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***Esi47*, a Stress-Inducible Protein Kinase Gene from
Lophopyrum elongatum Is Involved in Plant Hormone Signaling**

Wei Shen

A Thesis

in

The Department

of

Biology

**Presented in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy at
Concordia University
Montreal, Quebec, Canada**

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ABSTRACT

Esi47, a Stress-Inducible Protein Kinase Gene from *Lophopyrum elongatum* Is Involved in Plant Hormone Signaling

Wei Shen, Ph.D.
Concordia University, 2000

The cDNA and genomic DNA clones for the salt stress and abscisic acid (ABA) inducible gene *Esi47* were isolated from the salt-tolerant tall wheatgrass species *Lophopyrum elongatum*. The *Esi47* gene encodes a protein kinase with highest amino acid sequence similarity to the NAK group of protein serine/threonine kinases. *Esi47* and the NAK group protein kinases have a central catalytic domain flanked by short amino and carboxyl terminal non-catalytic domains. The NAK group was named after the first gene isolated in this group, the *Arabidopsis thaliana* *NAK* gene (Hardie, 1999; Moran and Walker, 1993). However, none of the genes in this group has been implicated to be involved in plant signaling pathways except for the *Arabidopsis* gene *ARSK1*, which has only been shown to be inducible by salt stress and ABA in plant roots (Hwang and Goodman, 1995). The protein phosphorylation activity of the *Esi47* gene product was demonstrated in *Escherichia coli*. *Arabidopsis* plants transformed with *Esi47* did not show any enhanced salt stress tolerance. Approximately 60 *Arabidopsis* genes previously shown to be regulated by salt stress or ABA were evaluated for altered expression in the transgenic plants by northern analysis, but none were found to be affected by *Esi47* overexpression. However, transient expression of *Esi47* in barley aleurone suppressed the induction of the barley gene for α -amylase by the plant hormone gibberellin, which is an ABA antagonist. This indicates that the *Esi47* gene is involved in plant hormone signaling. Analysis of the primary structure of the *Esi47* gene revealed that it contains in

the 5'-untranslated region of its mRNA a small upstream open reading frame that has been demonstrated to mediate the ABA regulation of *Esi47* expression in an independent study. Three *Arabidopsis* homologs of *Esi47* were identified and different members of this clade of genes showed differential patterns of regulation by salt stress and ABA in *Arabidopsis* roots and leaves. At least one of the *Arabidopsis* homologs, *F8A24.12*, contains a small open reading frame in its 5'-untranslated region, indicating that the unusual regulatory mechanism identified in *Esi47* may be widely conserved. The identification of the *Arabidopsis* genes homologous to *Esi47* would facilitate further functional characterization of this type of gene in plant stress and hormone signaling pathways in a model plant species.

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ABBREVIATIONS

ABA, abscisic acid

ABI (abi), abscisic acid insensitive

ABRC, abscisic acid response complex

ABRE, abscisic acid responsive element

AP2, APETALA2

bHLH-ZIP, basic helix-loop-helix zipper

BLAST, Basic Local Alignment Search Tool

bZIP, basic leucine zipper

cADPR, cyclic ADP-ribose

CAM, Crassulacean acid metabolism

CaMV, Cauliflower Mosaic Virus

CDK, cyclin-dependant kinase

cDNA, complementary DNA

CDPK, Ca^{2+} /calmodulin-dependant protein kinase

CE, coupling element

CK1, casein kinase I

CK2, casein kinase II

CNA, catalytic subunit of calcineurin

CNB, regulatory subunit of calcineurin

DAG, diacyl glycerol

DRE, dehydration responsive element

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid

EGTA, ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid

Esi, early salt-stress induced

EST, expressed sequence tag

GA, gibberellin (gibberellic acid)

GAP, glyceraldehyde 3-phosphate dehydrogenase

GBF, G-box-binding factor

GSA, glutamate- γ -semialdehyde

GST, glutathione-S-transferase

GUS, β -glucuronidase

IP₃, inositol 1,4,5-triphosphate

IPTG, isopropyl β -D-thiogalactopyranoside

Lea, late embryogenesis abundant

LRR, leucine-rich repeat

MAP, mitogen-activated protein

MAPK, mitogen-activated protein kinase

MAPKK, mitogen-activated protein kinase kinase

MAPKKK, mitogen-activated protein kinase kinase kinase

MBP, myelin basic protein

MDH, malate dehydrogenase

ME, malic enzyme

MIP, major intrinsic protein

MOPS, 3-(N-morpholino)propanesulfonic acid

MS, Murashige and Skoog

NAD(P), nicotinamide adenine dinucleotide (phosphate)

ORF, open reading frame

P5C, Δ^1 -pyrroline-5-carboxylate

P5CR, Δ^1 -pyrroline-5-carboxylate reductase

P5CS, Δ^1 -pyrroline-5-carboxylate synthase

PA, phosphatidic acid

PAGE, polyacrylamide gel electrophoresis

PCR, polymerase chain reaction

PEP, phosphoenopyruvate

PEPC, phosphoenopyruvate carboxylase

PGM, phosphoglyceromutase

PI-PLC, phosphoinositide-specific phospholipase C

PLC, phospholipase C

PLD, phospholipase D

PP2B, type 2B phosphatase

PP2C, type 2C phosphatase

PPDK, pyruvate orthophosphate dikinase

PTGS, posttranscriptional gene silencing

RLK, receptor-like kinase

SDS, sodium dodecyl sulfate

SNRK, SNF1-related protein kinase

uORF, upstream open reading frame

UTR, untranslated region

PART I. INTRODUCTION

Both plants and animals face a common challenge, that is, the ever-changing environment. Environmental conditions are not always favorable for the growth and survival of living organisms. Plants are immobile and cannot avoid adverse changes in local environments, such as periods of high or low temperatures, drought or increasing salinity, and heavy metals. The fact that many plant species or genotypes are able to survive unfavorable environmental changes means they possess certain tolerance mechanisms acquired through evolution.

How plants respond and tolerate environmental stresses is a fundamental question in plant biology. Their adaptation to adverse conditions involves genetic, biochemical, physiological and even morphological changes. Stress tolerance is also extremely important for agriculture. Due to the ongoing nearly explosive increase of the world population, the demand for food, mostly produced or originating from land-grown crops, is ever increasing. Meanwhile, the acreage of arable lands is shrinking due to the improper maintenance of these lands as well as the consequence of expanding land use for industry, human accommodation and other human activities. Efforts are being made to raise crop yields in the lands affected by unfavorable conditions and to expand suitable arable lands without clearing more forests. Genetic improvement of crop species is done by traditional breeding and more recently by genetic modification through gene transfer by modern recombinant DNA techniques. For this purpose, an extensive understanding of the biochemical and physiological mechanisms of stress response and tolerance and their genetic determinants is essential.

Soil salinity, which is one of the serious problems in agriculture throughout the world, affects about one-third of irrigated cropland. The cause of this problem could be insufficient precipitation and irrigation with poor quality water resulting in the accumulation of ions retained by soil. Salinity in soil inhibits plant growth, reduces crop yield, restricts the choice of species that can be grown on affected soil, and under extreme conditions can render land unsuitable for cultivation (Boyer, 1982; Epstein et al., 1980).

Salt stress affects plant growth in two ways (Serrano and Gaxiola, 1994). First, a number of ions, such as Na^+ , are toxic to many biochemical processes when they accumulate in plant tissues. They also can disturb the transportation and homeostasis of other ions, such as K^+ and Ca^{2+} , which are important for plant nutrition and cellular functions (Binzel et al., 1988). In addition, low osmotic potential resulting from high salt concentration in the soil causes cellular water loss, which in turn would reduce cell turgor, increase intracellular concentrations of solutes, disrupt membrane integrity and denature proteins. Severe water loss also inhibits photosynthesis and the resulting excess of excitation energy can produce reactive oxygen intermediates that would also damage membranes and enzymes (Smirnoff, 1993). Reduced water potential also often is caused by stresses like drought and low or freezing temperatures (for reviews see Bohnert et al., 1995; Bray, 1997; Ingram and Bartels, 1995). We can expect these stresses to elicit some similar responses from plants. In fact, salt stress and other low water potential stresses all induce elevated levels of the plant hormone abscisic acid (ABA). A large number of genes are upregulated by all of these stresses (for reviews, see Bohnert et al., 1995; Bray, 1997; Ingram and Bartels, 1995; Shinozaki and Yamaguchi-Shinozaki, 1997). In the

following literature review. progresses in studying the plant response to drought and low temperatures will be included along with those concerning salt stress.

1. PHYSIOLOGY OF SALT STRESS RESPONSE AND TOLERANCE IN PLANTS

The plant response to salt stress and related water deficit stresses is complex and involves many genetic and biochemical processes which in turn lead to certain adaptive cellular or physiological changes that contribute to adaptation or tolerance. The degree of stress tolerance varies greatly among different species and even among different genotypes within a particular species. Plant species can be categorized into glycophytes, facultative halophytes and halophytes according to their salinity sensitivity and their habitats. Glycophytes are very sensitive to salt stress and cannot survive and grow in the presence of high salinity; facultative halophytes usually grow in low-salinity conditions but can tolerate and survive occasional high-salinity stress; while halophytes have high-salinity environments as their normal habitats. Such variations in salt stress tolerance may be due to the difference in the ability of a plant to undertake certain essential adaptive responses. So far a number of biochemical and cellular processes have been implicated as playing roles in salt stress tolerance (for reviews, see Bohnert et al., 1995; Ingram and Bartels, 1996). Examples of these changes include selective uptake of non-toxic ions such as K^+ to balance osmotic pressure across plasma membranes, sequestration of Na^+ in vacuoles, redistribution of Na^+ to older tissues, accumulation of compatible osmolytes as osmoprotectants, regulation of stomatal closure, scavenging of reactive oxidants, and activation of molecular chaperones to repair denatured

macromolecules. These responses are likely to be regulated by signaling systems involving the second messenger Ca^{2+} and the plant hormone ABA whose levels are often found to be elevated by water deficit stresses.

1.1. Transportation and Distribution of Na^+ in Response to Salt Stress

Under salt stress conditions, plant cells encounter high osmotic pressure caused by the high external ion concentrations. In a common strategy of osmotic adjustment adopted by most plant species, plants increase ion uptake from the external environment and synthesize and accumulate compatible organic solutes to balance the water potentials across the plasma membrane to minimize water loss. The toxicity of the high cytosolic concentration of ions such as Na^+ is attenuated by compartmentation of these ions into the vacuole. In a way, the ability of a plant species to reach the balance of water potential by osmotic adjustment without accumulation of Na^+ in the cytosol determines its level of salt-tolerance. Therefore, the ion transportation across the plasma and tonoplast membranes is important for the adaptation to salt stress.

Plant roots may selectively increase K^+ uptake in response to salt stress. Ion homeostasis is essential for plant growth (reviewed by Niu et al., 1995). K^+ is one of the most important nutrients for plants whereas Na^+ is toxic to cells. K^+ also serves as an osmoticum. Plant roots keep a high intracellular K^+ concentration of 100 to 200 mM even though the external K^+ concentration is in the micromolar range in most soils; in contrast the Na^+ concentration inside a root cell is usually 1 to 10 mM (Binzel et al., 1988). Na^+ is a competitor for K^+ uptake and it is likely that they share mechanisms in transportation across the plasma membrane. Two mechanisms have been suggested for

the K^+ uptake in plant roots: a high affinity one and a low affinity one. K^+ and Na^+ transport across root cell plasma membranes is thought to be mediated by ion cotransporters and channels (Niu et al., 1995).

A number of genes responsible for the K^+ uptake in root cells have been cloned and their gene products characterized. The K^+ - Na^+ symporter HKT1 isolated from wheat has a high affinity for K^+ and is responsible for K^+ uptake at low K^+ concentrations (Rubio et al., 1995). The inward-rectifying K^+ channel AKT1 in *Arabidopsis thaliana* is able to mediate K^+ uptake at both low and high K^+ concentrations (Hirsch et al., 1998). In addition, AtKUP1 is a dual-affinity K^+ transporter and its activity of both low and high affinity K^+ uptake can be inhibited by Na^+ at concentrations higher than 5 mM (Fu and Luan, 1998; Kim et al., 1998).

High external Na^+ concentration blocks K^+ uptake and initiates low-affinity Na^+ uptake by HKT1. This function of Na^+ uptake may play a role in the Na^+ toxicity in plants, since mutations in HKT1 confer salt tolerance in yeast cells (Rubio et al., 1995). While HKT1 is not Ca^{2+} -sensitive in Na^+ uptake, a recent report described the observation of a partially Ca^{2+} -inhibited, weakly voltage-dependent, nonselective monovalent cation channel activity mediating toxic Na^+ influx through the plasma membrane of wheat root cells, thus accounting for the Ca^{2+} -inhibition-sensitive portion of the Na^+ influx in wheat roots (Davenport and Tester, 2000).

Na^+ efflux across the plasma membrane of plant root cells is probably through Na^+ - H^+ antiporters (Niu et al., 1995). Although the Na^+ transport is yet to be clearly defined, a high K^+/Na^+ ratio is nevertheless essential for plant cells. Mutation studies in *Arabidopsis* have suggested that K^+ uptake is important for plant cells to tolerate salt

stress. The *sos1*, *sos2* and *sos3* mutants of *Arabidopsis* are deficient in K^+ uptake at low K^+ concentrations and hypersensitive to salt stress (Liu and Zhu, 1997; Wu et al., 1996; Zhu et al., 1998). The degrees of salt-tolerance of these mutants correlate with the K^+ but not the Na^+ contents within cells (Zhu et al., 1998). The *sos3* mutant needs increased Ca^{2+} for proper K^+ nutrition and K^+/Na^+ selectivity (Liu and Zhu, 1997). In fact the *SOS3* gene encodes a Ca^{2+} -binding protein that interacts with and activates a protein kinase encoded by the *SOS2* gene (Halfter et al., 2000; Liu et al., 2000; Liu and Zhu, 1998). Therefore, the *SOS3* gene provides a link of the three cations, K^+ , Na^+ and Ca^{2+} , in K^+ nutrition and Na^+ tolerance. A recent report showed that the *SOS1* gene product has similarity to bacterial or fungal Na^+-H^+ antiporters (Shi et al., 2000). Moreover, this gene is induced by salt treatment and the observation that such upregulation is abated in the *sos3* or *sos2* mutant indicates *SOS1* is controlled by the *SOS3/SOS2* regulatory pathway. There is also evidence showing that plant cell cultures maintain a high K^+/Na^+ ratio in adaptation to salt stress. For example, salt-adapted tobacco cells have elevated capacity for K^+ uptake compared to the wild-type cells and salt does not inhibit K^+ uptake in the adapted cells (Watad et al., 1991).

Plant root cells may adopt another mechanism involving K^+ transport in response to water deficit stresses. K^+ as an essential nutrient whose uptake from the soil is followed by transport to other parts of the plant through xylem. Stele is the location of exit for the radial transport of ions from the epidermis to the xylem. The stelar cell outward-rectifying K^+ channel SKOR has been characterized to function in K^+ export to shoots (Gaymard et al., 1998). ABA, as a plant hormone involved in water stress responses, was found to be able to significantly reduce the K^+ outward channel activity in

maize stelar cells (Roberts, 1998). This was supported by the observation that the expression of the gene for the SKOR K^+ channel in *Arabidopsis* was strongly inhibited by ABA (Gaymard et al., 1998). These data indicate that reduction of K^+ export might be a mechanism of adaptation of plants during water stress to maintain high K^+ concentration in root cells.

Exclusion of Na^+ from cytoplasm through plasma membrane. To reduce Na^+ concentration in the cytoplasm, Na^+ efflux from the plant is necessary in environments of high salinity. One way is to export Na^+ from root cells through the plasma membrane. This may be mediated by the plasma membrane Na^+-H^+ antiporters driven by the proton gradient as a result of the increased plasma membrane H^+ -ATPase activity (for review, see Niu et al., 1995). Increased activities of Na^+-H^+ antiporters and H^+ -ATPases were observed in several cases. Elevated gene expression upon salt stress is probably responsible for increased H^+ -ATPase activity. The H^+ -ATPase gene expression or activity is positively correlated with the degree of salt tolerance observed in comparisons of glycophytes and halophytes. The plasma membrane H^+ -ATPase inhibitor sodium vanadate stimulates net Na^+ uptake in root cells of maize. The effect of vanadate apparently reflects reduced Na^+ efflux.

Vacuolar compartmentation of ions. Both halophytes and glycophytes reduce cytosol salt content by selectively sequestering ions in the vacuole. Salt-adapted plant cells are able to compartmentalize Na^+ and Cl^- into the vacuole, thus reducing the ionic toxicity of Na^+ in the cytosol but still maintaining intracellular osmotic pressure (Binzel et al., 1988). Although it is not well understood how Cl^- enters the vacuole, vacuolar Na^+-H^+ antiporters may be responsible for transporting Na^+ into the vacuole.

Overexpression of *AtNHX1*, the *Arabidopsis* homolog of the yeast vacuolar Na^+/H^+ antiporter gene *Nhx1*, in *Arabidopsis* plants increased their salt tolerance (Apse et al., 1999). These transgenic plants showed higher vacuolar antiporter activity and Na^+ contents than the wild-type plants. The H^+ gradient used to drive the Na^+ efflux into vacuoles is generated by the vacuolar H^+ -ATPase, and probably also by the H^+ -translocating inorganic pyrophosphatase (for review, see Niu et al., 1995). Increased transcript levels of the vacuolar H^+ -ATPase subunits A, B and c were observed in roots and leaves of the facultative halophyte *Mesembryanthemum crystallinum* upon salt treatment (L6w et al., 1996). These data support the importance of vacuolar Na^+ sequestration in the adaptation of plants to saline conditions.

Redistribution of Na^+ into old leaves. Some monocot cereals, for example, rice (Yeo et al., 1985), are found to have high Na^+ concentration in old leaves of salt-stressed plants relative to levels in young leaves. It is not clear if this phenomenon reflects an adaptive mechanism since a similar Na^+ distribution was also observed in plants of both the salt sensitive Chinese Spring wheat and the salt tolerant *Lophopyrum elongatum* × Chinese Spring wheat amphiploid (Colmer et al., 1995).

1.2. Regulated Water Permeability of Plasma Membrane and Tonoplast

The control of water loss from the cytoplasm, the maintenance of cell turgor and the prevention of plasmolysis are critical for plants to survive salt stress and water deficit stress. Biological membranes can have a much higher degree of water permeability than what can be explained by simple diffusion. High water transportation has been shown to be mediated by cell membrane water channels, called aquaporins, which are widely

distributed among plants and animals as a subgroup of the membrane-located major intrinsic proteins (MIP. for reviews. see Chrispeels and Maurel. 1994; Maurel. 1997). The plant aquaporins have a molecular mass of about 28 kD with six transmembrane domains and both amino and carboxyl termini facing the cytoplasm. The plasma membrane aquaporins and the tonoplast aquaporins in plants are distinct from each other in their amino acid sequences and form two clades in their phylogenetic tree (Yamada et al.. 1995).

