segregation ratio revealed that one line had a single insertion in its genome and could be used for future analysis. Thirty other T0 plants were treated with *A. tumefaciens* carrying the construct containing the mutated suORF and with the first intron deleted. The seeds were not screened for positive transformants.

Also, the investigation of the promoters of two other *Esi47* homologue genes *F12E4.50* and *T7F6.28* using the GUS reporter gene was initiated. Twenty-four plants were used for *A. tumefaciens*-mediated T-DNA transfer for each of the two promoter constructs. The T1 seed was not screened for positive transformants.

CHAPTER 4. DISCUSSION

4.1. *Arabidopsis thaliana*, a general view of a plant genome

The *Arabidopsis* genome project (Arabidopsis Genome Initiative, 2000) gave a global view of the relative importance of protein kinases. Over 25,000 genes were found after the DNA sequencing analysis of the entire genome and 860 genes were found to have a ser/thr kinase catalytic domain. The protein kinases play a central role in the regulation of the signaling pathways and in cross talk between different pathways. The role of protein kinases is particularly well studied in yeast and in human systems in the regulation of cell division, but little is known about the major functions of the plant protein kinases.

4.2. The F8A24.12 protein kinase could weakly interact with targeted proteins

To characterize the F8A24.12 ser/thr protein kinase, the yeast two-hybrid system was used to look for protein-protein interactions with the F8A24.12 as bait to screen an *Arabidopsis* cDNA library from mature leaves and roots. Unfortunately, the screening was unfruitful and no confirmed candidate proteins were shown to interact with the F8A24.12 ser/thr kinase. D. Elias previously had used the same yeast two-hybrid system to screen the same cDNA library to look for proteins interacting with the *L. elongatum* Esi47 protein kinase (data not shown). Of the 5.47×10^5 yeast transformants screened, 21 putative candidates were isolated, among which 6 clones were confirmed to interact with Esi47. They all encoded an Oxygen-Evolving Enhancer Protein 1 precursor (OEE1) from phototosystem II. In this study, although 3.7×10^5 colonies were screened no

interactions were evident. This may be due to problems that appeared at the experimental level. The library reamplification used an unusually long growth period, overnight in liquid culture and represents the second amplification in our laboratory. This may have diminished the diversity of the library. To confirm that any candidate from the library could interact with the bait protein, the number of screened yeast tranformants should be higher than what was done in this experiment. The original cDNA library contained $1.9x10^6$ independent clones, which is substantially larger than the number of clones tested in this experiment. Also it is possible that F8A24.12 interaction with a target protein is weaker than that of Esi47 or that the level of transcription of the targeted protein is very low and its cDNA is represented as a small proportion of the library. It would be good to test for F8A24.12-OEE1 interaction to confirm the Esi47 OEE1 interaction as Esi47 and F8A24.12 are thought to be homologues.

4.3. *F8A24.12* gene expression is mostly localized in vascular tissues and in actively dividing tissues

A. tumefaciens-mediated T-DNA transfer into the plant genome allows the production of transgenic lines and an *in vivo* system for gene promoter analysis to examine the pattern of expression of the corresponding gene through a reporter gene. This approach can give a more precise tissue specific localization of gene expression than Northern blot analysis. The GUS histochemical assays on the transgenic *Arabidopsis* lines expressing the *F8A24.12* promoter-GUS fusion demonstrated the specificity of the localization of *F8A24.12* gene expression. *F8A24.12* expression was observed at every stage of development that was assayed, namely in the embryo of mature seed,

germinating seed, seedlings and flowering plant. The GUS localization was more precisely observed in the root tip of the embryo of mature and germinating seeds (Figures 2B and 2A), but also at the vegetative and flowering stage where the GUS expression always was found to be highly expressed in the root tips, particularly in the root meristematic zone and in the elongation zone but not in the cells constituting the root cap (Figures 2D and 2E). After a longer incubation of the seedling in the GUS staining buffer, the vascular tissues revealed expression in the roots and the leaves (Figure 2C). At flowering, expression was observed in the cells of sepals and in the stigmatic tissues of the pistil for mature and immature pistils (Figure 2G). During seed development, the stigmatic cells at the tip of the silique, derived from the pistil always showed visible GUS expression but at a lower level (Figure 2H). Furthermore, GUS expression appears strongly in the abscission zone of the petals and sepals at the base of the silique (Figure 2H). This expression persists much beyond the time of petal and sepal abscission. Specific staining also appears at the surface of contact between the trichome and the leaf that can be considered as an abscission zone too (Figure 2I). Abscission occurs in many species during plant development or in response to tissue damage and stress when entire organs such as leaves, flowers and fruit are shed, though in *Arabidopsis* the shedding of petals and sepals are one of the few abscission events to take place. The phytohormone ethylene plays a major role in abscission (Eyal *et al.*, 1993). The *ACS2* gene that encodes the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which is part of the rate-limiting step in ethylene biosynthesis (Kende *et al.*, 1993) has a strong level of expression in the petals and sepals and in the flower abscission zone (Liang and Theologis, unpublished), similar to what was observed for

F8A24.12, thus could implicate the *F8A24.12* gene in the ethylene response in abscission. The physiology of the stigma, composed of outer papillae, is similar to an abscission zone in that the plant cell wall is degraded to produce canals through which the pollen tubes grow (Hong *et al.*, 2000). Ethylene also modulates plant defense response genes such as *PR-1*, which like many defense related genes including the antimicrobial enzyme, chitinase ß-1-3 glucanase also is expressed specifically in the abscission zone (del Campillo *et al.*, 1992) and mature pistils (Harikrishna *et al.*, 1996). Moreover, ethylene induces enzymes such as polygalacturonase (PG) that degrade the pectin-rich middle lamellae between cells (del Campillo *et al.*, 1990) in the abscission zone. Several tomato genes encoding PGs, *TAPGs 1*, *2*, and *4*, were found to be expressed in abscission zones of the leaves and flowers and in the pistils. The function of each PG and their timing of expression is different, *TAPG 4* is expressed much earlier during leaf and flower abscission than *TAPG 1* and *2* (Kalaitzis *et al.*, 1997). The study of the promoters of the three PGs expressing the GUS reporter gene revealed that *TAPGs 1*, *2* and *4* expression is localized in the upper one-third of the pistil including the stigma and the style in fully opened flowers (Hong *et al.*, 2000). Another member of the PG gene family *TPG7* was shown to have a similar pattern of expression except that it was expressed in both mature and immature pistils (Hong and Tucker, 2000). The pattern of expression of the gene *F8A24.12* is similar to that of *TPG7* and points to a possible role of the protein kinase in cell separation. The cell separation zone of the stigma and the style do not have mechanical barriers and can be attacked by pathogens. This supports the idea that PGs play a role in plant defense and the accumulation of their products of degradation, oligogalacturonides, may produce a physical barrier to protect against pathogen attacks

(Kalaitzis *et al.*, 1997). Several roles can be attributed to the F8A24.12 ser/thr kinase and may be dependent on the developmental stage of the plant and environmental changes. It possibly could be part of a signaling pathway involved in plant response to wounding or plant defense where F8A24.12 would regulate directly or indirectly specific wound- and defense-induced genes. As the F8A24.12 protein kinase is localized in the cells of the meristematic and elongation zone of the roots, in the cells of the abscission zone of the sepals and petals and also in the stigmatic tissues of the pistil, which are highly dividing areas perhaps F8A24.12 is involved in the mechanism of cell division.