Aquaporins facilitate water flow. symplasmic or transcellular. through nonvascular plant tissues in addition to the apoplastic water flow. On the other hand. they are involved in cell volume adjustment and play a role in osmoregulation (Maurel. 1997). Since most of the space of a plant cell is occupied by the vacuole. the extremely high permeability of the tonoplast conferred by tonoplast aquaporins allows instant water flow into or out of the vacuole to buffer osmotic fluctuations in the cytoplasm upon changes in apoplastic water potentials. therefore. avoiding the collapse or swelling of the cytoplasm (Maurel et al.. 1993). The plasma membrane water current may be reduced by inactivation of aquaporins during water deficit stress to avoid water loss. Phosphorylation of aquaporins is thought to activate their water transport activity (Johansson et al.. 1998). Phosphorylation levels of the spinach plasma membrane aquaporins PM28A and PM28B decrease in response to reduced water potentials although the transcript levels of the genes remain unchanged (Johansson et al.. 1996. 1998). The phosphorylation of PM28A at its carboxyl terminus requires Ca^{2+} and is probably catalyzed by a protein kinase C. Such regulation allows quick activation and inactivation of the aquaporin. Other plasma membrane aquaporins are regulated through

transcript levels, for example, the mRNA levels of the ice plant *M. crystallinum* plasma membrane aquaporin genes *MipA* and *MipC* decrease significantly in both leaves and roots when stressed with salt (Yamada et al., 1995). In contrast, the *Arabidopsis* gene *Rd28* shows high similarity to plant aquaporin genes but its expression is upregulated by drought (Yamaguchi-Shinozaki et al., 1992). Any adaptive role of *Rd28* by its way of regulating water transport in response to stress remains to be determined by further investigation.

1.3. Accumulation of Compatible Organic Solutes in Cytoplasm as Osmoprotectants

One aspect of osmotic adjustment in response to salt stress is the accumulation of compatible organic solutes in the cytoplasm of plant cells which raises intracellular osmotic pressure and prevent water loss. The concentration of such solutes can exceed 1 M but does not interfere with cellular functions. Plant cells accumulate compatible solutes in response to all osmotic stresses, such as drought and low temperatures, as well as to salt stress. In addition, at high concentrations some of these solutes can stabilize proteins and membranes when cells experience low water potential, and therefore, function as osmoprotectants in adaptation to stresses. Common compatible organic solutes are categorized into three groups: amino acids, betaines and related compounds, and sugar alcohols (for reviews, see Delauney and Verma, 1993; McNeil, 1999; Stoop, 1996). Different plant species may accumulate a particular kind of compatible organic solute in response to stress. Enhanced activities of the enzymes involved in the biosynthesis pathways for these solutes and elevated expression of the genes for these

enzymes are often associated with the stress response of a plant species. Overexpression of the related genes in transgenic plants can confer enhanced stress tolerance in such plants (Kavi Kishor, 1995).

Amino acids. Amino acids, represented by proline, are the most widely distributed osmoprotectants in plants as well as in other classes of organisms such as bacteria. In addition to its role in osmotic adjustment, proline may also function to stabilize proteins and subcellular structures (for review, see Delayney and Verma, 1993). The increase of proline concentration in plant cells upon stress is due to the increased synthesis of proline. In plants proline is synthesized through the intermediate glutamate- γ -semialdehyde (GSA). GSA is spontaneously converted to Δ^1 -pyrroline-5-carboxylate (P5C), which is then reduced to proline by P5C reductase (P5CR). During normal growth conditions, plants synthesize proline from ornithine by ornithine- δ -aminotransferase. When plants are stressed the ornithine pathway is repressed and the accumulation of proline is the result of the increased conversion from glutamic acid to GSA by P5C synthase (P5CS). P5CS is a bifunctional enzyme: the γ -glutamyl kinase activity phosphorylates glutamic acid to become glutamyl- γ -phosphate which is then converted to GSA by the GSA dehydrogenase activity.

Although the P5CR activity increases when plants are treated with salt, this step is not rate limiting for proline biosynthesis. On the other hand, P5CS is subjected to feedback inhibition by proline, the product of the pathway. There is some indication that stress tolerance conferred by proline accumulation in some plants might be due to the reduced sensitivity of P5CS to proline feedback inhibition. Moreover, environmental stresses and ABA induce the expression of the gene for P5CS as well as the production of

proline (Yoshida et al., 1995). These data indicate that P5CS is the rate-limiting step in proline synthesis when plants are placed under stress conditions. Two P5CS genes, *AtP5CS1* and *AtP5CS2*, have been identified in *Arabidopsis* (Strizhov et al., 1997; Yoshida et al., 1995). Both genes are induced by osmotic stresses and ABA but have differential tissue specificity in expression.

In some plant species, accumulation of proline does not always correlate with stress tolerance, which means that proline accumulation is not sufficient for stress tolerance. In many other plant species, however, evidence has been accumulating to demonstrate that proline does have an adaptive role in response to stress although it is still not clear if it is due to its role in osmotic adjustment or its function of stabilizing proteins and subcellular structures. The *P5CS* gene was overexpressed in transgenic tobacco plants in which proline production was increased and osmotolerance was enhanced (Kavi Kishor et al., 1995). Expression of an antisense gene of *P5CS* in *Arabidopsis* reduced proline production and osmotic stress tolerance, which could be compensated for by addition of external L-proline but not by D-proline (Nanjo et al., 1999). Additional support for the role of proline in salt stress tolerance came from the overexpression of a mutant form of P5CS which has lost its susceptibility to feedback inhibition by proline. Expression of this gene in transgenic tobacco plants resulted in higher cellular proline concentrations under both normal and high salinity conditions and enhanced salt tolerance (Hong et al., 2000).

Betaines and related compounds. Betaines are amino acid derivatives in which the nitrogen atom is fully methylated. Some of these quaternary ammonium compounds commonly found in plants are glycinebetaine, prolinebetaine and β -alaninebetaine.

Choline-*O*-sulfate and 3-dimethylsulfoniopropionate are not betaine compounds but are structurally and functionally similar to betaines. The concentration of certain betaines increases when plants are under osmotic stresses and low temperature stress due to the elevated expression of the genes involved in the biosynthesis of these compounds (for review, see McNeil et al., 1999). Accumulation of betaines, which can be over 1 M, occurs in the cytoplasm and chloroplasts but not in vacuoles.

The function of betaines and related compounds in osmoprotection is not fully understood but a theory has been proposed. Exclusion of these compounds from the water layer in contact with protein surfaces may result in the thermodynamically favored structures for proteins, most probably their native, folded conformations (Timasheff, 1992). This is in contrast to the effects of solutes such as NaCl, which would interact directly with protein surfaces and cause protein unfolding and denaturation. Exclusion from the protein surface may also explain the cryoprotectant and heat-protectant properties of these compounds (Winzor et al., 1992).

In plants studied so far, glycinebetaine is synthesized from choline, which is oxidized to betaine aldehyde by choline monooxygenase. Betaine aldehyde is then oxidized to become glycinebetaine by betaine aldehyde dehydrogenase. Accumulation of glycinebetaine has been shown to have an adaptive role in stress tolerance in some plant species. Comparison of the salt-sensitive wheat and the amphiploid from a cross of wheat and the salt-tolerant wheatgrass *L. elongatum* provides genetic indication that glycinebetaine accumulation may contribute to salt tolerance since the amphiploid accumulates significantly more glycinebetaine in young leaves than wheat in response to salt stress (Colmer et al., 1995). Maize plants with a single locus mutation, which

abolishes glycinebetaine synthesis, are less salt stress tolerant than wild-type plants (Saneoka et al., 1995). Transgenic expression of the genes for enzymes involved in glycinebetaine synthesis in plant species that normally do not accumulate glycinebetaine increases stress tolerance of these plants. For example, *Arabidopsis* plants transformed with the *codA* gene for choline oxidase from the bacterium *Arthrobacter globiformis*, which converts choline to glycinebetaine, accumulate glycinebetaine and are more tolerant to salt and cold stresses (Hayashi et al., 1997).

Sugar alcohols. Sugar alcohols are widely distributed among various groups of organisms including bacteria, plants, insects, and even mammals. Some sugar alcohols, such as mannitol, are major photosynthetic products in many plant species and their accumulation may function as energy storage. On the other hand, these compounds may also contribute to osmotic stress response and tolerance as compatible solutes or osmoprotectants (for review, see Stoop et al., 1996). In salt-stressed celery plants photosynthetic sucrose is suppressed, which leads to a massive shift in partitioning of fixed carbon to mannitol (Everard et al., 1994). Expression of the bacterial gene *mtlD*, which encodes mannitol-1-phosphate dehydrogenase that is able to convert mannose to mannitol, in transgenic tobacco plants resulted in the production and accumulation of mannitol (Tarczynski et al., 1992). Transformed plants had better growth properties than the wild-type plants under saline conditions (Tarczynski et al., 1993).

1.4. Stomatal Closure in Response to Water Loss Stresses

Stomata are microscopic pores distributed on the surface of aerial parts of plants. Stomatal pores are surrounded by a pair of guard cells. Guard cell turgor controls the

opening or closure of the stomatal aperture. Opening of stomatal aperture allows plants to take up CO_2 for photosynthesis. On the other hand, the stomatal pore is the passage for water transpiration; therefore, its regulation is critical for plant water status. It has been observed that low water potentials caused by osmotic stresses such as drought would lead to closure of the stomatal aperture to prevent further water loss. Light, CO_2 , humidity, temperature, plant water status and hormones all serve as signals for the opening or closure of the stomata (for review, see Assmann and Shimazaki, 1999).

Stomatal closure occurs when a reduced concentration of solutes in guard cells causes water loss and deflating of the cell that changes cell shape and reduces the size of the stomatal aperture. K^+ , Cl^- and some organic solutes are critical for controlling the guard cell turgor. For closing the stomatal pore, these solutes should be exported from within the guard cell to an external space. The vast majority of the guard cell K^+ is stored in the vacuole. For releasing K^+ from the guard cell, K^+ should cross both the tonoplast membrane and the plasma membrane. The K^+ flux from vacuole to cytoplasm is probably mediated by a voltage-independent K^+ channel which is activated by the elevated cytosolic Ca^{2+} concentration (Ward et al., 1995). However, it is not known what is responsible for the Cl^- transportation across the tonoplast membrane into the cytosol. On the other hand, release of cytosolic ions to the extracellular space is through the outward rectifying K^+ channel and anion channels. These channels are activated in an ABA-dependent manner. Elevation of ABA levels increases cytosolic Ca^{2+} concentration. Inhibition of the guard cell plasma membrane H^+ -ATPase by the elevated cytosolic Ca^{2+} causes depolarization of the plasma membrane, increasing the driving force for the anion channels and leading to further depolarization. Evidence of the role of

ABA in increasing cytosolic Ca^{2+} concentration and activation of anion channels came from the *Arabidopsis* ABA-insensitive (*abi*) mutants *abi1* and *abi2* in which guard cell Ca^{2+} rise was reduced. anion channels were impaired and stomatal closure failed in response to ABA treatment (Allen et al., 1999; Pei et al., 1997). The outward-rectifying K^+ channels are activated as a result of the depolarized plasma membrane potential. Also, cytosolic Ca^{2+} inhibits inward-rectifying K^+ channels, which are involved in K^+ influx during stomatal opening. These concerted activities of the ion transportation across the plasma membrane underscore the ABA-mediated ion efflux leading to the stomatal aperture closure.

Elevation of the guard cell cytosolic Ca^{2+} concentration might be dependent on cyclic ADP-ribose (cADPR). Microinjection of cADPR into guard cells causes the release of vacuolar Ca^{2+} into the cytosol and consequently a reduction in guard cell turgor (Leckie et al., 1998). On the other hand, microinjection of the cADPR production antagonist nicotinamide potentially blocked of blockage of ABA-induced stomatal closure, which is in agreement with the observations that cADPR is a mediator in ABA signaling.

Other regulators involved in the ABA-mediated stomatal closure include phosphoinositide-specific phospholipase C (PI-PLC), phospholipase D (PLD), protein kinase, protein phosphatase, protein farnesyltransferase and actin filaments. Protein phosphorylation may play a key role in transducing the signals from the environment and ABA since the two ABA-insensitive (ABI) genes *ABI1* and *ABI2* encode protein phosphatase 2C (Leung et al., 1994, 1997; Meyer et al., 1994). A protein kinase gene has been isolated from *Vicia faba*, a dominant mutant form of which renders guard cells

insensitive to ABA-induced closure due to the abolished ABA activation of plasma membrane anion channels (Li et al., 2000). Also, protein farnesylation is important in ABA-mediated stomatal movement. The *Arabidopsis* ABA hypersensitive gene *ERL1* encodes the β -subunit of heterodimeric protein farnesyltransferase, which adds a 15-carbon farnesyl lipid to specific proteins. Mutations in *ERL1* or inhibition of its gene product cause superinduction of plasma membrane anion channel activity and stomatal closure by ABA (Pei et al., 1998). Moreover, reduced wilting during drought stress was observed in the *erl1* mutant plants. Inactivation by mutation suppresses *abi1* and *abi2* mutations, suggesting that the *ERL1* gene product acts downstream of the *ABI1*- and *ABI2*-encoded phosphatases. PLD activity in guard cells and the accumulation of its product, phosphatidic acid, which was shown to be able to inhibit the activity of the inward rectifying K^+ channel and induce stomatal closure (Jacob et al., 1999). Similarly, there is evidence showing a role for PI-PLC in the ABA-mediated cytosolic Ca^{2+} -dependent stomatal closure (Staxén et al., 1999). In addition, microfilaments in guard cells have been implicated in ABA-mediated stomatal closure. Stabilizing guard cell actin filaments and thereby inhibiting their redistribution and movement with actin antagonists such as phalloidin abolished stomatal closure induced by ABA in *Commelina communis* epidermal tissues (Kim et al., 1995).

Most studies focused on the ABA-mediated stomatal closure in plants in response to water deficit stresses such as drought. The importance of ABA in regulating stomatal closure was demonstrated in the *Arabidopsis* *abi* mutants *abi1* and *abi2* which fail to close their stomatal aperture during drought stress (Roelfsema and Prins, 1995). In contrast to water loss stresses, Na^+ -induced stomatal closure has been observed only in

certain salt-tolerant halophyte plants. In many non-halophyte plants, whose normal habitats are low salinity environments, Na^+ induces stomatal opening (for review, see Zeiger, 1983). The high salinity induced stomatal closure may be specific to Na^+ since KCl does not produce such an effect. Such a property of the halophytes may represent a unique salt tolerance mechanism in which Na^+ transportation into leaves is prevented through reduced transpiration by decreasing stomatal aperture. There is evidence showing the Na^+ signal controls the K^+ currents across the guard cell plasma membrane. In the halophytic species *Aster tripolium*, uptake of Na^+ in guard cells downregulates K^+ uptake, probably mediated through an increase of cytosolic Ca^{2+} concentration, which inactivates the inward-rectifying K^+ channels (Véry et al., 1998). This phenomenon was not observed in the related non-halophytic species *Aster amellus*.

1.5. Switching from C_3 Photosynthesis to Crassulacean Acid Metabolism

Crassulacean acid metabolism (CAM) is the pathway of photosynthetic carbon fixation that some plant species use in adaptation to CO_2 limitation, for example, in marine and desert environments (for reviews, see Cushman and Bohnert, 1997, 1999). Typically, plants with CAM open their stomata at night for external CO_2 uptake and primary fixation to phosphoenolpyruvate (PEP) to form oxaloacetate, which is subsequently reduced to malate and stored in the vacuole. PEP carboxylase (PEPC) catalyzes the CO_2 fixation and NAD(P)-malate dehydrogenase (MDH) converts oxaloacetate to malate. PEP is supplied from the glycolysis of storage carbohydrates in the chloroplast. During the day the stomata are closed and the vacuolar malate is exported into the cytosol to be decarboxylated to pyruvate by NAD(P)-malic enzyme

(ME). This process liberates CO₂ which is then reassimilated through ribulose-1.5-bisphosphate carboxylase/oxygenase and the photosynthetic carbon reduction cycle in the chloroplast. Pyruvate is used for gluconeogenesis to regenerate storage carbohydrates.

The nocturnal CO₂ fixation by CAM allows plants to minimize daytime water loss due to the higher temperature. Therefore, CAM displays a considerable competitive advance over other photosynthesis mechanisms when plants are faced with certain environmental stresses, such as drought and high salinity, which impose water limitation. Indeed, some facultative CAM plant species switch from C₃ photosynthesis to CAM when exposed to drought or high salinity. Such a species is the extensively studied common ice plant *M. crystallinum*, from which many genes involved in and related to CAM have been isolated and characterized (Cushman and Bohnert, 1997, 1999).

The key enzyme in CAM, PEPC, is encoded by a small gene family of up to four members in *M. crystallinum* and other CAM plant species. Only one gene family member, *Ppc1*, is involved in CAM. That gene is also induced by environmental stresses such as high salinity (Cushman et al., 1989). Other enzymes associated with CAM are encoded also by gene families and at least one member of each family is CAM-specific and stress-inducible. These enzymes include NADP-ME, NAD(P)-MDH, enolase, phosphoglyceromutase (PGM) and the subunits of the vacuolar H⁻-ATPase (Cushman and Bohnert, 1997, 1999). Enolase and PGM convert 3-phosphoglycerate derived from glycolysis in the chloroplast to PEP for CO₂ fixation at night. The activity of vacuolar H⁻-ATPase might provide the driving force for the transport of cytosolic malate into the vacuole. In addition, activities of starch-degrading enzymes also increase substantially during CAM induction in *M. crystallinum* probably because increased rates of starch

turnover might be necessary for CAM (Paul et al., 1993). However, some single-copied genes in *M. crystallinum*, for example, *Ppdk1* for pyruvate orthophosphate dikinase (PPDK) and *GapC1* for NAD-glyceraldehyde 3-phosphate dehydrogenase (GAP), both involved in starch metabolism, are necessary for both C₃ photosynthesis and CAM, and are also inducible by high salinity stress.

The coordinated upregulation of gene expression or enzyme activity during stress induction of CAM in the facultative CAM plant species like *M. crystallinum* indicates that these plants have evolved to utilize CAM as a way of adaptation to stress. Certain common mechanisms may be responsible for the regulation of these genes.

Transcriptional activation might be the major control of these CAM genes whose mRNA levels are elevated within a few hours after the onset of stress. Indeed, parts of the promoter regions required for stress induction in the *M. crystallinum* genes *Ppck1* and *GapC1* share extensive sequence similarity and contain sequences resembling the DNA binding motif for the MYB class of transcription factors at similar locations, indicating the two genes share the same mechanism of regulation (Schaeffer et al., 1995). There are also preliminary studies on posttranscriptional, translational and posttranslational regulations of the CAM-associated genes (Cushman and Bohnert, 1997, 1999). One interesting aspect is that the PEPC activity is regulated by reversible phosphorylation/dephosphorylation controlled by the Ca²⁺-independent PEPC kinase in *M. crystallinum*, which itself is induced by salt stress (Li and Chollet, 1994). On the other hand, in non-stress conditions, the nocturnal nature of CO₂ fixation by CAM suggests a pattern with circadian rhythms for the regulation of the related genes or their

products. This has been observed in the activities of PEPC and PEPC kinase (Carter et al., 1991; Nimmo et al., 1987).