4.4. *F8A24.12* is induced differentially by salt and MeJA

The gene *F8A24.12* was demonstrated by Northern blot analysis of salt-treated *Arabidopsis* to have a peak of gene induction in the roots after 6 hr treatment and a peak of induction in leaf tissue after 24 hr treatment. Unlike the regulation of *Esi47* in *L. elongatum*, *F8A24.12* did not show any induction in *Arabidopsis* after treatment with exogenously applied ABA (Shen *et al.*, 2001). Six transgenic lines 63, 61, 111, 15, 16 and 202 in which the GUS reporter gene is regulated by 2.05 kb of the *F8A24.12* promoter plus the 5'UTR region, were tested using the GUS histochemical assays and showed salt-induced GUS expression in lines 15 and 111 after 6 hr and 24 hr in the leaves (data not shown). A similar experiment with exogenously applied MeJA showed that GUS expression increased in lines 15, 111, 202 and 63 after 12 hr, 24 hr and 36 hr in the leaves but not after 6 hr (data not shown). These results showed a potential role of MeJA and more probably of salt on the regulation of *F8A24.12* gene. In addition, these results show a strong difference of GUS expression between the transgenic lines. Such

variation is thought to be due to the differences in the DNA surrounding the different sites of the T-DNA insertion in the plant genome.

Some of these results were confirmed by GUS fluorometric assays on the same six transgenic lines treated for 6, 12, 24 and 36 hr with salt or MeJA. The statistical analysis ANOVA taking into account treatment, transgenic line and time of treatment indicated that MeJA caused a small but significant induction of the *F8A24.12* promoter plus 5'UTR construct of 45% and 57% respectively after 24 hr and 36 hr of treatment (Tables 2 and 3). Measurements taken at the time points 6 and 12 hr showed induction of 48% and 44%, respectively, but the increases were only statistically significant at the 0.10 level. NaCl treatment also caused a significant induction of the expression construct of 61.2% after 24 hrs of treatment (Tables 6 and 7). The results of the salt treatment are in agreement with the Northern blot analysis of *F8A24.12* expression in roots and shoots of salt-treated hydroponically grown *Arabidopsis* (Shen *et al.*, 2001). In that work, a strong peak of salt induction was detected in roots after 6 hr treatment and a smaller peak of induction in the leaves after 24 hr treatment. In my experiments, the whole plant was tested with mixed roots and shoots. Shoots represent more than 80% of the plant mass thus the salt effect was mostly measured for the shoots which may explain the observed induction after 24 hr treatment but not after 6 hr treatment. The *F8A24.12* expression was detected by histochemical assays in the roots of treated and untreated plants and no salt induction was visually observed after treatment (data not shown). The plant growth conditions used in the work described here were very different from those of Shen *et al.* (2001). Here, plants were grown in Petri dishes with high humidity, the roots were

illuminated and the plants were younger than those used for the Northern analysis in Shen's work.

In this experiment, the difference of the level of expression and timing of gene induction of *F8A24.12* after salt stress or exogenously applied MeJA suggests that MeJA and NaCl may act in the same pathway and the later response to NaCl may be due to the accumulation of jasmonates (Moons *et al.*, 1997). The biosynthesis of oxylipins such as JA are derived from the catabolism of fatty acids and accumulate in the plant cell in response to genetically programmed and environmental signals or elicitors (Creelman and Mullet, 1997). During water deficit stress caused by salt, drought or cold, JA accumulates in response to mechanical perturbations such as turgor reduction (Creelman and Mullet, 1997). This reinforces the hypothesis that the *F8A24.12* salt stress induction occurs through JA signaling. To check this hypothesis, the level of MeJA in the plant should be measured after a salt treatment. In addition, the level of expression of *F8A24.12* could be measured after NaCl treatment in plants previously treated with cycloheximide (a JA inhibitor) and also in the JA insensitive *Arabidopsis* mutants jar1, jin1, jin4 and coi1 that are defective in JA-dependent response or in the JA *Arabidopsis* mutant opr3 defective in the 12-oxo-phytodienoate (OPDA) reductase required for JA biosynthesis (Creelman and Mullet, 1997). Little is known about the JIPs and their interactions within specific JA-dependent signalling pathways to which they belong. The screening for mutant plants that show normal growth in the presence of high JA levels allowed the isolation of the previously cited JA insensitive mutants and from their analysis, the determination of different JA functions in plant development and in response to biotic and abiotic stresses. JA modulates seed germination, root growth,

protein storage during vegetative development, fruit ripening and pollen viability. High JA levels are found in zones of cell division, young leaves, and reproductive structures. Moreover, JA regulates wounding, defense response to pathogen an insects, stress-related growth inhibition (Xie *et al.*, 1998) foliar senescence, foliar abscission and ethylene biosynthesis (Reymond and Farmer, 1998).

4.5. Putative functions of the F8A24.12 NAK protein kinase

Several functions can be imputed to the F8A24.12 protein kinase. The characteristic pattern of expression with the specific localization of the GUS reporter gene driven by the *F8A24.12* promoter supports the idea that the F8A24.12 protein kinase could play a role in JA-dependent signaling pathways such as the control of flowering, pollen development, fruit development, leaf senescence, cell differentiation, separation and division, abscission and also root growth inhibition (Staswick and Howell, 1992) during stress conditions (Ellis and Turner, 2002). The studies reported here implicate the F8A24.12 protein kinase in several JA-dependent signaling pathways and warrant further investigation into the possible role of *F8A24.12* in development and the stress response. As a component of the JA-dependent response, the F8A24.12 protein kinase would either lead directly to the activation of other downstream proteins by phosphorylation or indirectly to the induction of genes through activation of transcription factors. All hypotheses regarding the function of *F8A24.12* under JA-dependent induction should consider the effect of others phytohormones such as ethylene. Increasing evidence demonstrates that the effects of different phytohormones are interrelated and may act synergistically or antagonistically to regulate specific genes. Such interaction varies

among different plant species and stimuli. The biosynthesis of phytohormones and their accumulation in plant cells at a precise concentration regulates the specificity of the adapted response at the appropriate developmental stage or environmental change (Moons *et al.*, 1997).

To further elucidate F8A24.12 functions, other *Arabidopsis* NAK protein kinase family members have to be investigated. The analysis of *Arabidopsis* transgenic plants carrying the *F12E4.50* or *T7F6.28* promoter should be done soon. Moreover, transgenic plants overexpressing these three genes would allow one to see changes in expression of other genes and define the signaling pathways to which they belong.

4.6. The suORF, a regulatory mechanism for gene expression

The post-transcriptional regulation of gene expression includes factors that affect transcript stability. At the translational level, the regulation takes place from the passage of mRNA to the synthesized protein. The 5'UTR region has a central function in these steps of gene regulation. The sequencing of several eukaryotic organisms has brought attention to the presence of specific elements such as suORFs in the 5'UTR region of a few genes that would play a control role at the post-transcriptional and translational level (Vivela *et al.*, 1998). In yeast, the presence of an upstream ORF (uORF) in the 5'UTR region of a gene reduces its mRNA stability. This was demonstrated by integrating an uORF in the chloramphenicol acetyltransferase gene, which caused the rapid degradation of its message (Oliveira *et al.*, 1995). It also has been observed that stress-induced genes like the *Yap1* and *Yap2* genes encoding transcription factors involved in the resistance to metal ions, are differentially regulated by uORFs. Moreover, two uORFs, uORF1 and