Exogenous application of ABA stimulates the activities of PEPC and many other CAM-associated enzymes and triggers CAM, consistent with the fact that stress increases endogenous amounts of ABA during CAM induction (Cushman and Bohnert, 1997, 1999). However, ABA itself might not be sufficient to induce CAM, but rather it requires other stimuli such as high light intensity. In addition, the importance of protein phosphorylation/dephosphorylation and Ca^{2+} /calmodulin has been implicated in the signaling for stress induced CAM by using specific enzyme inhibitors or Ca^{2+} chelators (Cushman and Bohnert, 1997, 1999; Taybi and Cushman, 1999), but no details of a signaling pathway has been described so far.

1.6. Damage Control Mechanisms

Salt stress and other water deficit stresses surely have damaging effects on cellular structures and cellular, biochemical and physiological processes due to the high Na^+ concentration and low water potentials. Plants have evolved active biochemical and physiological mechanisms to protect cellular structures and macromolecules and to repair any damage caused by the stress. The effects of the stress are therefore minimized to sustain cellular functions. So far most of the evidence for the existence of such mechanisms has come from the molecular studies of stress induced genes. The enzymes and proteins these genes encode have been implicated in cellular damage control (for reviews, see Bray, 1993, 1997; Bohnert et al., 1995; Ingram and Bartels, 1996).

Detoxification of active oxygen species and other cytotoxins. High salinity and other osmotic stresses may cause stomatal closure and reduced CO₂ uptake that in turn leads to the channeling of reducing equivalents to the production of active oxygen species rather than to CO₂ fixation (Price and Hendry. 1991; Smirnov. 1993). A number of antioxidant enzymes have been implicated in the involvement in stress response. Catalase reduces H₂O₂ to O₂ and may play an important role in antioxidant defense against environmental stresses. In maize, ABA and osmotic stress induce expression of one of the catalase genes, *Cat1*, and such regulation is likely mediated by H₂O₂ itself since H₂O₂ can also enhance *Cat1* mRNA levels (Guan et al., 2000). The pea genes coding for the cytosolic ascorbate peroxidase and Cu/Zn-superoxide dismutase, enzymes involved in the removal of cellular H₂O₂ in plants, are upregulated during drought stress and following recovery from the stress (Mittler and Zilinskas, 1994). The activities of these two enzymes and the tolerance to superoxide radicals are enhanced in the *Arabidopsis* recessive photoautotrophic salt tolerant mutant, *psl1*, which, unlike the wild-type plants, shows elevated tolerance to salt stress under moderate light intensities (Tsugane et al., 1999). This confirms a role for the scavenging of active oxygen species in adaptation of plants to salt stress although such potential for tolerance is usually blocked in the wild-type plants.

The transcript level and the enzymatic activity of glyoxalase I, an enzyme involved in cell division and growth, are elevated in plants in response to salt and other abiotic stresses. Overexpression of the *Brassica juncea* gene for glyoxalase I in tobacco results in the increased tolerance to salt and methylglyoxal, a cytotoxic compound produced in metabolically active cells which can be detoxified by glyoxalase (Veena et

al., 1999). Salt and other osmotic stresses also upregulate genes for other enzymes able to detoxify certain cytotoxins or reactive oxygen species. Such enzymes include the soluble epoxide hydrolase (Kiyosue et al., 1994) and glutathione-S-transferases (Kiyosue et al. 1993; van der Kop. 1996). However, their exact roles in salt stress response and tolerance remain to be elucidated.

Proline and related compounds may also act as scavengers of free radicals. It was observed that accumulation of proline by blocking proline feedback inhibition on P5CS resulted in the reduction of salt stress induced increase of malondialdehyde, a lipid peroxidation product, in transgenic tobacco plants (Zhong et al., 2000). As previously mentioned, these transgenic plants have improved salt stress tolerance.

Stabilization of cellular structures and macromolecules. During seed maturation several types of highly hydrophilic proteins with distinct primary structures accumulate to high abundance (Baker et al., 1988). These proteins, designated LEAs for late embryogenesis abundant, accumulate at the desiccation stage in seed development. Moreover, many genes for such proteins are upregulated by high salinity, dehydration, low temperatures, as well as by ABA, during vegetative growth (for reviews, see Bray, 1997; Ingram and Bartels, 1996; Skriver and Mundy, 1990). Therefore, their expression pattern indicates that the LEA proteins have a role in water deficit stress response and tolerance. The LEA proteins are grouped according to their amino acid sequence similarities and the occurrence of specific motifs. Common features of these proteins are 1) they are hydrophilic throughout the length of the polypeptide; 2) they usually have biased amino acid compositions, namely, high contents of hydrophilic and charged amino

acids, such as glycine and glutamic acid, and nearly no cysteine and tryptophan; 3) many of them contain repeated amino acid sequences.

In a plant species, each LEA group is usually encoded by a multigene family, and each gene in a family may have its specific spatial and temporal expression pattern. A comparative study showed that the protein levels of Group 2 LEA (dehydrin) and Group 3 LEA proteins are higher in the roots of salt-tolerant rice varieties than in the roots of a sensitive rice variety (Moons et al., 1995). A similar correlation also was found for the transcript levels for the dehydrin gene in drought-tolerant and drought-sensitive lines of sunflower (Cellier et al., 1998). The barley Group 3 LEA gene *HVA1* has been expressed in rice and improved the salt and drought stress tolerance of the transgenic plants (Xu et al., 1996). However, the exact functions of the LEA proteins are still unknown. Their biased amino acid compositions and the repeated sequence motifs suggest that they do not have any enzymatic activities. Nevertheless, the amino acid compositions and sequences and the predicted secondary structures of the LEA proteins sometimes strongly suggest their possible functions in stress response and tolerance. The Group 1 LEA (Em) proteins are composed of over 70% random coil and, therefore, are heavily hydrated, so that they may function as water binding proteins to maintain minimal water supply in case of cellular water loss (McCubbin and Kay, 1985). The hydrophilic nature and the random coil structure of the LEA proteins, especially dehydrins, enable their shape to conform to and coat other proteins or cellular structures. It makes them excellent candidates for macromolecular compatible solutes to solvate proteins and cellular structures to prevent them from crystallization or aggregation and maintain their natural folding in environments of low cellular water potentials or high concentrations of solutes

(Baker et al., 1988; Close, 1996). Dehydrins are suggested to function as cryoprotectants, probably also due to their solvation property, during the acclimation to freezing stress which leads to decreased water potential (Danyluk et al., 1998). The Group 3 LEA proteins contain an 11-amino-acid motif, sometimes repeated, which could exist as amphipathic helices, forming intramolecular helical bundles and presenting a surface of charged amino acid residues capable of binding ions (Baker et al., 1988; Dure III, 1993). Such salt bridges may prevent the crystallization of proteins and cellular structures caused by high cellular ionic strength during salt and water deficit stresses.

Protein repair and degradation. Upon salt or water deficit stresses, the low cellular water potential or high ionic strength causes many proteins to denature. Damaged proteins should either be repaired or degraded. Dehydrins may possess molecular chaperone functions. This is based on the possibility that dehydrins bound with ordered water molecules are able to preferentially exclude solutes from the surface of unfolded or partially unfolded proteins and, therefore, to drive them back to a folded state or at least to inhibit their further unfolding (Close, 1996). Exposed hydrophobic parts of denatured proteins might interact with dehydrins through their amphipathic lysine-rich segments, which occur as repeated motif in dehydrin proteins.

On the other hand, irreversibly denatured proteins should be removed and degraded for turnover of amino acids for the synthesis of new proteins. Many genes for protein degradation are upregulated by high salinity and water deficit stresses (for reviews, see Bray, 1997; Ingram and Bartels, 1996). Examples are the *Arabidopsis rd19A* and *rd21A* genes for cysteine proteases, the *UBQ1* gene for ubiquitin extension protein, the *ERD1* gene for the regulatory subunit of the ClpA protease, and the pea *15a*

gene for a possible protease. It is also possible that some of these proteases can remove a piece of peptide from certain proteins thereby activating these proteins: while specific protease inhibitors may regulate the activities of the proteases. since many genes for protease inhibitors are also induced by stress (Ingrama and Bartels, 1996).

2. SALT AND WATER DEFICIT STRESS SIGNALING

There must be additional, yet to be uncovered, strategies certain plant species may invoke in their resistance to high salinity or other water deficit stresses. Some responses mentioned above may be used by most of the plant species. nevertheless there is a wide variation in salt tolerance among plant species. It is also possible that a particular strategy of adaptive response is sufficient for a certain species or variety to survive stress. The variation in plant response, including the rapidity of the response, raises the question of how the stress signals are perceived and transduced to regulate gene expression and protein or enzyme activity, which are responsible for the response. Some stress-induced genes encode transcription factors and, therefore, may regulate the expression of other sets of genes: pathways may cross-talk: moreover, some genes and proteins have redundant functions. In this section, focus will be on protein kinases and their involvement in stress signaling. Other signaling molecules such as the GTP-binding GTPases have not yet been demonstrated to be involved in stress signaling in plants.

2.1. Perception of Salt Stress Signals

Both Na^+ and decreased cellular water potential can serve as signals of salt stress. So far there has been no report of a sensor in plants for increased Na^+ concentration.

Several mechanisms have been proposed for sensing water loss in a plant cell. for example, decrease or loss of turgor, change in cell volume or membrane area, loss of membrane stretch, or, alteration in cell wall-plasma membrane interaction (Bray, 1997). One step closer to finding an osmosensory mechanism in plants is the cloning and characterization of a transmembrane histidine kinase gene, *ATHK1*, from *Arabidopsis* (Urao et al., 1999). Histidine kinase is one of the components in the so-called two-component osmosensor complex originally found in bacteria. The membrane-bound histidine kinase is autophosphorylated in response to changes in osmotic pressure in the environment and subsequently relays the phosphate group to the second component, the response regulator, which triggers the downstream signaling events (for review, see Urao et al., 2000). *ATHK1* is able to suppress the osmosensitive phenotype of the yeast mutant, *sln1*, which lacks a functional *Sln1* gene for the histidine kinase in the osmosensor two-component system. Thus, *ATHK1* is a plausible candidate for sensing environmental water deficit stresses in plants.

2.2. Abscisic Acid Signaling and Its Interaction with Other Plant Hormones in Stress Response

The evident involvement of ABA in salt and water deficit stresses is due to the fact that ABA is able to induce similar responses, including gene expression, to what these abiotic stresses could induce and that ABA can induce stomatal closure and sometimes improve stress tolerance. ABA is a sesquiterpenoid synthesized from mevalonic acid probably via certain forms of carotenoids. The pathway for ABA synthesis in plants has been defined with the help of plant ABA-deficient mutants (for

review, see Giraudat et al., 1994). ABA reaches high levels during seed development and thus promotes seed maturation desiccation tolerance. In dry seeds, ABA, although its level is low, maintains seed dormancy and prevents precocious germination. In vegetative growth ABA levels are elevated when plants encounter abiotic stresses such as high salinity and water deficit, and trigger downstream responses through ABA-dependent pathways (Shinozaki and Yamaguchi-Shinozaki, 1997). It is not known how the ABA level increases in response to salt and water deficit stresses. But genes for 9-*cis*-epoxycarotenoid dioxygenase, an enzyme involved in the ABA biosynthesis pathway have been reported to be induced by water deficit stresses in several plant species including maize, bean and cowpea (Iuchi et al., 2000; Qin and Zeevaart, 1999; Tan et al., 1997). In cowpea, the transcript level of the gene, *VuNCED1*, was observed to accumulate earlier than the increase of the ABA level (Iuchi et al., 2000). There are also stress signaling pathways that are ABA-independent. The two classes of stress response pathways can be demonstrated by the regulation pattern of a particular stress-induced gene and may work together in plants to respond to stress and make adaptation. Some of the stress responses involving ABA were previously discussed. Moreover, many ABA-inducible genes have not been functionally assessed in the stress response and for stress adaptation.

ABA receptors have not been identified in the plant cell. However, much progress has been made in deciphering ABA signal transduction by the study of ABA response mutants, especially in *Arabidopsis* (for reviews, see Leung and Giraudat, 1998; McCourt, 1999). Only those mutants for which the gene products are defined will be discussed here.

Type 2C protein phosphatases function as negative regulators in ABA signaling. One type of ABA response mutants is the *abi* mutants, which have been isolated from *Arabidopsis* from screens of seeds that resist germination inhibition by ABA. In *Arabidopsis* two classes of *abi* mutants, *abi1-1* and *abi2-1*, have been identified, both classes of mutations are semidominant. The mutations in *abi1-1* and *abi2-1* have similar effects in causing resistance to ABA inhibition of seed germination, failed stomatal closure in response to ABA and drought stress, and abolished gene induction by ABA and stress (Leung et al., 1997). The affected genes, *ABI1* and *ABI2*, respectively, encode functionally homologous type 2C protein phosphatases (PP2C: Leung et al., 1994, 1997; Meyer et al., 1994). The expression patterns of the two genes are also similar in that they are expressed in most of the plant tissues although the *ABI2* mRNA is detected at relatively low levels in leaves. Both genes are also induced by external ABA and sorbitol treatments in wild-type plants and in the *abi2-1* mutant but not in the *abi1-1* mutant (Leung et al., 1997). Therefore, the two genes are functionally redundant to a certain degree. The *abi1-1* mutant has a G180Q mutation in *ABI1* and the PP2C activity of *ABI1* is reduced but not abolished. Intragenic revertants of *abi1-1* have been obtained: they carry missense mutations in the *ABI1* gene such that the PP2C activity was abolished (Gosti et al., 1999). These revertant alleles are recessive to the wild type allele *ABI1* and cause ABA-hypersensitivity phenotypes since they are more sensitive to ABA inhibition of seed germination and more resistant to drought stress. Thus, the wild type *ABI1* is proposed to be a negative regulator of ABA response. Since the *ABI1* gene is induced by ABA, the action of *ABI1* is elicited by ABA to form a negative feedback loop (Gosti et al., 1999). The protein phosphatase activities of *ABI1*

and ABI2 indicate protein phosphorylation/dephosphorylation plays critical roles in ABA and stress signaling.

Transcription factors are involved in ABA response. Another class of *abi* mutation is represented by the maize *viviparous-1* (*vp1*) and *Arabidopsis abi3* mutants (for reviews, see Leung and Giraudat, 1998; McCourt, 1999). The corresponding affected genes, *VP1* and *ABI3*, respectively, encode homologous transcription factors that are expressed exclusively in seeds. This explains the fact that these mutations only affect seed development and germination and the developmental expression of seed storage protein and LEA protein genes. However, ectopic expression of *ABI3* in transgenic *Arabidopsis* causes hypersensitivity to ABA in vegetative plant tissues and causes genes normally expressed specifically in seeds to be induced by ABA in leaves (Parcy and Giraudat, 1997). *VP1* has been demonstrated to activate the promoter of *Em*, the wheat gene for a Group 1 LEA protein, probably by the binding of the G-box-like ABA responsive elements, and by facilitating the binding of basic leucine zipper (bZIP) factors to this promoter (Hobo et al., 1999; McCarty et al., 1991; Razik and Quatrano, 1997).

Two additional classes of *abi* mutants, *abi4* and *abi5*, have been isolated from *Arabidopsis* and their phenotypes are similar to those of the *abi3* mutants in that the effects of the mutations are mainly in seed development and germination. The corresponding genes, *ABI4* and *ABI5*, like *ABI3*, also encode transcription factors. *ABI4* is homologous to a plant specific family of transcriptional regulators containing an APETALA2 (AP2) DNA binding domain (Finkelstein et al., 1998), while *ABI5* encodes a bZIP transcription factor (Finkelstein and Lynch, 2000). According to the expression analyses, the activities of the *ABI4* and *ABI5* genes may not be confined to the seed.

Transcripts for *ABI4* and *ABI5* were also found in seedlings of the wild-type *Arabidopsis* plants. Moreover, the ABA-induction of the *Arabidopsis AtEm1* gene in seedlings was abolished in the *abi5* mutant. *ABI1*, *ABI2*, *ABI3*, *ABI4* and *ABI5* itself may regulate the expression of *ABI5* since the *ABI5* mRNA levels are diminished in seeds of the corresponding mutants. Furthermore, ectopically expressed *ABI3* enhances the vegetative expression of *ABI5* and renders it ABA-inducible. This indicates the two genes function in the same pathway, and *ABI3* is upstream of *ABI5*.

Protein farnesylation negatively regulates ABA response. The *era* mutants isolated from *Arabidopsis* are a class of ABA-responsive mutants which have enhanced response to external ABA treatment. In the *era* mutants, seeds are not able to germinate at external ABA concentrations that would permit wild-type seeds to germinate. The gene affected in the *eral* mutants encodes the β -subunit of a protein farnesyl transferase (Cutler et al., 1996). Since the *eral-1* mutant allele is a T-DNA insertion mutation and its function is fully lost, the wild-type *ERAI* gene may play a role of negative regulation in ABA signaling through farnesylation of other signaling proteins. In addition to the expression of *ERAI* in flower buds, the gene is also expressed in guard cells; therefore, the function of *ERAI* in ABA response is not restricted in seed development. Loss of the *ERAI* gene or application of protein farnesylation inhibitors results in ABA hypersensitivity in guard cell anion channel activation and stomatal closure (Pei et al., 1998). Moreover, the *eral* mutants showed less transpirational water loss under drought stress.

Interaction of signaling between ABA and other plant hormones. Some plant hormones have antagonistic effects in plant development or in response to environmental

cues: for example, ABA promotes seed dormancy and prevents germination whereas gibberellin (GA) helps to break seed dormancy and induces germination. It is evident that signaling pathways for different plant hormones interact with each other since screening for suppressor mutants which are deficient in synthesis of or response to one hormone often turned out to have defects in synthesis of or response to another hormone (for review, see McCourt, 1999). In barley aleurone cells, where hydrolytic enzymes are produced and secreted for the breakdown of seed storage macromolecules for nutrients for seed germination, the GA induction of genes for those hydrolytic enzymes such as α -amylase is blocked by the external application of ABA (for reviews, see Lovegrove and Hooley, 2000; McCourt, 1999). Transient expression of an ABA-induced wheat protein kinase gene *PK4B.1* in barley aleurone diminished the GA induction of the genes for α -amylases and a cysteine proteinase (Gómez-Cadenas et al., 1999). PLD also functions in the ABA-mediated inhibition of α -amylase activities. Application of ABA increased the PLD activity and application of the PLD reaction product, phosphatidic acid, inhibited α -amylase production in barley aleurone protoplasts (Ritchie and Gilroy, 1998). Moreover, expression of the barley gene *HvSPY*, a homologue of the negative *Arabidopsis* GA-response regulator gene *SPY*, stimulates the expression of an ABA-induced dehydrin gene promoter in barley aleurone and inhibits the GA-induction of the α -amylase gene (Robertson et al., 1998). Since *SPY* has similarity to Ser/Thr-*O*-linked *N*-acetylglucosamine transferases, it is possible that this type of protein glycosylation plays opposite roles in GA and ABA responses. Alternatively, the *SPY* genes may mediate ABA responses which cause inhibition of GA-mediated gene activation since *Arabidopsis spy1* mutant seeds are partially insensitive to ABA inhibition on germination

(Steber et al., 1998). Moreover, a GA-insensitive mutant, *sly1*, was isolated by screening for suppressors of the *abil-1* mutant (Steber et al., 1998).

Ethylene is also involved in ABA signaling. The ethylene signaling pathway defined by the *ETR1*, *CTR1* and *EIN2* genes inhibits ABA signaling in seeds since mutant alleles of the constitutive ethylene response mutants *ctr1* and the ethylene insensitive mutant *ein2* were isolated as an enhancer and a suppressor, respectively, of the *abil-1* mutant phenotypes (Beaudoin et al., 2000) and mutant alleles of *ein2* enhance ABA response (Ghassemian et al., 2000). Studies of such mutants have led to the findings that endogenous ethylene promotes germination by decreasing sensitivity to endogenous ABA and that the ABA inhibition of root growth requires a functional ethylene signaling pathway.

2.3. Cytosolic Ca^{2+} and Related Molecules as Second Messengers

There is evidence showing that second messengers, especially Ca^{2+} , are involved in transducing signals of salt stress and water deficit stresses. For example, Ca^{2+} is necessary for plants to maintain proper K^+/Na^+ selectivity across the plasma membrane during salt stress. Ca^{2+} is able to improve stress tolerance as shown in the *Arabidopsis sos* mutants. Increased cytosolic Ca^{2+} concentrations mediate ABA-induced stomatal closure in response to drought stress. Elevation of cytosolic Ca^{2+} concentration and Ca^{2+} channel activities are found necessary for the induction of stress-regulated genes such as *P5CS1* by salt or mannitol treatments in a study using a Ca^{2+} channel blocker and a Ca^{2+} chelator (Knight et al., 1997). Many signaling molecules and events involved in stress response rely on cytosolic Ca^{2+} . Two Ca^{2+} /calmodulin-dependent protein kinase (CDPK)

genes, *ATCDPK1* and *ATCDPK2*, have been isolated from *Arabidopsis* and shown to be induced by salt and drought stresses (Urao et al., 1994). *ATCDPK1* might function to regulate the expression of stress-induced genes such as *HVA1*, which encodes a Group 3 LEA protein (Sheen, 1996). In addition, a gene for the regulatory subunit of calcineurin, a class of Ca^{2+} /calmodulin-dependent type 2B protein phosphatase (PP2B), is found to be upregulated by water deficit stress in *Arabidopsis* plants (Kudla et al., 1999).

The magnitude, duration, speed and spatial organization of the changes in cytosolic Ca^{2+} concentration may define different signals for a particular stress response (for review, see Bush, 1995). Plant cells may uptake Ca^{2+} from the apoplast, or, Ca^{2+} may be released into the cytosol from intracellular stores such as vacuoles. Also, Ca^{2+} exclusion and sequestration mechanisms exist for maintaining Ca^{2+} homeostasis in the cytoplasm. cADPR is one of the signals used to release vacuolar Ca^{2+} through channels on the tonoplast during stress and ABA signaling. cADPR is able to mediate ABA responses, including activation of stress-responsive genes in a Ca^{2+} -dependent way and causes an increase in cytosolic Ca^{2+} concentration (Allen et al., 1995; Wu et al., 1997). This mechanism is confirmed in the ABA-induced stomatal closure (Leckie et al., 1998).

The Ca^{2+} influx through the plasma membrane may be through stretch-activated Ca^{2+} channels as demonstrated in guard cells responding to tension in the membrane caused by osmotic stress (Cosgrove and Hedrich, 1991). On the other hand, Ca^{2+} efflux from the cytoplasm may use a Ca^{2+} -pump ATPase in the plasma membrane and a Ca^{2+} - H^+ antiporter in the tonoplast (for review, see Niu et al., 1995). However, further characterization of the mechanisms mediating the Ca^{2+} fluxes across the plasma membrane and the tonoplast during salt stress response is necessary.

It should be noted that not all of the signaling processes in stress response involve Ca^{2+} . Cytoplasmic pH possibly serves as second messenger in a Ca^{2+} -independent manner. For example, ABA can induce alkalinisation of the cytoplasm of guard cells and thus activate the plasma membrane outward-rectifying K^+ channels (Irving et al., 1992).

Inositol 1,4,5-trisphosphate (IP_3), a well-established signal molecule in animal cells, has been demonstrated in plants to induce Ca^{2+} release from intracellular stores and participate in Ca^{2+} - and ABA-mediated stress responses (Allen et al., 1995). *AtPLC1*, a gene for PI-PLC, which catalyzes the reaction to produce IP_3 , is induced by dehydration and salt stress (Hirayama et al., 1995). ABA- and stress-responsive genes can be induced by microinjection of IP_3 into plant cells, and such induction can be blocked by the IP_3 receptor inhibitor heparin (Wu et al., 1997). However, heparin does not affect the cADPR-mediated gene activation by ABA. Therefore, IP_3 may be involved in a secondary ABA response.

2.4. Phospholipase D and Its Products

The previous section discussed the involvement of phospholipids in stress and ABA signaling in plants demonstrated by phospholipase C (PLC) and its product IP_3 . In addition, phosphatidic acid (PA), which has already been recognized in animals as a second messenger associated with early events in certain cellular signaling processes, is also emerging as an important signal molecule in stress response in plants. PA is the product of the enzyme PLD and can also be produced through phosphorylation of the PLC product diacyl glycerol (DAG) by DAG kinase. There is evidence that PLD is an important component in ABA and stress signaling (for review, see Wang, 1999). PLD

activity is induced within minutes by dehydration in the resurrection plant *Craterostigma plantagineum* (Frank et al., 2000). In which case it seems that the activation of the PLD activity is mediated by a G protein but not by ABA. since the G protein-activating peptide mastoparan mimics dehydration in PLD activation. whereas ABA shows no effects on the PLD activity. However, the transcript level of one of the cloned PLD genes in this plant, *CpPLD-2*, is induced by both dehydration and ABA. Increases in the promoter activity and mRNA levels of genes for PLD and in the PLD protein level and enzymatic activity were reported from *Arabidopsis* and castor bean (Fan et al., 1997; Xu et al., 1997). Water deficit stress induced increase of PA was also observed in salt stressed green alga *Chlamydomonas moewusii*, partly due to the PLD activity and partly due to the DAG kinase activity (Munnik et al., 2000). In addition, stress also causes increased levels of diacylglycerol pyrophosphate, which is produced from further phosphorylation of PA by PA kinase and may also be a second messenger. Several studies showed PLD and PA are involved in ABA and stress signaling, for example, as mentioned earlier. PLD and PA mediate the ABA responses and actions in barley aleurone cells (Ritchie and Gilroy, 1998) and the stomatal closure induced by ABA (Jacob et al., 1999). The gene encoding one of the PLD forms, *PLD α* , is induced by ABA and ethylene, and has been implicated in the ABA and ethylene promoted senescence of detached *Arabidopsis* leaves (Fan et al., 1997).

2.5. Protein Phosphorylation and Dephosphorylation

Protein kinases. The activity of a protein can be regulated through reversible phosphorylation. Phospho-modifications of proteins are catalyzed by protein kinases and

protein phosphatases. Eukaryotes also use protein phosphorylation for perception of signals and for relaying and amplifying signals through a kinase cascade within a cell. Protein kinases catalyze phosphorylation by adding a phosphate group to the hydroxyl group of the side chains of an amino acid residue. The phosphate group at the γ position of an ATP molecule is the phosphate donor in this reaction. Protein His kinases were discussed earlier in this thesis. Protein Ser/Thr kinases and protein Tyr kinases are the two major classes of protein kinases categorized according to the specific amino acid residue that they phosphorylate. There is sequence similarity between the two classes and both contain common invariant amino acid residues at particular positions (Hanks et al., 1988). The catalytic domain of a protein kinase can be divided into 11 subdomains. Protein Tyr kinases actually represent a small group of kinases found in yeast and animals but not yet in plants, although there are reports demonstrating the existence of protein Tyr phosphorylation activity.

Each protein kinase has its specific protein substrates. There are 860 protein kinase genes in *Arabidopsis* (The Arabidopsis Genome Initiative, 2000). Such a large number, together with the specific spatial and temporal expression pattern, allows each protein kinase to regulate fairly specific cellular processes. Protein kinases are therefore probably the most important family of proteins involved in cellular signal transduction. The mitogen-activated protein (MAP) kinase pathways have been well characterized in yeast and animals and shown to mediate quite a number of signaling events ranging from cell growth to environmental stress response. MAP kinases have also been found in plants, although so far none of them has been described in a well-characterized signaling pathway (for review, see Hirt, 1997). However, the tomato *Pto* disease resistance gene

which encodes a protein kinase other than a MAP kinase has been well characterized in signal transduction in plants. The *Pto* gene controls the specific resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* which causes the speck disease in susceptible tomato plants (Martin et al., 1993). Resistance begins with the interaction of *Pto* with the pathogen elicitor, which is the product of the avirulence gene of the pathogen, *AvrPto* (Scofield et al., 1996; Tang et al., 1996). The *Pto* protein kinase then phosphorylates one of its substrates, *Pti4*, an AP2 domain transcription factor. The phosphorylation of *Pti4* by *Pto* enhances its ability to bind the GCC-box in the promoters of certain *PR* pathogen resistance genes and to activate their expression, thereby preventing the onset of infection (Gu et al., 2000).

The many protein kinases in plants can be classified into subfamilies according to their structures, substrate specificities, cellular functions and requirements for particular co-factors or other proteins for activation (Hardie, 1999). The major protein kinase groups found in plants are: 1) CDPKs, which contain a calmodulin-like domain and require Ca^{2+} for activation; 2) the SNF1-related protein kinases (SNRKs), which have sequence similarity to the yeast SNF1 protein kinase; 3) the receptor-like kinases (RLKs), which span the plasma membrane, receive signals by interacting with extracellular ligands thereby activating the intracellular kinase domain and initiating downstream signaling events; 4) the MAP kinases (MAPKs); 5) the MAPK kinases (MAPKKs), which have dual specificity and activate MAPKs by phosphorylation of both Thr and Tyr residues in MAPKs; 6) the MAPKK kinases (MAPKKKs), which activate MAPKKs; 7) the cyclin-dependent kinases (CDKs), which are activated by binding by cyclins and are involved in controlling cell growth and division. Other groups include the casein kinases

I (CK1), the casein kinases II (CK2), the CTR/Raf-like subfamily, the LAMMER subfamily, the S6 kinase homologues, the PVPK-1 subfamily, and the NAK subfamily. There are also some protein kinases which do not fit into any of the above groups.

Plant protein kinases in many of these subfamilies have been shown to be related to salt and water deficit stress or ABA responses. The most apparent indication of their involvement in stress signaling is that their genes are upregulated by salt treatment or drought. Activation of certain kinases in response to stress or ABA also has been demonstrated by phosphorylation activity assays with specific protein substrates. The stress-induced protein kinase genes cloned or protein kinase activities detected in responsive to stress so far demonstrate that protein kinases are involved in a wide range of stress signaling processes.

The wheat protein kinase gene *PKABA1* is the first plant kinase gene discovered to be up-regulated by ABA and water deficit stresses (Anderberg and Walker-Simmons, 1992; Holappa and Walker-Simmons, 1995). As mentioned earlier, it has been shown in the barley aleurone tissues to repress the GA-induction of α -amylase genes and a cysteine proteinase gene. The repression requires the kinase activity of PKABA1 since the null-mutant form deficient in ATP-binding did not have such effects (Gómez-Cadenas et al., 1999). It is not known yet if PKABA1 is involved in other aspects of the ABA and stress responses.

Two CDPK genes from *Arabidopsis*, *ATCDPK1* and *ATCDPK2*, are induced by drought and high salinity (Urao, et al., 1994). In a transient expression assay in maize leaf protoplasts, ATCDPK1 or ATCDPK1a, which is 96% identical to ATCDPK1 in amino acid sequence, was able to activate the promoter of the barley ABA-inducible

HVA1 gene in the absence of ABA (Sheen, 1996). The activation was dependent on the kinase activity of the CDPKs and could be abolished in the presence of a PP2C, which has been shown to inhibit the ABA-induction of the *HVA1* promoter in the maize leaf protoplast. Therefore, ATCDPK1 and ATCDPK1a are positive regulators in ABA signaling, whereas PP2C is a negative regulator (Gosti et al., 1999).

The *Arabidopsis SOS2* gene encodes an SNF1-related protein kinase and is induced specifically in roots by salt stress (Liu et al., 2000). The regulatory carboxyl terminal domain of the SOS2 protein interacts with the Ca^{2+} -binding protein SOS3, which is similar to the yeast calcineurin B subunit, and is thus activated in a Ca^{2+} -dependent manner as assayed with synthetic peptide substrates for SNF1 (Halfter et al., 2000). Mutations in *SOS3* caused hypersensitivity to salt stress, and abolished the salt induction of the putative Na^+/H^+ antiporter gene *SOS1* in both roots and leaves (Shi et al., 2000). The *sos2* mutants are also hypersensitive to salt stress, but in these mutants the induction of the *SOS1* gene is abolished only in leaves but not in roots. It is possible that a homologous gene of *SOS2* is responsible for the salt induction of *SOS1* in roots. The *sos1* mutants are impaired in Na^+ tolerance and K^+ nutrition. The possibility that SOS1 functions as a plasma membrane Na^+/H^+ antiporter mediating Na^+ efflux makes the SOS3/SOS2/SOS1 pathway the most interesting stress signaling pathway discovered so far.

MAP kinase activities have been detected in assays for phosphorylation of the MAP kinase specific substrate myelin basic protein (MBP) in ABA-treated barley aleurone protoplasts (Knetsch et al., 1996), salt-treated alfalfa suspension cells (Munnik et al., 1999) and osmotic stressed tobacco suspension cells (Hoyos and Zhang, 2000;

Mikołajczyk et al., 2000). Interestingly, the tobacco cells demonstrate two MAP kinase-like activities, the activation of both are Ca^{2+} - and ABA-independent. The 48-kD kinase is the salicylic acid-induced kinase, whereas the 40- or 42-kD kinase has similarity to the *Arabidopsis* ASK1 protein kinase. The *Pisum sativum* MAP kinase homolog PsMAPK is very similar to the yeast MAP kinase Hog1p, which is involved in the yeast osmo-regulatory pathway, and its expression in a yeast *hog1* mutant rescued its osmo-sensitivity phenotype (Pöpping et al., 1996). Also, by complementation of the salt-sensitive phenotypes of yeast mutants, a GSK/shaggy-like protein kinase gene (Piao et al., 1999) and a gene for a homolog of the yeast Dbf2 protein kinase (Lee et al., 1999) were cloned and both of the genes are stress regulated. Other stress inducible protein kinase genes identified include the RLK gene *RPK1* from *Arabidopsis* (Hong et al., 1997), the NAK protein kinase subfamily gene *ARSK1* from *Arabidopsis* (Hwang and Goodman, 1995), and the *V. faba* guard cell specific kinase gene *AAPK*, which has been shown to be Ca^{2+} -independent and to regulate the ABA-mediated anion channels and stomatal closure (Li and Assmann, 1996; Li et al., 2000). In addition, a protein kinase activity has been shown to be stimulated by water deficit stress in the elongation zone of maize primary roots with casein and histone as substrates (Conley et al., 1997).

Protein kinase inhibitors. Protein kinase activities are also regulated in plant cells through phosphorylation by other protein kinases or dephosphorylation by protein phosphatases. They can also be inactivated by interaction with proteinaceous protein kinase inhibitors. So far very few reports have focused on the significance of kinase inhibitors in stress and ABA signaling. A tobacco gene encoding a 14-3-3 protein, a class of protein kinase C inhibitors found in animals, has been isolated and shown to be

downregulated by salt stress in tobacco cell cultures (Chen et al., 1994). However, there is no indication that plants possess protein kinase C-like activities or genes. The *Arabidopsis* CDK inhibitor gene, *ICK1*, is induced by ABA and such induction coincides with the decrease in Cdc2-like histone H1 kinase activity (Wang et al., 1998). Their study demonstrated a role of CDK inhibitors in cell cycle regulation but no indication of their participation in stress signaling. Perhaps more protein kinase inhibitors will be found to play roles in stress or ABA signaling with techniques for detecting protein-protein interaction assays such as the yeast two-hybrid system.

Protein phosphatases. Phosphorylation of proteins by protein kinases is reversible and dephosphorylation of phosphorylated proteins is catalyzed by protein phosphatases (for review, see Luan, 1998). The importance of protein phosphatases in stress and ABA signaling is demonstrated by the ABA response genes *ABI1* and *ABI2*, both of which encode protein Ser/Thr phosphatases 2C, although their substrates are so far unknown. The other type of protein Ser/Thr phosphatase implicated in stress and ABA signaling is calcineurin, which is Ca^{2+} /calmodulin dependent and belongs to the PP2B class. Calcineurins are heterodimeric and composed of a catalytic subunit (CNA) and a regulatory subunit (CNB). In yeast calcineurins mediate adaptation to high salinity stress through regulation of Na^+ efflux and K^+ uptake. The *Arabidopsis* CNB gene *AtCBL1* is induced by drought, cold and wounding (Kudla et al., 1999). Coexpression of a truncated form of the yeast *CNA2* gene for CNA and the yeast gene *CNB1* for CNB confers salt stress tolerance in transgenic tobacco plants (Pardo et al., 1998). Two *Arabidopsis* genes, *STZ* and *STO*, which encode zinc-finger-like transcription factors, can complement the yeast mutants deficient of the genes for CNA and CNB, increase the

Ca²⁺ sensitivity of the yeast mutants which are more tolerant to elevated Ca²⁺ levels than the wild-type yeast, and increase salt stress tolerance in wild-type yeast (Lippuner et al., 1996). This offers an indirect evidence for the involvement of plant calcineurin genes in stress tolerance.

Although protein tyrosine phosphorylation activities have not been conclusively demonstrated in plants except for the case of MAPKK, protein tyrosine phosphatase activities do exist in plants. The salt stress induced *Arabidopsis* gene *AtPTP1* encodes a functional protein Tyr phosphatase which is able to remove the phosphate group from the phosphotyrosine residues in casein and MBP (Xu et al., 1998). It is not known if any MAPK is its proper substrate, however, it points to the probable existence of protein tyrosine phosphorylation in plants.