uORF2, in the 5'UTR of the *Yap2* gene have different impacts on gene expression. The ribosomal machinery binds to the 5'-cap region of the mRNA and scans the 5'UTR region until it recognizes the first start codon (AUG). But, more than AUG recognition is required for translation initiation. Many other factors including the context sequence adjacent to the AUG, for example, a purine at the -3 postion (more particularly a A) from a start codon is favorable to the translation initiation (Joshi *et al.*, 1997). The context sequence at the 3' end of the uORF was found to be crucial at the level of regulation. The uORF2 has a higher G/C content right after the stop codon than does uORF1 and this promotes a more efficient termination of ribosomal scanning and thus inhibits translation initiation of the major ORF and accelerates the mRNA decay (Vivela *et al.*, 1998). In addition, the presence of secondary structures such as a stem-loop downstream of the uORF can block the translation of the gene (Vivela *et al.*, 1999). The *Arabidopsis* gene *ATB2*, which is downregulated by the signal molecule sucrose, has small ORFs in its 5'UTR region and the removal of these blocks the repressive effect of the sucrose on gene expression (Smeekens, 2000). The uORFs are often found in stress responsive genes that are specifically activated, thus they may act as inhibitors to translation under non-stressed conditions (Vivela *et al.*, 1999). Fluorometric assays on transgenic lines containing the GUS reporter gene driven by the *F8A24.12* promoter and 5'UTR with the mutated ATG of the suORF reported here showed that lines with the mutated suORF construct showed no significant difference between plants treated with NaCl or MeJA and control plants. The *F8A24.12* gene is the first case that suggests such regulation for salt stress or MeJA treatment. To establish whether the suORF regulates gene expression at the level of translation, it would be critical to compare the changes in mRNA levels

with those for the level of protein accumulation in response to stress and hormone treatment. A mutation in the ATG of the uORF of the *Esi47* gene abolished the repressive effect of this suORF and obliterated the ABA responsivness of this gene promoter plus 5'UTR region (Shen *et al.*, 2001). The data presented here for the *F8A24.12* gene showed a similar effect on gene expression. The loss of the suORF coincides with the loss of responsiveness to both salt and MeJA treatments. Though the general pattern is similar between mutations in the suORF of *Esi47* and *F8A24.12* the signaling molecules were quite different, ABA in one case and MeJA in the other case. *Esi47* and *F8A24.12* are homologues though they are found in very distantly related species. *Esi47* is from the monocot *L. elongatum* and the latter gene is from the dicot *Arabidopsis*. These data suggest that the regulatory role of suORFs is widespread and evolutionarily diverse. The *Arabidopsis* gene *F12E4.50* is a duplicate gene of *F8A24.12* that has a different pattern of expression than *F8A24.12*, and has been shown to be responsive to ABA treatment. The pattern of control of *F12E4.50* would be an important object of investigation for the regulation of the ABA response in *Arabidopsis*. It is not known if *F12E4.50* contains a suORF because a full length cDNA is not yet available.

The basal level of GUS expression in transgenic lines with the mutated suORF was higher than that in lines with intact 5'UTRs. This was similar to expression levels that were measured with intact and mutated suORFs with *Esi47* in transient expression assays with corn callus (Shen *et al.* 2001). The similarity is striking, though it is not possible to conclude that the differences in basal levels of expression with intact and mutated F8A24.12 constructs was significant due to the relatively small number of transgenic lines evaluated. A higher basal level of expression in the lines with the mutated suORF

and the inducibility of the constructs with the intact suORF suggest that the regulation of *F8A24.12* mediated by the suORF is controlled by a repressor that associates with the suORF and that can be inactivated by the MeJa signaling pathway. There were 10 single insertion lines developed for intact suORF and 24 lines for the mutated suORF construct in the work described here. Thus, it would be exciting to expand this comparison to a larger population to investigate the basal level of expression in transgenic plants with the two constructs. The comparison between the two constructs also could be done with transient expression assays in plant cells transformed by electroporation in which very large sample sizes could be tested.

The regulation of gene expression that is mediated by the suORF of *F8A24.12* is an exciting area for continued research. A promising avenue for continued work would be to identify the binding proteins that might associate with the suORF, via biochemical purification or mutant screening. To proceed with these approaches it would be fruitful to develop a system in which the MeJa induction affects are more pronounced. Thus it would be important to investigate the effects of multiple copies of the suORF, and perhaps it would be fruitful to investigate the suORFs in the related protein kinase gene *T7F6.28*, or possibly in *F12E4.50*.

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