3. GENE EXPRESSION IN RESPONSE TO SALT AND WATER DEFICIT STRESSES AND ABSCISIC ACID

The environmental signals perceived and processed by the plant cell change the levels of activities of the genes and gene products, which determine the overall biochemical and physiological responses of the plant. Transcriptional activation or repression is the most fundamental level at which the activity of a gene is regulated, whereas posttranscriptional regulation provides additional levels of control on the expression of some genes. Moreover, the developmental program of the plant may have a critical influence on how a gene is regulated by environmental signals by providing specific intracellular environments in certain tissues at certain developmental stages. Collections of stress-inducible genes have been isolated by screening cDNAs derived

from stressed plants (Gulick and Dvořák, 1990, 1992; Kiyosue et al., 1994; Yamaguchi-Shinozaki et al., 1992) along with hundreds of other such genes cloned in many studies. In addition, changes in the profiles of *in vitro* translated proteins from mRNAs isolated from salt-stressed plants have been observed by two-dimensional SDS polyacrylamide gel electrophoresis (PAGE) and confirmed the importance of transcript regulation in stress response (Gulick and Dvořák, 1987; Ramagopal, 1987). The roles of many of these stress-regulated genes have been discussed earlier in this thesis. For many other genes whose biochemical functions are only predicted based on the sequence similarity of their encoded products to other proteins, only speculations can be made regarding their integration into stress response and adaptation physiology, or there is no functional indication at all from their deduced amino acid sequences. Nevertheless, these genes have provided excellent materials for the regulation studies, almost all of which have focused on the elevation of transcript levels of the genes. Activation of stress induced genes also provided a basis for obtaining mutants with altered stress responses. A number of mutants selected from *Arabidopsis* seeds containing the firefly luciferase reporter gene under control of the stress-inducible promoter of the *rd29A* gene displayed an enhanced response or loss of response to different stimuli such as salt, drought, low temperature and ABA (Ishitani, et al., 1997). Analysis of these mutants should identify players in the stress response and ABA signaling pathways.

3.1. Transcription Factors

At the molecular level transcriptional activation or repression of a gene requires the interaction of transcription factors with *cis*-acting elements in the promoter region of

a gene either by direct binding or through interaction with other bound transcription factors. Such interaction promotes or blocks the initiation of transcription by RNA polymerase along the promoter of a gene. Transcription factors are the link between the processed signals and the alteration of gene expression. They are localized in or translocated into the nucleus and activation of the genes encoding them often precedes the activation of their target genes in the corresponding tissues. Few stress related transcription factors have been demonstrated to promote the expression of specific genes. In some cases only the binding to DNA sequences by such proteins has been demonstrated. Moreover, no transcription repressors involved in stress and ABA response have been identified.

Basic leucine zipper factors. The bZIP transcription factors are characterized by a carboxyl terminal domain containing a region rich in basic amino acid residues and an adjacent leucine-zipper motif in which leucine residues occur every seven positions. The ZIP region which forms a coiled coil is involved in protein dimerization which is necessary for DNA binding by the basic region. One class of the bZIP factors recognizes the G-box-like elements which contain a core sequence of ACGT. These bZIP factors are involved in the activation of certain stress and ABA induced genes through binding to G-box-like ABA response elements (ABREs). Some of these G-box-binding factors (GBFs), such as the wheat EmBP (Razik and Quatrano, 1997) or the rice TRAB1 (Hobo et al., 1999b), confer the ABA-inducibility to the *Em* gene by interaction with both the *cis*-element ABRE and the transcription factor VP1/ABI3, which is involved in ABA response (McCarty et al., 1991). It is not clear if such gene activation is of any significance in the vegetative growth of plants in response to ABA and stress since the

ABI3/VP1 and *Em* genes are specifically expressed during seed development. The *Arabidopsis* gene for a GBF, GBF3, is itself induced by ABA in cell suspension cultures. GBF3 is able to bind to the promoter of the ABA-responsive gene for alcohol dehydrogenase, *Adh*, through the G-box element in *in vitro* assays (Lu et al., 1996). However, it remains to be determined whether the interaction of GBF3 and the G-box in the *Adh* promoter is responsible for the ABA induction of the *Adh* gene *in vivo*. The rice bZIP GBF, OSBZ8, is able to bind to G-box-containing sequences and its gene expression in rice seedlings is induced by dehydration and ABA (Nakagawa et al., 1996). Therefore, stress and ABA activation of genes in vegetative growth may also employ the interaction between a bZIP transcription factor and the G-box-like elements in the promoter of the gene. Two bZIP factors, DPBF-1 and DPBF-2, were isolated from carrot in a yeast one-hybrid system screen with the promoter of the carrot ABA-responsive LEA gene *Dc3* as bait (Kim et al., 1997). These bZIPs represent another class of bZIPs that recognize DNA sequences containing the E-box ACACNNG core and also have weak binding activity to the G-box sequence CACGTG, which agrees with the E-box consensus sequence. Finally, the ABA response gene *ABI5* also encodes a bZIP factor with high similarity to DPBF-1. *ABI5* is required not only for the developmental expression of certain LEA genes but also for the ABA induction of the *Arabidopsis* *Em*-like gene, *AtEm1*, during vegetative growth. It is not clear if *ABI5* activates *AtEm1* directly. (Finkelstein and Lynch, 2000).

Proteins containing the APETALA2 DNA binding motif. Two *Arabidopsis* proteins are able to bind to the dehydration responsive elements (DREs), with a core sequence of PuCCGAC, found in some stress and ABA induced genes such as *rd29A*

(Liu et al., 1998). These proteins, DREB1A and DREB2A, can activate a promoter containing the DREs in leaf protoplasts and raise the basal levels and the degree of ABA and stress induction of the *rd29A* gene in transgenic *Arabidopsis* plants. They contain the AP2 motif found in many plant proteins with DNA binding activities. The AP2 motif binds specifically to DNA sequences containing the GCC-box, with a core sequence of GCCGCC, or the DREs. It contains an 18-amino acid core region which may form an amphipathic α -helix that could be involved in protein-protein interaction to facilitate DNA binding (Okamoto et al., 1997). Interestingly, the *DREB1A* gene is induced specifically by low temperature but not by salt, drought, or ABA, whereas the *DREB2A* is induced by all these stresses and ABA. It is proposed that the two genes represent two distinct stress response pathways: one is for low temperature and is ABA-independent, and the other is for water deficit stresses such as high salinity and drought (Liu et al., 1998). Overexpression of the *DREB1A* gene in *Arabidopsis* improves the tolerance of the transgenic plants to both freezing and drought stresses, probably because the set of genes controlled by DREB1A overlap with those involved in water deficit stress tolerance. *CBF1* (*DREB1B*) is another cold stress induced gene which encodes a DRE-binding, AP2 domain-containing protein in *Arabidopsis* (Stockinger et al., 1997). In addition, the *Arabidopsis* ABA response gene *ABI4* also encodes an AP2 domain protein and its expression occurs both during seed development and in vegetative tissues, although its function in gene regulation remains to be investigated (Finkelstein et al., 1998).

MYB and MYC proteins. The MYB transcription factors contain two to three imperfect helix-turn-helix repeats, R1, R2 and R3, and exist widely in all eukaryotes, as large gene families. The MYB gene families are particularly large in plants. One of the

MYB genes from *Arabidopsis*, the dehydration, salt and ABA induced *Atmyb2*, has been characterized as being able to bind the MYB recognition sequence TAACTG and to activate promoters containing this sequence, for example, that of the stress and ABA induced *rd22* gene (Abe et al., 1997; Urao et al., 1993). The *rd22* gene can also be activated by rd22BP1, a MYC class of transcription factor (Abe et al., 1997). MYCs contain a basic helix-loop-helix-zipper (bHLH-ZIP) domain at their carboxyl termini, where the ZIP is involved in forming homodimers or heterodimers and the bHLH mediates binding to DNA containing the CANNTG consensus sequence for MYCs. The gene for rd22BP1 is induced by dehydration and salt stresses and by ABA. Therefore, that both MYB and MYC type transcription factors may mediate the stress and ABA induction of the *rd22* gene poses the question of whether the two classes of transcription factors work in concert or differentially. In addition, in a study which characterized 100 *Arabidopsis* R2R3 type MYB genes, 19 were shown to be induced by ABA and 10 by drought, which means that this class of transcription factors is important to water deficit stress and ABA responses (Kranz et al., 1998).

Other transcription factors involved in Stress and ABA responses. Genes for the homeodomain leucine zipper proteins CPHB-1 and CPHB-2 from the resurrection plant *C. plantagineum* are both induced rapidly by desiccation but differentially induced by ABA. They have been shown to be able to form heterodimers and bind to DNA containing the sequence CAAT(C/G)ATTG (Frank et al., 1998). Alfin1, an alfalfa protein which is a zinc-finger transcription factor, binds to a specific DNA sequence in the salt induced gene *MsPRP2*. Overexpression of the *Alfin1* gene in alfalfa not only enhances the expression of *MsPRP2* but also improves the salt stress tolerance (Winicov

and Bastola, 1999). Finally, the ABA response gene products ABI3/VP1 are proteins containing transcription activation domains and have been shown to activate the promoters of the wheat *Em* gene and the maize *Cl* gene (Kao et al., 1996; McCarty et al., 1991).

3.2. *Cis*-Acting Elements

DNA sequence elements located in specific positions in the promoter region of a gene determine how this gene is transcriptionally regulated through the binding of proper *trans*-acting factors, which have been already discussed. In many cases, the occurrence of a responsive element-like sequence in the promoter of a gene is not sufficient for activation by certain signals. The specific transcriptional activation of a gene may rely on the interaction of multiple elements in its promoter and a number of transcription factors from different signaling pathways. Moreover, the interaction among the transcription factors may be equally important in this aspect. The combinational effects of these *cis*-elements and *trans*-factors therefore determine the magnitude of the activation of a gene and the temporal and spatial occurrence of the activation.

G-box-like abscisic acid responsive elements and their coupling elements.

The G-box element, CACGTG, has been found in many gene promoters mediating gene activation. In the promoters of some genes which are responsive to ABA, an element of (C/T)ACGTGGC has been shown to be able to mediate ABA response (for reviews, see Busk and Pagès, 1998; Shinozaki and Yamaguchi-Shinozaki, 1997). Some sequences that are slightly different but contain the ACGT core also confer ABA-responsiveness. A gene may have more than one copy of the ABRE-like sequences in its promoter. A

detailed study was done on the barley ABA-induced gene *Hva22* by transient assays of the activities of modified promoters in response to ABA in barley aleurone tissues (Shen and Ho, 1995). There are three copies of ABRE-like sequences in the *Hva22* promoter and only the two closer to the transcription start confer ABA responsiveness and do so in an additive manner. Each of the two functional ABREs needs a downstream coupling element (CE) for full induction by ABA. One of these, CE1, with a sequence of TGCCACCGG, is also found in many other ABA inducible genes. An ABA response complex (ABRC), composed of an ABRE and its CE, may determine the response to a specific environmental signal. An additional ABRC was found in the promoter of the barley ABA induced gene *Hva1*, where the coupling element, CE3, has the sequence of ACGCGTGCCTC (Shen et al., 1996). CE3 is apparently similar to ABRE and was demonstrated to be interchangeable with and functionally equivalent to ABRE within an ABRC (Hobo et al., 1999a). Both ABREs and CE3 have been shown to bind to the bZIP transcription factor TRAB1 (Hobo et al., 1999a). So far there is no direct evidence showing an ABRE or an ABRC mediates salt and water deficit stress responsiveness of a gene.

Dehydration responsive elements. An analysis of the promoter of the salt, drought, low-temperature and ABA induced *Arabidopsis* gene *rd29A* revealed a DNA sequence, TACCGACAT, conferring inducibility by dehydration or salt stress to the gene in transgenic tobacco plants (Yamaguchi-Shinozaki and Shinozaki, 1994). This DRE may function by itself for providing responsiveness specifically to dehydration, high salinity, or low temperature, but not to ABA. Since the *rd29A* gene also has an ABRE-like sequence in its promoter and is responsive to ABA, its expression might be induced

independently by ABA mediated by this putative ABRE and a yet to be defined CE. The DRE resembles the GCC-box of *cis*-acting elements which have a core sequence of GCCGCC and interact with the AP2 class of plant transcription activators. Indeed, two *Arabidopsis* proteins containing the AP2 motif, DREB1A and DREB2A, are able to interact with the *rd29A* DRE and mediate the stress response (Liu et al., 1998). As mentioned earlier, the *DREB1A* gene responds specifically to cold stress while the *DREB2A* gene is induced by dehydration and high salinity but not by low temperature. Thus three independent signaling pathways all lead to the activation of the *rd29A* gene: 1) from cold stress to *DREB1A* to the DRE in *rd29A*; 2) from water deficit stress to *DREB2A* to the DRE in *rd29A*; and 3) from water deficit stress to ABA to a bZIP to the putative ABRE in *rd29A*. The DRE was also found to bind to the *Arabidopsis* CBF1/DREB1B, which is closely related to DREB1A and its gene is regulated by low temperature (Liu et al., 1998; Stockinger et al., 1997).

MYB and MYC recognition elements. The consensus of the MYB transcription factor recognition sequence is (C/T)AAC(G/T)G, or C(A/C)GTT(A/G) in complementary orientation; whereas the consensus of the MYC protein recognition sequence is CANNTG. A 67-bp fragment in the promoter region of the *Arabidopsis* dehydration, salt and ABA induced gene *rd22* is sufficient to confer responsiveness to dehydration and ABA treatments to a reporter gene in transgenic plants (Iwasaki et al., 1995). This DNA fragment contains two putative MYC recognition sites, one MYB recognition site, as well as an ACGT sequence. Both the proximal MYC site, CACATG, and the MYB site, TGGTTAG, are necessary for the full induction of the *rd22* promoter by dehydration (Abe et al., 1997). A composite promoter containing that 67-bp *rd22*

promoter fragment can be activated by the MYB protein encoded by the stress and ABA induced gene *Atmyb2* (Abe et al., 1997). The proximal MYC site in this fragment can interact with the stress induced gene product rd22BP1, which is a MYC protein, and thereby the promoter is activated (Abe et al., 1997).

Other *cis*-acting elements. The sequence CGTGTCGTCCATGCAT in the maize *C1* gene is necessary and sufficient for ABA induction. The sph-element-like sequence contained in this sequence, CGTCCATGCAT, is necessary for the activation of *C1* by VPI (Kao et al., 1996). Four tandem copies of a 21-bp sequence Hex III, that is, TTCGGCCACGCGTCCAATCCG, confers both gene expression in mature tobacco seeds and responsiveness to drought and salt stresses and ABA in transgenic tobacco plant seedlings (Lam and Chua, 1991). The Motif III sequence, GCCGCGTGGC, is necessary for ABA induction of the rice *rab16B* gene (Ono et al., 1996). In the promoter region of the carrot ABA-inducible gene *Dc3* a consensus sequence, ACACNNTG, occurred four times and each copy was shown to bind two bZIP proteins, DPBF-1 and DPBF-2 (Kim et al., 1997). It is interesting that this element is similar to the G-box-like elements and both classes of the elements are recognized by bZIP proteins. Some DNA sequences have been shown to bind to nuclear protein extracts, they include Motif IIa and Motif IIb, CCGCCGCGCCTG and CCGCCGCGCTG, respectively, from the rice ABA responsive gene *rab16A* (Mundy et al., 1990) and the (A/G)(A/G)CCC(A/G)(A/G) sequences from the ABA inducible promoter of the resurrection plant *C. plantagineum* gene *CDeT27-45* (Nelson et al., 1994).

3.3. Chromatin Structures

Genomic DNA wraps around the histone proteins to form nucleosomes which are the basic units of chromatin. The accessibility of RNA polymerase and transcription factors to the promoter region of a gene is affected by the presence of nucleosomes. Therefore, remodeling of chromatin structure provides a level of regulation of expression. It is observed that the *Arabidopsis* ABA and desiccation induced *Adh* gene is associated with different nucleosome structures in induced and uninduced conditions (Vega-Palas and Ferl, 1995). Binding of a transcription factor to the *Adh* promoter is weaker *in vivo* than *in vitro*. This indicates that the nucleosome structure inhibits the binding *in vivo* (McKendree et al., 1990). Also, the binding of EmBP-1, a bZIP protein, to ABREs is affected by the positions of the nucleosomes in *in vitro* assays (Niu et al., 1996). The fact that the linker histone H1 gene, *His1-3*, from *Arabidopsis* is inducible by drought reinforced the possibility that altered chromatin structures are involved in regulating stress related genes since linker histones binds to the external surface of nucleosomes thus linking adjacent nucleosomes and contributing to the determination of chromatin structure (Ascenzi and Gantt, 1997).

3.4. Posttranscriptional Regulation

Regulation of the activity of a gene at the posttranscriptional levels includes transcript processing, transcript stability, translation efficiency, protein modification, and protein turnover. Very few studies have addressed these gene regulation mechanisms. For the salt-induced alfalfa gene *MsPRP2* the mRNA stability is important for the accumulation of the transcripts (Deutsch and Winicov, 1995). Translational control of

genes with constant mRNA levels has been observed in the desiccation tolerant moss *Tortula ruralis* where, in response to slow dehydration, some mRNA species were sequestered into messenger ribonucleoprotein particles (mRNPs) which inhibit the translation of the trapped mRNAs (Wood and Oliver, 1999).

Protein phosphorylation is the major mechanism for posttranslational modification of certain proteins and it has already been discussed in the section for protein phosphorylation in stress signaling. Other mechanisms of protein modification, for example, farnesylation and glycosylation, may also play a role in regulation of the activity of a protein. In addition, activation of a protein could result from peptide cleavage by a specific protease, while protease inhibitors could regulate the activities of proteases. Several genes for proteases and protease inhibitors that are induced by salt, drought and low temperature stresses and ABA have been identified as discussed previously (for review, see Ingram and Bartels, 1996). Moreover, these proteases may function in the controlled degradation of certain proteins thereby regulating their activities in a cell.

4. TECHNICAL DEVELOPMENTS IN THE RESEARCH ON STRESS SIGNALING

Developments in genomics studies in the past decade have revolutionized the way researchers conduct their studies in almost every aspect of plant molecular genetics and its application in plant biochemistry and plant physiology. The completion of the sequencing of the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000) and the release of the rice genome sequence draft to the public will make it possible to view

the gene compositions of the two organisms. These sequence data, together with the availability of expressed sequence tags (ESTs) from *Arabidopsis* and many other species including rice, maize and soybean, substantially reduced the effort in identifying genes of special interest. It is also possible to build a phylogenetic tree for any of the multigene families from *Arabidopsis* and rice and thus to learn the differential gene expression patterns and the specific and redundant activities of the related genes.

With the powerful technique of DNA microarray or DNA chip, expression of thousands of genes can be characterized at the same time, instead of a few at a time by the classical northern blot hybridization analysis. Such global views of gene expression provide new clues as to how plants respond to environmental and developmental cues and open new avenues for advances in related research. On the other hand, development in proteomics studies will not only parallel microarray data, but also detect gene regulation at post-transcriptional levels. Currently, the available techniques in proteomics include two-dimensional electrophoresis, the yeast two-hybrid system for protein-protein interaction, mass spectrometry and other analysis tools. In addition, determining the three-dimensional structures of proteins would help to understand how proteins work in relation to other molecules in a plant cell and may suggest activity for the proteins with unknown functions, for example, the dehydrins and other LEA proteins.

Manipulation of the expression of a gene should give clues to the functions the gene may have in stress response and signaling. Altering the expression of a gene in a plant provides important genetic evidence for the activity of the gene. Collections of thousands of *Arabidopsis* T-DNA insertion mutants are available and can be screened by polymerase chain reaction. However, these mutants do not cover the complete set of the

genes of the *Arabidopsis* genome. Alternative ways to obtain null mutant lines are by overexpression of antisense sequences, by cosuppression resulting from the expression of the sense sequences, or by dsRNA-mediated gene silencing. In overexpression studies, constitutive expression of a gene may have toxic effects on plant cells. Efforts have been made to establish systems in which genes may be activated under the control of inducible promoters, for example, promoters that can be activated by ethanol, dexamethasone, or by heat shock.

Those gene manipulation techniques rely on the possibility to transform a particular plant species with foreign DNA. *Arabidopsis* can be readily transformed with the *in planta* infiltration technique with *Agrobacterium tumefaciens* for DNA delivery. This technique is simple and efficient and gives almost no somatic segregation. But for other plant species more laborious methods of transformation are used, such as regeneration of plants from callus or other tissues infected by *Agrobacterium* or bombarded with DNA-coated particles. Plant transformation is also important for the application of the research results in improving stress tolerance traits of many crop species.

5. THE TALL WHEATGRASS *LOPHOPYRUM ELONGATUM*

5.1. *Lophopyrum elongatum* Is Highly Salt Tolerant

My thesis is based on the results I have obtained from the experimental research on the salt stress induced protein kinase gene *Esi47* from the wild tall wheatgrass *Lophopyrum elongatum* (Host) A. Löve [synonym *Elytrigia elongata* (Host) Nevski.

Agropyron elongatum Host]. *L. elongatum*, which naturally occurs in the littoral zones and salt marshes of the Mediterranean region, is highly salt tolerant. Like many other wild wheatgrass species, the *L. elongatum* plants are able to survive and set seed while growing in a hydroponic solution containing 500 mM NaCl (McGuire and Dvořák, 1981). Phylogenetically, *L. elongatum* is a very close relative of wheat *Triticum aestivum*. Many grass species in the Triticae are polyploids. For example, wheat is a hexaploid ($2n = 6x = 42$) with a genome formula of AABBDD, where AA, BB and DD represent the genomes of the ancestral diploid parental species of wheat. But *L. elongatum* is a diploid ($2n = 2x = 14$) and its genome formula is EE, which indicates another derivative of the ancestral grass genome. Being a diploid makes it a convenient species for genetic studies. More importantly, since the homologous chromosomes of the grass species are interchangeable, *L. elongatum* serves as an excellent source of genetic materials for breeding for salt stress tolerance of wheat, a salt sensitive species. The availability of the amphiploid between *L. elongatum* and the Chinese Spring wheat variety, the complete set of the *L. elongatum* chromosome subtraction lines derived from this amphiploid and the complete set of *L. elongatum* chromosome substitution lines in the Chinese Spring background have already contributed to the cytogenetic studies on the salt stress tolerance of these grass species. That the amphiploid is more salt tolerant than Chinese Spring wheat indicating that the genetic determinants for the stress tolerance in *L. elongatum* can be transferred and expressed on the wheat background (Omielan et al., 1991). In addition, the *L. elongatum* chromosome 3E contributes more than the other chromosomes to the elevated stress tolerance when the substitution lines are compared. Due to the close genetic relationship between *L. elongatum* and wheat, genetic

modification of wheat by transformation with the genes responsible for salt tolerance from *L. elongatum* would cause less impact on other physiological and agronomical traits of wheat than would genes from an unrelated species.

L. elongatum is a facultative halophyte. Physiological studies which compared the salt-tolerant *L. elongatum* × wheat amphiploid and the salt-sensitive wheat suggest that salt tolerance might be due to the exclusion of Na⁺ and Cl⁻ and inclusion of K⁺ in general (Omielan et al., 1991). What also makes a contribution is the differential distribution of solutes in such a way that the plants maintain low Na⁺ and high K⁺ levels and accumulate glycine betaine in young leaf tissues (Colmer et al., 1995). Moreover, *L. elongatum* shows tolerance not only to NaCl, but also to other mineral salts such as Mg²⁺, K⁺ and SO₄²⁻, as well as sea water probably by additional mechanisms (Dvořák and Ross, 1986).

5.2. The *Early Salt-Stress-Induced Genes* from *Lophopyrum elongatum*

Changes in gene expression in response to salt treatment have been observed in the *L. elongatum* × Chinese Spring wheat amphiploid roots but not in the leaves as revealed by two-dimensional electrophoresis of proteins translated from mRNAs (Gulick and Dvořák, 1987). Eleven salt-induced cDNA species were cloned from the roots of *L. elongatum* treated with 250 mM NaCl for 6 h from a differential screen (Gulick and Dvořák, 1990, 1992). The corresponding genes are designated *Early salt-stress-induced (Esi)* genes. All these genes respond to NaCl treatment rapidly and, except for *Esi47*, reach maximal induction within 6 h. The transcript levels for most of the genes drop to pretreatment levels 24 h after the onset of stress. However, the mRNA levels of *Esi3*,

Esi14 and *Esi47* stay high for at least 3 days. All the cDNAs give rise to single bands in northern hybridization analysis, except for *Esi18* which detects at least three transcript bands, suggesting a possibility that this gene represents a multigene family. The salt induction of the *Esi* genes is specific in roots rather than in shoots, except for *Esi15*, which can also be induced by salt in shoots (Galvez et al., 1993). All these genes are also induced by ABA and osmotic stress created by mannitol treatment in a similar way to the treatment by salt. Therefore, these *Esi* genes may be responsible for the responses of the plant to hyperosmotic stress. Osmotic shock may be the signal for such responses since three steps of incremental increases of salt concentration to 250 mM renders much less or almost no induction of the genes.

All these *Esi* genes also exist in each set of the wheat genomes and have been mapped in the *L. elongatum* and wheat chromosomes (Dubcovsky et al., 1994). There is only a single copy of all of the *Esi* genes in each haploid genome except for *Esi2* and *Esi18*, with two and at least five copies, respectively, in each genome. Remarkably, the salt induction rates of all these *Esi* genes in the salt tolerant *L. elongatum* are higher than those in the salt sensitive Chinese Spring wheat (Galvez et al., 1993). Such positive correlations between the levels of gene induction and the degrees of stress tolerance suggest certain active roles of these genes in adaptation to stress. Since these *Esi* genes are not unique to *L. elongatum*, it may be possible that the difference in stress tolerance between *L. elongatum* and wheat is due to differential gene regulation in the two genomes.

The first cDNA clones for the *Esi* genes contained mostly short cDNA inserts (Gulick and Dvořák, 1992). DNA sequencing of these cDNAs and the subsequent

isolation of longer or full-length cDNAs revealed the identities of most of these *L. elongatum* *Esi* genes (Table 1). As expected, *Esi18* is one member of the dehydrin multigene family. Five member genes in this family have been isolated and all of them are salt inducible (Gulick and Dvořák, 1992; W. Shen and P.J. Gulick, unpublished data). *Esi35* is also a dehydrin gene but its product shows extensive dissimilarity to those typical dehydrins such as the rice Rab16 and the barley Dhn dehydrins (Gulick and An, 1993); instead, *Esi35* is similar to the wheat acidic dehydrin wcor410, which has been implicated in low-temperature tolerance (Danyluk et al., 1998).

Esi3 encodes a small protein, 54 amino acid residues in length, with two putative membrane-spanning hydrophobic segments (Gulick et al., 1994). Genes for identical or very similar proteins have been isolated from barley and *Arabidopsis* and shown to be induced specifically by low temperature but, interestingly, not by salt, drought, or ABA (Capel et al., 1997; Goddard et al., 1993). It has been suggested that the *Esi3*-like proteins may stabilize membranes or to protect membrane proteins (Capel et al., 1997).

The gene product of *Esi28* is an actin depolymerizing factor (Shen et al., 1999). A nearly identical gene from wheat, *Wcor719*, is inducible by low temperature (Danyluk et al., 1996). The involvement of actin filament state in regulating membrane ion channels has been implicated in the light-induced stomatal opening in guard cells (Hwang et al., 1997). The root specific induction of the *Esi28* gene by salt implies that this gene may be one of the regulators in controlling the permeability of the root cell membrane ion channels.

KCl induces three *Esi2*-like transcripts from *L. elongatum* roots instead of only one from the NaCl treated roots and there are two loci for the *Esi2*-like genes in the *L.*

Table 1. The Early salt-stress-induced genes from *Lophopyrum elongatum*.

Gene	Product	mRNA size (kb)	GenBank accession No.	Note
<i>Est2</i>	unknown function	1.2	-	Pro-rich and a domain resembling part of a nodulin
<i>Est3</i>	small hydrophobic protein	0.6	U00966	two membrane-spanning segments
<i>Est4</i>	N.D.	1.0*	-	partial cDNA
<i>Est14</i>	serine protease	1.4*	-	partial cDNA
<i>Est15</i>	N.D.	1.1*	-	partial cDNA
<i>Est18</i>	dehydrins	0.7, 0.7, 1.1, 1.5, 2.1*	-	a multigene family with five members cloned
<i>Est28</i>	actin depolymerizing factor	0.9	AF196350	
<i>Est32</i>	N.D.	1.3*	-	partial cDNA
<i>Est35</i>	acidic dehydrin	1.2	L19419	
<i>Est47</i>	protein kinase	1.9	AF131222	
<i>Est48</i>	aspartate aminotransferase	1.0*	-	partial cDNA

The identities of the gene products were defined by comparing the DNA sequences of each gene to those in the GenBank database with TBLASTX searches. The sizes of the mRNAs were determined according to the sizes of the full length cDNA inserts or coding region prediction from the genomic DNA sequences, except for those indicated by a star (*), which were estimated from northern blot analysis (Gulick and Dvořák, 1992). The references for some of the genes are: Gulick et al., 1994 (*Est3*); Shen et al., 1999 (*Est28*); Gulick and An, 1993 (*Est35*). N.D., not determined.

elongatum genome (Dubcovski et al., 1994; Galvez et al., 1993). Accordingly, two *Esi2*-related genes have been isolated and at least one of them is induced by salt stress. The patterns of salt regulation of the *Esi2* genes remain to be determined. The proteins encoded by the two *Esi2* genes are rich in proline at their carboxyl termini; and the amino termini resemble part of the nodulin proteins (P.J. Gulick, unpublished data). Further characterizations need to be carried out before any functional speculation could be made for *Esi2*s in stress response and tolerance.

Esi47 encodes a protein Ser/Thr kinase (this study) and *Esi14* encodes a serine protease (P.J. Gulick, unpublished data), both groups of proteins have been discussed in earlier sections. The gene product of *Esi48* is aspartate aminotransferase, however, no functional speculations could be made so far for a role of this enzyme in salt and water deficit stress response in plants (W. Shen and P.J. Gulick, unpublished data). The identities for the remaining three *Esi* genes, *Esi4*, *Esi15* and *Esi32*, are yet to be determined.

5.3. *Esi47* May Be Involved in Stress Signaling

My research for this thesis has been focused on the structural and functional characterization of the protein kinase gene *Esi47* and its potential role in stress signaling. I isolated both full-length cDNA and genomic DNA clones for this gene. Sequences of the clones revealed that the gene possesses unique features such as a 5'-untranslated region (UTR) intron and a short upstream open reading frame (uORF). The kinase activity of *Esi47* was demonstrated in the bacterium *Escherichia coli*. Biolistic introduction of this gene into the barley aleurone tissue caused suppression of the GA

induction of the gene for α -amylase. I also studied the expression patterns of the three *Arabidopsis* homologs of the *Esi47* gene and found that these genes are differentially regulated by salt stress and ABA in *Arabidopsis* roots and leaves. At least one of these *Arabidopsis* homologs, *F8A24.12*, also contains a 5'-UTR intron and a small uORF. Identification of the *Arabidopsis Esi47* homologs would make it possible for the future manipulation of their expression in efforts to further decipher the role of the *Esi47*-like genes in stress response and adaptation.

PART II. MATERIALS AND METHODS

1. PLANT GROWTH

1.1. *Lophopyrum elongatum*

In this study, *Lophopyrum elongatum* (Accession C) was grown hydroponically. Seeds of *L. elongatum* were surface sterilized with 12% (w/v) sodium hypochloride and approximately 0.5% (v/v) Tween 80 for 15 min and rinsed with sterile water extensively. Treated seeds were mounted onto water-soaked, autoclaved filter paper supported by slant boards. Seeds were imbibed at 4°C for 3 days and then moved to greenhouse for germination. Seedlings with 5- to 10-cm long roots were transplanted to a modified Hoagland solution, which contains 3 mM KNO₃, 1 mM NH₄H₂PO₄, 0.5 mM MgSO₄, 5.5 mM Ca(NO₃)₂, 25 μM KCl, 12.5 μM H₃BO₃, 1 μM MnSO₄, 1 μM ZnSO₄, 0.25 μM CuSO₄, 2 μM H₂MoO₄ and 50 mg/l [(O₂CCH₂)₂NCH₂CH₂N(CH₂CO₂)₂]FeNa·2H₂O (Fe-EDTA, Aldrich), pH 6.5 (Gulick and Dvořák, 1987). Constant aeration was provided. For salt treatment, a concentrated NaCl solution was added to the hydroponic solution with agitation. The final concentration of NaCl was 250 mM and the duration of treatment was 6 h.

1.2. *Arabidopsis thaliana*

The *Arabidopsis thaliana* Colombia ecotype strain Col-0 and transgenic lines CS6265 (expressing the *Agrobacterium tumefaciens tms2* gene for amidohydrolase) and CS8035 (expressing the *Brassica napus FAD3* gene for omega-3 fatty acid desaturase)

were used in this study. The seeds were obtained from the Arabidopsis Biological Resource Center.

Plants for seed production, transformation and salt tolerance assays were grown in greenhouse in a composite containing equal parts of black earth, peat moss, perlite and vermiculite and watered with 1 g/l of the fertilizer Plant-Prod 20-20-20 (Plant Products Co.) if necessary.

For RNA extraction for assessing the function of the *Esi47* transgene in *Arabidopsis*, seeds were surface sterilized in the same way as *L. elongatum* seeds. Sterilized seeds were plated in Petri dishes containing a medium of 1 × Murashige and Skoog (MS) salts, 1 × Gamborg's B-5 vitamins, 1% sucrose, and 1.2% agar. The plates were kept at 4°C for 3 days and moved to growth room under constant illumination of an average light intensity of 5000 lux at 22°C. After four days, the seedlings were transferred to 250-ml flasks containing 75 ml of a liquid medium of 1 × MS salts and 1% sucrose. Each flask contained 50 seedlings and was placed on a shaker rotating at 100 rpm under constant lights. After growing for 6, 10, 14, or 18 more days, the plants were collected and frozen in liquid N₂. Some the plants were treated with 250 mM NaCl for 6 h before being frozen.

For RNA extraction from the wild-type plants for studying gene regulation pattern of the *Arabidopsis Esi47*-homologs, plants were grown hydroponically in a modified Hoagland solution according to Gibeaut et al. (1997), which contains 1.25 mM KNO₃, 1.5 mM Ca(NO₃)₂, 0.75 mM MgSO₄, 0.5 mM KH₂PO₄, 50 μM KCl, 50 μM H₃BO₃, 10 μM MnSO₄, 2 μM ZnSO₄, 1.5 μM CuSO₄, H₂MoO₄, and 2.64 mg/l [(O₂CCH₂)₂NCH₂CH₂N(CH₂CO₂)₂]FeNa·2H₂O (Fe-EDTA, Aldrich), pH 6.0. The light

intensity was approximately 5000 lux and the temperature was 22°C. The photoperiod for the first 5 weeks of growth was 8 h light and 16 h dark, and plants were then switched to constant light for 2 days before treatments with either 250 mM NaCl or 100 µM abscisic acid (ABA, a mixture of isomers, Sigma). The durations of treatments were 2, 6 and 24 h, respectively; control plants were not treated. Roots and leaves were collected separately.

2. BASIC MOLECULAR BIOLOGY TECHNIQUES

2.1. Routine Techniques

Most procedures involving recombinant DNA techniques and molecular characterizations were carried out according to Sambrook et al. (1989) and Innis et al. (1990). Sometimes the instructions from the providers of enzymes or chemicals were also followed. These procedures include enzymatic reactions with restriction endonucleases and DNA and RNA modifying enzymes, polymerase chain reaction (PCR) amplification, agarose gel electrophoresis of DNA, DNA purification by phenol extraction and ethanol precipitation, restriction mapping and transformation of *Escherichia coli* cells. The *Pfu* polymerase (Stratagene) was used for PCR when the amplified DNA fragments were used for cloning. Plasmid DNAs were prepared with the columns from the QIAprep Spin Miniprep Kit or the QIAGEN Plasmid Midi Kit (Qiagen). Restriction or PCR DNA fragments were gel-purified with the QIAEX II Gel Extraction System (Qiagen).

2.2. Plant DNA Extraction

L. elongatum nuclear DNA was extracted from leaves that had been cut into 1- to 2-cm pieces. Seventy-five ml of H buffer (10 mM Tris base, 10 mM EDTA, 80 mM KCl, 1 mM spermidine and 1 mM spermine, pH 9.4) was added to each 10 g of tissues. The leaves were then homogenized in a blender at low speed three times, 10 sec each time. After filtering through eight layers of cheesecloth, the extracts were mixed with 0.025 volume of 20% (w/v) Triton X-100 diluted in H buffer by gentle swirling. The mixture was kept on ice for 10 min and then centrifuged at $1670 \times g$ for 20 min. Pellets were suspended in 5 ml of H buffer containing 0.5% (w/v) Triton X-100 with the help of a paintbrush. Once suspended, another 25 ml of the same solution was added and centrifuged again. This time the pellets were suspended in 1 ml of 2 mg/ml proteinase K (Boehringer Mannheim) in PDIB buffer (50 mM Tris base, 100 mM EDTA and 100 mM NaCl, pH 8.0). Later, 10 ml of PDIB buffer containing 0.5% SDS, preheated to 65°C, were added. After the proteinase K treatment at 65°C for 20 min, DNA was extracted with an equal volume of phenol/chloroform (1:1) and precipitated with the addition of 3 M NaAc, pH 5.2, and ethanol. DNA precipitates were collected with a glass rod, washed several times in 70% (v/v) ethanol, briefly dried and finally dissolved in 5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA would not completely dissolve into the buffer and, therefore, aliquots of the supernatant were used for subsequent experiments.

2.3. Plant RNA Extraction and Poly(A⁺)-RNA Purification

Plant tissues were ground in liquid N₂ into fine powers. To each gram of powdered tissue, 5 ml of RNA extraction buffer [0.2 M Na₂B₄O₇, 30 mM EGTA, 2% (w/v) SDS and 1% sodium lauryl sarcosine, pH 9.0] and 5 ml of phenol/chloroform (1:1) was added. The mixture was shaken vigorously several times during an incubation of at least 10 min at room temperature (approximately 22°C). After centrifugation supernatant fluid was extracted once more with phenol/chloroform and twice with chloroform. RNA was precipitated by the addition of an equal volume of 4 M LiCl and incubation at 4°C for 16 to 24 h. The RNA pellet was collected by centrifugation at 12000 × g for 1 h at 4°C, washed once with 2 M LiCl and dissolved in 10 mM Tris-HCl, pH 7.5. RNA was precipitated again by adding one-tenth volume of 3 M NaAc, pH 6.0, and 2.5 volumes of 95% (v/v) ethanol and kept at -20°C for 16 to 20 h. The RNA was recovered by centrifugation and washed in 70% (v/v) ethanol. Finally, extracted RNA was dissolved in water to a concentration of approximately 1 mg/ml.

Poly(A⁻)-RNA was purified from total RNA extracts with poly(U) Sephadex (Gibco BRL) columns. Poly(A⁻)-RNA was bound to the column in a solution containing 0.2 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.2% SDS, eluted with 90% (v/v) formamide, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.2% SDS. To the fractions of eluted RNA, NaCl was added to a final concentration of 0.4 M. Poly(A⁻)-RNA was precipitated by adding 2 volumes of 95% (v/v) ethanol and stored at -80°C for at least 16 h. Samples were centrifuged, and the precipitates were washed in 70% (v/v) ethanol and dissolved in water.

2.4. Bacterial Phage DNA Extraction

A 50-ml *E. coli* XL-1 Blue (P2) (Stratagene) culture in LB medium containing 10 mM MgSO₄ and 0.2% maltose was grown with shaking at 37°C till its A_{600nm} reached around 0.5 O.D. Two hundred µl of a λ phage solution with a titre of at least 10¹¹ pfu/ml was added to the bacterial culture. The mixture was left at room temperature for 10 min and incubated at 37°C with shaking for about 5 h till the culture became clear. The phage culture was then chilled in ice. One ml of chloroform was added and the culture was shaken briefly and left on ice for 10 min. Then 3 g of NaCl was added to the phage solution, and the solution was incubated at room temperature with shaking for 20 min and centrifuged at 12000 × g for 30 min. To the supernatant fluid 100 µg of each of DNase I and RNase A was added and incubated at 37°C for 30 min. To precipitate phages, 3.75 g of polyethylene glycol (average molecular weight 8000) was added and the mixture was shaken on a rotary shaker at room temperature for 1 h, incubated on ice for 30 min and centrifuged at 12000 × g for 30 min. The phage pellet was suspended in 500 µl SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCl, pH 7.5, and 0.01% gelatin), 5 µl 10% (w/v) SDS and 5 µl 0.5 M EDTA, pH 8.0, and incubated at 65°C for 10 min. After phenol/chloroform (1:1) extraction and chloroform extraction, phage DNA was precipitated with the addition of NaAc and ethanol, washed in 70% (v/v) ethanol, briefly dried and dissolved in TE buffer.

2.5. Radioactive Labeling of DNA Probes

Twenty-five ng of DNA fragments were used for radioactive labeling with the Multiprime DNA Labeling System (Amersham). Each reaction included random

hexamer oligonucleotides, the large fragment of the DNA polymerase I (the Klenow enzyme) and 50 μCi of [α - ^{32}P]dCTP (3000 Ci/mmol, ICN). Labeling was carried out at room temperature for 3 h. Unincorporated nucleotides were removed by passing the labeling mixture through a MicroSpin S-200 HR spin column (Pharmacia). The total radioactivity of labeled probe was usually between 1×10^7 to 1×10^8 cpm. Labeled DNA fragments were boiled for 5 min before being added to hybridization solution.

2.6. Southern Blot Hybridization

Restriction enzyme digested DNA fragments were separated in 0.7% agarose gels and transblotted to the Hybond N⁻ nylon filters (Amersham) with 20 \times SSC (3 M NaCl, 0.3 M sodium citrate). After being fixed at 80°C for 2 h, the filters were prehybridized in 5 \times SSC, 5 \times Denhardt's solution (1 \times solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.5% SDS and 100 $\mu\text{g}/\text{ml}$ denatured herring sperm DNA at 65°C for 1 h. For hybridization, denatured ^{32}P -labeled DNA fragments were added to the prehybridization solution and incubated at 65°C for 16 to 20 h. Filters were washed in 1 \times SSC, 0.1% SDS at room temperature several times. Finally the filters were washed twice, 15 min to 1 h each time, in 1 \times SSC, 0.1% SDS at 65°C. Autoradiography was done at -80°C with the Lightening Plus intensifying screen (DuPont).

2.7. Northern Blot Hybridization

Total RNA was extracted from *Arabidopsis* seedlings grown as described above and dissolved in water. Twenty μg of each sample were dried and dissolved in 9 μl of a solution containing 1 part of 10 \times MOPS buffer (0.2 M MOPS, pH 7.0, 80 mM NaAc,

and 10 mM EDTA), 3.5 parts of formaldehyde, 10 parts of formamide, and 5.5 parts of water. After RNA was dissolved, it was denatured by incubation at 65°C for 15 min. Then 1 µl of a solution containing 50% (v/v) glycerol, 1 mM EDTA, pH 8.0, and 0.25% bromophenol blue was added. Such RNA samples were separated in 1% denaturing agarose gels containing 1 × MOPS buffer and 2.2 M formaldehyde. After electrophoresis in 1 × MOPS buffer, the RNA gel was washed in 20 × SSC for 20 min. RNAs were transblotted to the Hybond N nylon filters (Amersham) and fixed at 80°C for 2 h. The filters were prehybridized in 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution, 0.5% SDS and 100 µg/ml denatured herring sperm DNA at 42°C for 1 h. A denatured ³²P-labeled DNA probe was added to the prehybridization solution and hybridization was carried out at 42°C for 16 to 20 h. Procedures for filter washing and autoradiography were the same as those for Southern analysis, except that the final two washings were in 1 × SSC and 0.1% SDS at 50°C. The probes for the *Arabidopsis* genes were the ³²P-labeled restriction fragments of the entire cDNA inserts of the expressed sequence tag (EST) clones which are listed in Table 2 and were obtained from the Arabidopsis Biological Resource Center.

2.8. DNA Sequencing

Nucleotide sequence of DNA was determined by the dideoxyribonucleotide termination method with the Sequenase DNA sequencing kit (USB). [α -³⁵S]dATP (1000 Ci/mmol, ICN) was used for labeling. Occasionally, DNA samples were also sent to the Centre for Applied Genetics at the Hospital for Sick Children, Toronto, Ontario, for automated sequencing. Sequences of large DNA fragments were determined with the

Table 2. *Arabidopsis thaliana* genes analyzed for regulation by *Esi47* in transgenic *Arabidopsis* by northern blot hybridization

Gene	GenBank accession No.	Gene product	EST clone
A. Genes upregulated by stress or ABA			
<i>ABI1</i>	X77116	type 2C protein phosphatase	250D22T7
<i>ABI2</i>	Y08965	type 2C protein phosphatase	166I6T7
<i>ARSK1</i>	L22302	NAK group protein kinase	166N10T7
<i>ATCDPK2</i>	D21806	CDPK	182C10T7
<i>ATCP1</i>	AF009228	calcium binding protein	305H10T7
<i>ATECP31</i>	D64139	Group 5 LEA protein	153B6T7
<i>ATECP63</i>	D64140	Group 3 LEA protein	ATTS2716
<i>ATHB12</i>	AF079587	homeobox Leu zipper protein	215K8T7
<i>ATMEKK1</i>	D50468	MAPKKK	23A12T7
<i>ATMPK3</i>	D21839	MAPK	77D1T7
<i>ATP5CS1</i>	X86777	P5CS	ATTS3925
<i>ATP5CS2</i>	Y09335	P5CS	G2E1T7
<i>ATPIP5K</i>	AB005902	phosphatidylinositol-4-phosphate 5-kinase	ATTS3354
<i>ATRR1</i>	AB010915	two component response regulator	ATTS0721
<i>ATRR2</i>	AB010916	two component response regulator	103N10T7
<i>ATSEH</i>	D16628	soluble epoxid hydrolase	96B7T7
<i>CBL3</i>	AF076253	calcineurin subunit B	97E24T7
<i>DI19</i>	X78584		181N5T7
<i>DI21</i>	X78585		162M5XP
<i>DREB2.1</i>	AB007790	AP2 domain transcription factor	ATTS4926
<i>ERD1</i>	D17582	regulatory subunit of ClpA protease	E7E10T7
<i>ERD5/PRO1</i>	U59508	Pro dehydrogenase	65F8XP
<i>ERD6</i>	D89051	sugar transporter	206M14T7
<i>ERD10/LIT29/LIT45</i>	X77614	dehydrin	185C8T7
<i>ERD11</i>	D17672	GST	206N21T7
<i>ERD13</i>	D17673	GST	211H3T7
<i>ERD14</i>	D17715	dehydrin	34A9T7
<i>ERD15</i>	D30719		E12H3T7
<i>GSK1</i>	AF019927	GSK/shaggy-like protein kinase	212D21T7
<i>GST8</i>	AJ012571	GST	202N7T7
<i>ICK1</i>	AF001949	CDK inhibitor	156J24T7
<i>KLN1</i>	X51474	antifreeze protein	ATTS0371
<i>KLN2/COR6.6</i>	X62281	antifreeze protein	103L16T7
<i>PROT2</i>	X95738	Pro transporter	203L17T7

(To be continued)

Table 2.

Gene	GenBank accession No.	Gene product	EST clone
<i>RAB18</i>	X68042	dehydrin	104G6T7
<i>Ran1</i>	AF017991	GTP binding protein	194L7T7
<i>RD17/COR4⁻</i>	X59814	dehydrin	159B22T7
<i>RD19A</i>	D13042	thiol protease	H10D10T7
<i>RD21A</i>	D13043	thiol protease	187D5T7
<i>RD22BP1</i>	AB000875	MYC-like protein	G5C4T7
<i>RD28</i>	D13254	transmembrane channel protein	222N5T7
<i>RD29A/LIT65</i>	D13044		ATTS3065
<i>RD29B/LIT⁻8/LIT140/COR⁻8</i>	D13044		H5B12T7
<i>SIMP</i>	AF003728	plasma membrane intrinsic protein	ATTS1629
<i>STZ</i>	X95573	zinc finger protein	186P13T7
<i>XERO1</i>	X64199	dehydrin	194D19T7
<i>XERO2/LIT30</i>	X77613	dehydrin	ATTS0613
?	AF004393	tonoplast intrinsic protein	88K23XP
?	AJ002597	membrane associated protein	171H24T7
?	X66023	Group 1 LEA protein	158D2T7
<i>FXA24.12</i>	AC015985	NAK group protein kinase	F3A9T7
<i>F12E4.50</i>	AL162751	NAK group protein kinase	311H3T7
<i>TF6.28</i>	AC005770	NAK group protein kinase	193H7T7
<u>B. Genes downregulated by stress or ABA</u>			
<i>ATGRP2B</i>	U39072	Gly-rich protein	ATTS2494
<i>CCR1/ATGRPs</i>	L04171	RNA binding Gly-rich protein	172M6T7
<i>CCR2/ATGRP⁻</i>	L04172	RNA binding Gly-rich protein	212I11T7
<i>DR4</i>	X78586	Kunitz protease inhibitor	35C10T7
<u>C. Control genes</u>			
<i>ACT2</i>	U41998	actin	E7C1T7
<i>FPS1</i>	U80605	farnesyl diphosphate synthase	F1A1T7
<i>HMG-beta2</i>	Y14073	high mobility group 1 protein	163J9T7
<i>18S rRNA</i>	X16077	18S rRNA	40F8T7

Sequences of some of the genes may have multiple accession numbers but only one of them is listed. The references for the genes could be obtained from their GenBank entries. Genes that have not been named are indicated by "?s" in the first column. The EST clones were provided by the Arabidopsis Biological Resource Center.

combination of the following strategies: 1) using synthetic oligonucleotides designed to hybridize at specific positions; 2) making restriction fragment subclones in which the sequence to be determined was made proximal to the cloning sites of the vector, thus, oligonucleotides corresponding to the vector could be used for sequencing; and 3) making nested serial deletions of a DNA fragment with Exonuclease III and the mung bean nuclease. The sequences of both strands of cDNA and genomic DNA for *Esi47* were determined. For checking ligation products, only the sequence of one of the two strands was determined.

3. LIBRARY CONSTRUCTION AND SCREENING

3.1. Screening the *Lophopyrum elongatum* cDNA Library for *Esi47* cDNA Clones

The cDNA library was previously constructed in the λ Uni-ZAP XR vector (Stratagene) with mRNAs from the *L. elongatum* roots treated with 250 mM NaCl for 6 h. The library was amplified by a factor of 1000. A total of 1×10^6 pfu were plated in ten 15-cm Petri dishes and screened for *Esi47* cDNA clones. Plaques were lifted, with duplicates, from the plates onto Hybond N nylon filters. The filters were denatured in 1.5 M NaCl and 0.5 M NaOH for 2 min, neutralized in 1.5 M NaCl and 0.5 M Tris-HCl, pH 8.0, for 5 min, washed in 0.2 M Tris-HCl, pH 7.5, and $2 \times$ SSC for 30 sec, and finally baked at 80°C for 2 h to fix DNA.

The probe used for hybridization was a 250-bp *EcoRI-SalI* restriction fragment corresponding to the 5'-end of the original partial-length *Esi47* cDNA insert (Gulick and Dvořák, 1990, 1992). The *EcoRI* site is in the vector pJET2 and the *SalI* site is within the

cDNA insert. The procedures for prehybridization and hybridization were the same as those for the Southern analysis. After hybridization, the filters were finally washed twice in $0.5 \times$ SSC and 0.1% SDS at 65°C. Positive plaques were purified two to three rounds until homogeneity was reached. The plasmids within the λ phage DNA were rescued with the helper phage ExAssist and the *E. coli* strain SOLR (Stratagene) according to the protocol from the supplier. The cDNAs were recovered in the vector pBluescript SK- (Stratagene), inserted at the *EcoRI* and *XhoI* sites with their 5'-ends at the *EcoRI* site. There is an adaptor sequence of GGCACGAG between the *EcoRI* site and the 5'-ends of the cDNAs. The cDNA clones were designated Esi47-(number) and are listed in Table 3.

3.2. Construction of the *Lophopyrum elongatum* Genomic DNA Library and Isolation of Genomic Clones for *Esi47*

L. elongatum genomic DNA was partially digested with *Sau3A* to a point that most of the DNA fragments were in the range of 10 to 20 kb. The ends of such DNA fragments were filled in with dGTP and dATP and then ligated with the two arms of the λ Fix II vector (Stratagene). The recombinant DNAs were packaged with the Gigapack Gold packaging extracts (Stratagene).

Approximately 1×10^6 pfu of this library were screened for *Esi47* genomic DNA clones. The procedures were the same as those for cDNA screening except that the DNA fragment used as probe was the Esi47-7 cDNA, which contains the protein kinase open reading frame (ORF) and the 3'-untranslated region (UTR). Positive plaques, Esi47-G1 and Esi47-G3, were purified and phage DNAs were prepared for restriction mapping. DNA fragments corresponding to the *Esi47* gene were identified by Southern analysis.

Table 3. Plasmids isolated and constructed in this study.

Plasmid	Description	Vector
A. <i>Esi47</i> cDNA clones		
Esi47-3	full-length cDNA	pBluescript SK-
Esi47-7	partial cDNA, but contains the entire protein kinase ORF	pBluescript SK-
Esi47 ^{K124Q} (or Esi47M)	same as Esi47-7, but contains the K124Q mutation	pBluescript SK-
B. <i>Esi47</i> genomic DNA subclones		
Esi47-GX	a 3.3-kb <i>Xba</i> I fragment including 2-kb of the promoter and part of the transcribed region	pBluescript SK-
Esi47-GK	a 4.3-kb <i>Kpn</i> I fragment including most of the transcribed region and the 3'-flanking region	pBluescript SK-
C. Expression of <i>Esi47</i> in <i>E. coli</i>		
GST-Esi47 (or GST47)	fusion of GST with the Esi47 kinase	pGEX-2T
GST-Esi47 ^{K124Q} (or GST47M)	fusion of GST with the K124Q mutant form of the Esi47 kinase	pGEX-2T
GST-Esi47CD (or GST47KD)	fusion of GST with the catalytic domain of the Esi47 kinase	pGEX-2T
D. Expression of <i>Esi47</i> in <i>Arabidopsis</i>		
35S-Esi47 (or 35S47)	<i>Esi47</i> kinase ORF controlled by the CaMV 35S promoter	pBI121
35S-Esi47 ^{K124Q} (or 35S47M)	the K124Q mutant form of the <i>Esi47</i> kinase ORF controlled by the CaMV 35S promoter	pBI121
E. Expression of <i>Esi47</i> in barley aleurone		
Act1-Esi47 (or ACT47)	<i>Esi47</i> kinase ORF controlled by the rice <i>Act1</i> gene promoter and first intron	pCOR113
Act1-Esi47 ^{K124Q} (or ACT47M)	the K124Q mutant form of the <i>Esi47</i> kinase ORF controlled by the rice <i>Act1</i> gene promoter and first intron	pCOR113

Act1 is the rice gene for actin-1. Sources of the vectors: pBluescript SK-, Stratagene; pGEX-2T, Pharmacia; pBI121, Clontech; pCOR113, McElroy et al. (1991). ORF, open reading frame; GST, glutathione-S-transferase; CaMV, cauliflower mosaic virus.

Proper DNA restriction fragments from Esi47-G1 were subcloned into the vector pBluescript SK-. Subclone Esi47-GX has a 3.3-kb *XhoI* fragment containing 2-kb of the promoter region and part of the transcribed region; subclone Esi47-GK has a 4.3-kb *KpnI* fragment containing most of the transcribed region and the 3'-flanking region. The subclones are listed in Table 3.

4. PRIMER EXTENSION TO DETERMINE TRANSCRIPTION INITIATION SITE

The primer to be extended was the antisense primer ESI47PA (ACAACCGACCAACCAACTGCTC), which is 64-bp downstream of the 5'-end of the longest cDNA insert Esi47-3. Ten pmole of the primer was end-labeled in a 10- μ l mixture containing 50 μ Ci [γ - 32 P]ATP (3000 Ci/mmol, ICN) and 6 units of T4 DNA kinase at 37°C for 30 min. Labeled oligonucleotides were purified by phenol extraction and ethanol precipitation. The total radioactivity of the labeled primer was usually about 1×10^7 cpm. Approximately 5×10^6 cpm of the labeled primer was used in the reverse transcription of 1.5 μ g of poly(A⁻)-RNA derived from *L. elongatum* roots treated with 250 mM NaCl for 6 h. The primer and RNA were mixed with 1.5 μ l of the 5 \times reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM MgCl₂, and 50 mM DTT) in a volume of 13 μ l. The mixture was heated at 68°C for 2 min and let to cool slowly to 37°C. Then dATP, dCTP, dGTP and dTTP were added to 1 mM each, along with 5 units of Moloney murine leukemia virus reverse transcriptase to make a final volume of 15 μ l. The reaction was carried out at 37°C for 1 h and then stopped by heating at 65°C for 10 min. Four μ l of the sample were electrophoresed in a 6% polyacrylamide sequencing gel.

5. PLASMID CONSTRUCTION

The plasmids constructed in this study for various purposes are listed in Table 3. The *Pfu* polymerase (Stratagene), which lacks the activity of adding a nucleotide to the 5'-end of a DNA fragment, was used for amplification of DNA fragments by PCR.

5.1. Expression of *Esi47* and Its Derivatives in *Escherichia coli* as Glutathione-S-Transferase Fusion Proteins

The *E. coli* strain PR745 (New England Biolabs) was used as host for plasmid DNA constructs to produce the fusion of glutathione-S-transferase (GST) and various forms of the *Esi47* protein kinase. The protease gene *lon* was deficient in this strain.

A DNA fragment containing the *Esi47* protein kinase ORF and the 3'-UTR was produced by PCR from the cDNA clone *Esi47-3*. The pair of primers used were *ESI47GEX* (AGCAGAAAGATCTATGCAGTGCTTC, the underlined letters indicate the *Bgl*II site) at the 5'-end of the ORF, and *ST-3'* (TAGGGCGAATTGGGTACCG) corresponding to the pBluescript SK- sequence close to the cloning region. The PCR products were digested with *Bgl*II. Gel-purified DNA fragment, with a *Bam*HI-compatible *Bgl*II end and a blunt end, was ligated to the *Bam*HI and *Sma*I (resulting in a blunt end) digested vector pGEX-2T (Pharmacia) to form the construct GST-*Esi47* (or GST47).

The K124Q mutation of the *Esi47* protein kinase was obtained by PCR from the GST-*Esi47* construct with the antisense primer *ESI47HINDIII* (CTCACAAGCTTGGGTGTC, the *Hind*III site underlined) at the translation termination

site and the sense primer ESI47MUT

(GCACCCTCGAGCCGCGCCGAAGCCTCGATGTCGCCATCcAGCAGCTCG, the *Xho*I site underlined). ESI47MUT contains an A to C single base change (indicated by the lowercase letter c) from the wild-type *Esi47* cDNA that would cause alteration of the Lys residue at position 124 to Gln (K124Q). Lys-124 is an absolutely conserved amino acid residue in the catalytic domain of protein kinases and is required for the catalytic activity of the kinases. The PCR product was digested with *Xho*I and *Hind*III. The gel-purified PCR fragment was ligated to the gel-purified larger fragment of the *Xho*I and *Hind*III digested plasmid GST-*Esi47* to form the construct GST-*Esi47*^{K124Q} (or GST47M).

The expression plasmid for the fusion protein of GST with the catalytic domain of the *Esi47* protein kinase was made by PCR amplification of a DNA fragment from the cDNA clone *Esi47-3*. The sense primer was ESI47KDN (TTCGGATCCCAGGAGCTCAAGAGC) containing a *Bam*HI site (underlined) and the antisense primer was ESI47KDC (TGGAATTCCAAGGTCAGTCTGTC) containing an *Eco*RI site (underlined). These primers would amplify the region encoding the catalytic domain of the *Esi47* protein kinase as shown in Figure 1 (see Part III). The PCR product was partially digested with *Bam*HI and *Eco*RI and the fragment containing the intact catalytic domain was gel-purified. The resulting DNA fragment was ligated to the *Bam*HI and *Eco*RI digested vector pGEX-2T to form GST-*Esi47*CD (or GST47KD).

5.2. Expression of *Esi47* or Its K124Q Mutant Form in *Arabidopsis thaliana*

Wild-type *Esi47* cDNA insert or its K124Q mutant form was liberated from the vector by digestion by digesting the *Esi47-7* cDNA clone or *Esi47M* (constructed in the same way from *Esi47-7* as for obtaining the GST-*Esi47*^{K124Q} from GST-*Esi47*) with *Xba*I and *Apa*I, which are located in the multiple cloning sites of pBluescript SK-. The cDNAs extend from position 187 to the poly(A)-tail and encompasses the protein kinase ORF and the 3'-UTR (see Figure 1 in Part III). *Apa*I-digested ends were made flat by treatment with T4 DNA polymerase. These fragments were used to replace the reporter gene for β -glucuronidase (GUS) in the vector pBI121 (Clontech) between the *Xba*I and T4 DNA polymerase-blunted *Sac*I sites. Thus, the *Esi47* cDNAs or the K124Q mutant form would be under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The resulting plasmids were designated 35S-*Esi47* (or 35S47) and 35S-*Esi47*^{K124Q} (or 35S47M) respectively.

5.3. Expression of *Esi47* in Barley Aleurone Tissues under the Control of the Rice Actin-1 Gene Promoter

The *Esi47* protein kinase ORF and its K124Q mutant form was amplified by PCR from the cDNA clones *Esi47-7* and *Esi47M*, respectively. The primers used were the sense primer *ESI47GEX* (AGCAGAAGATCTATGCAGTGCTTC, the *Bgl*II site underlined) and the antisense primer *ESI47CTER* (TCCATATCTAGAGTCAATTGTGTTC, the *Xba*I site underlined). The PCR fragments were digested with *Bgl*II, and the ends were made blunt by being filled by the Klenow enzyme, and digested again with *Xba*I. The gel-purified DNA fragments were ligated to

the *SmaI* (resulting in a blunt end) and *XbaI* digested vector pCOR113, which contains the promoter and the 5'-UTR intron of the rice actin-1 gene *Act1* (McElroy et al., 1991). The resulting plasmid constructs are designated Act1-Esi47 (or ACT47) and ACT-Esi47^{K124Q} (or ACT47M), respectively.

6. PROTEIN KINASE ACTIVITY ASSAY IN *ESCHERICHIA COLI* IN VIVO

A volume of 0.15 ml of overnight cultures of *E. coli* strains containing the GST and Esi47 protein kinase fusion constructs was added to 1.35 ml of fresh liquid LB medium. Subcultures were incubated at 37°C for 2 h with vigorous shaking. Such subcultures were duplicated for each strain. To one set of these subcultures 15 µl of 0.1 M IPTG was added for induction of the production of the fusion proteins, whereas 15 µl of water was added to the other set of the cultures as controls. The bacteria were incubated for an additional 1 h at 37°C. The cells were collected by centrifugation in a microcentrifuge at top speed for 30 sec. Pellets were washed twice in 0.5 ml of 20 mM Tris-HCl, pH7.5, and 150 mM NaCl, and resuspended in 100 µl of the same buffer. One µl of 0.1 M IPTG was added to the set of bacteria for fusion protein production and 2 µl of 50 µCi/µl of carrier-free [³²P]orthophosphate (ICN) was added to all the tubes. After incubation at room temperature for 1 h, the bacteria were centrifuged. Pellets were washed three times in 0.5 ml of 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl (PBS), and resuspended in 20 µl water. Eighty µl of the protein sample buffer [0.4 M Tris-HCl, pH 6.8, 16% (v/v) glycerol, 3.2% (w/v) SDS, 8% (v/v) β-mercaptoethanol and 0.002% bromophenol blue] were added and the bacteria were boiled for 10 min. Ten µl of these bacterial protein extracts were separated by SDS

polyacrylamide gel electrophoresis (PAGE) in a 10% gel. After electrophoresis protein bands were visualized by Coomassie Blue staining. The radio-labeled proteins were detected by autoradiography.

7. PURIFICATION OF GLUTATHIONE-S-TRANSFERASE FUSION PROTEIN

E. coli stains containing plasmids for expressing GST fusion proteins were subcultured in LB medium from overnight cultures in a ratio of 1 in 10. One liter of the subculture was grown at 37°C for 2 h and IPTG was added to 0.1 mM. The bacteria were continued grown at 37°C for 2 h before the cells were collected by centrifugation at 6000 × g for 10 min at 4°C. Nine ml of PBS were added to the bacterial pellet to suspend bacteria. Half a CompleteTM protease inhibitor cocktail tablet (Boehringer Mannheim) dissolved 0.5 ml water was added to the bacterial suspension, which was then sonicated 3 times, 5 sec each time. Triton X-100 (diluted in PBS) was added to 1% (w/v) and the mixture was centrifuged at 48000 × g for 10 min. About 12.5 ml of the supernatant, which is the soluble bacterial protein extract, were applied to a 0.5-ml column of glutathione-Sepharose resin (Pharmacia). The column was washed with PBS and the GST fusion proteins were eluted by 5 mM glutathione. Three 0.5-ml fractions were collected. Aliquots of 10 µl of the elution fractions and 2 µl of the crude extract were analyzed with SDS-PAGE in a 10% gel and proteins were stained with Coomassie Blue.

8. TRANSFORMATION OF *AGROBACTERIUM TUMEFACIENS* AND *ARABIDOPSIS THALIANA*

Plasmids 35S-Esi47 and 35S-Esi47^{K124Q} were amplified in *E. coli*, purified and transferred to *A. tumefaciens* strain GV3101 containing the plasmid pMP90. Fifty ml of GV3101 cultures grown at 28°C for 12 h were collected by centrifugation, washed in TE buffer, and resuspended in 5 ml of LB medium. An aliquot of 500 µl of the cells was frozen in liquid N₂, let thaw on ice and mixed with 1 µg of plasmid. The mixture was frozen in liquid N₂ for 5 min and placed immediately in 37°C water bath for 5 min. One ml of LB medium was added and the mixture was gently rotated for 4 h at room temperature. Finally, the bacteria were plated on LB plates containing 25 µg/ml gentamycin and 50 µg/ml kanamycin and incubated at 28°C for 2 days. Plasmids from the transformants were verified by restriction enzyme digestion.

Arabidopsis plants were transformed by the *in planta* vacuum infiltration method (Bechtold et al., 1993). *Arabidopsis* plants were grown in greenhouse till flower stems were about 5-cm long and clipped off. After 4 days, the plants were immersed in *A. tumefaciens* cells grown overnight at 28°C and suspended in the infiltration medium containing 0.5 × MS salts, 1 × Gamborg's B-5 vitamins, 5% (w/v) sucrose, 0.05% MES, 0.044 mM 6-benzylaminopurine and 0.02% Silwet L-70 (Lehle Seeds), pH 5.7. The A_{600nm} of the bacterial culture was about 0.8 O.D. A vacuum of 600 to 700 mm Hg for 1 min was applied to the plant dipped in bacteria. The infected plants were let grow in greenhouse and seeds were later collected. Transformants with kanamycin resistance were selected on a medium containing 1 × MS salts, 1 × Gamborg's B-5 vitamins, 1% sucrose, 30 µg/ml kanamycin and 0.8% agar, pH 5.7. Transformant lines which were

homozygous for a single transgene insertional locus were selected on the basis of 3:1 segregation for kanamycin resistance.

9. PLANT STRESS-TOLERANCE ASSAY

9.1. Root Elongation

This assay was done according to Wu et al. (1996). Seedlings were grown vertically on a medium containing 1 × MS salts, 1 × Gamborg's B-5 vitamins, 1% sucrose and 1.2% agar under continuous light for 4 days in growth room and transferred to MS plates containing 0, 50, 100, or 200 mM of NaCl. The plates were rotated 90° and seedlings were left to grow for another eight days. The lengths of elongated roots of six to nine seedlings from each treatment were measured and their averages were compared.

9.2. Watering Plants with NaCl-Containing Solution

In greenhouse, 3-week old soil-grown *Arabidopsis* plants were flooded with 1 g/l of the Plant-Prod 20-20-20 nutrient solutions containing NaCl at 0, 100, 133, 166, and 200 mM, respectively, for 3 days and the pots were let drain. Later the plants were only watered with minimal amounts of the Plant-Prod 20-20-20 solution (less than 5 ml for each plant and less often than every two days) to keep the soil wet. The growth and survival of the plants were observed every day.

Alternatively, the plants were divided into five sets and flooded with NaCl solution every day. The concentration of NaCl increased stepwise every 4 days. Each

plant set had a final NaCl concentration. The final NaCl concentrations were 0, 50, 100, 150 and 200 mM, respectively. The plants were watered with NaCl for a total of 24 days.

9.3. Water Loss of Detached Leaves

Leaves of 22-day old *Arabidopsis* plants were detached and weighed over a time course of 2 h. One leaf from each plant and a total of four plants of each line were measured. The relative leaf weights compared to those at the time of excision and their standard deviations were compared.

10. TRANSIENT EXPRESSION OF *ESI47* IN BARLEY ALEURONE

The procedures for DNA bombardment were carried out essentially according to Lanahan et al. (1992) and Shen et al. (1993). The effector plasmids were Act1-Esi47 and Act1-Esi47^{K124Q}, whose construction was described earlier in this section. In these plasmids, the Esi47 protein kinase ORF or its K124Q mutant form was under the control of the constitutively active promoter and 5'-UTR intron of the rice *Act1* gene for actin-1. Two reporter plasmids were used: one was Hva1-GUS (QS264; Straub et al., 1994) in which the promoter of the barley ABA-induced *Hva1* gene was fused to the reporter gene for GUS; the other was Amy-GUS (MBL022; Lanahan et al., 1992) in which the promoter of the barley gibberellin (GA)-induced low-pI α -amylase gene *Amy32b* was fused to GUS. Plasmid pAHC18 (Bruce et al., 1989), which contains the ubiquitin gene promoter-luciferase construct, was used as an internal control to measure the DNA delivery efficiency. Each bombardment included an effector plasmid, a reporter plasmid

and pAHC18; or as controls, only a reporter plasmid and pAHC18 were used in each bombardment.

Embryoless Himalaya barley half-seeds were imbibed for 2 days and their pericarp/testa layers were removed. Ten of these half-seeds were bombarded each time with a 2:1 molar ratio of reporter plasmid to pAHC18. The final DNA concentration used was 6 µg/µl. Ten µl of DNA were coated onto tungsten particles. When an effector plasmid was included, the molar ratio of effector plasmid to reporter plasmid was 1:1. After each bombardment, five of the half-seeds were treated with 20 µM ABA (in experiments with the Hva1-GUS reporter plasmid) or 1 µM GA (in experiments with the Amy-GUS reporter plasmid) for 24 h in a buffer containing 20 mM CaCl₂, 20 mM sodium succinate, pH 5.0, and 10 µg/ml chloramphenicol. The other five half-seeds were incubated in the same buffer but without the addition of plant hormones. The half-seeds were then homogenized in 800 µl of the grinding buffer (100 mM sodium phosphate buffer, pH 7.2, 5 mM DTT and 20 µg/ml leupeptin), and centrifuged at 12000 × g at 4°C for 10 min. For luciferase assay, 100 µl of supernatant fluid were mixed with 100 µl of 2 × luciferase assay buffer (1 × buffer is 30 mM Tris-SO₄, pH 7.7, 10 mM DTT and 1 mM EDTA). ATP was added to 2 mM immediately before measuring the luciferase activity in a luminometer. One hundred µl of 1 × assay buffer containing 1 mM luciferin were automatically injected to the reaction mixture upon placing the cuvettes in the machine. Emitted photons were counted for 10 sec. For GUS assay, 50 µl of aleurone tissue extract was diluted with 200 µl of GUS assay buffer [50mM NaHPO₄, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% (w/v) Triton X-100, 2 mM 4-methylumbelliferyl β-D-glucuronide and 20% (v/v) methanol] and

incubated at 37°C for 20 h. Fifty µl of the reaction mixtures were diluted in 2 ml of 0.2 M Na₂CO₃ and the fluorescence was measured in a fluorometer. The GUS activities were normalized against the luciferase activities of the corresponding samples. Four independent bombardments were done for each combination of the plasmid constructs.

11. COMPUTER-ASSISTED DATA ANALYSIS

11.1. Routine Tools

The molecular biology data analysis computer software package PC/GENE (IntelliGenetics) was used in this study for nucleotide and amino acid sequence storage and processing in a personal computer. The usage included sequence data entry, changing a nucleotide sequence into its complement sequence, translation of a nucleotide sequence into amino acid sequence, alignment of sequences, searching for signature sequences and molecular mass calculation. For searching the GenBank and other on-line databases for sequences similar to a given sequence, the World Wide Web based Basic Local Alignment Search Tool (BLAST, Altschul et al., 1990) services provided by the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) or the Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org/>) were used. These programs include the nucleotide sequence comparison (BLASTN), the amino acid sequence comparison (BLASTP), the comparison of a given nucleotide sequence translated in six reading frames to amino acid sequences with the sequence entries in the protein databases (BLASTX), the comparison of a given amino acid sequence with the sequence entries in the DNA databases translated in six reading frames to amino acid

sequences (TBLASTN), and the comparison of a given nucleotide sequence translated in six reading frames to amino acid sequences with the sequence entries in the DNA databases translated in six reading frames to amino acid sequences (TBLASTX).

11.2. Construction of Sequence-Based Phylogenetic Tree

The phylogenetic tree of Esi47 and its related plant protein kinases was based on the amino acid sequences of the catalytic domains of these kinases. The full-length amino acid sequence of the Esi47 protein kinase was used in a BLASTP search for its most similar protein kinases in the databases. The catalytic domain of Esi47 was determined according to Hanks et al. (1988) and Hanks and Quinn (1991). The catalytic domains of the Esi47-like kinases were then determined by aligning their full-length amino acid sequences with that of Esi47. These catalytic domain sequences were then aligned by the CLUSTAL computer program. This program is based on the method of Higgins and Sharp (1988) and is included in the PC/GENE package. The phylogenetic tree was thus built based on the multiple sequence alignment, again by the CLUSTAL program. Representatives of other groups of protein kinases from *Arabidopsis* (Hardie, 1999), along with the tomato disease resistance protein kinases Pto (Martin et al., 1993) and Pti1 (Zhou et al., 1995), were used as references on the tree. These kinases include the NAK group protein kinase ARSK1, the leucine-rich repeat (LRR) receptor-like kinases (RLK) ERECTA and RPK1, the S-domain RLK RLK1, the Raf-like protein kinase CTR1, the SNF1-related protein kinase (SNRK) Akin10, the Ca²⁺/calmodulin-dependent protein kinase (CDPK) CDPK1, the cyclin-dependent kinase (CDK) CDC2a, the mitogen-activated protein kinase (MAPK) MPK1, the mitogen-activated protein

kinase kinase (MAPKK) MAP2K α and the GSK3/shaggy-like protein kinase GSK1. The protein kinases that are the most similar to Esi47 are all from *Arabidopsis*, and they are F8A24.12, F12E4.50, T7F6.28, T9I4.2, T22N4.7, APK2b, APK2a, T22E16.110, NAK and APK1.

PART III. RESULTS

1. *Esi47* IS AN NAK GROUP PROTEIN SERINE/THREONINE KINASE GENE

1.1. The *Esi47* cDNA Contains a Protein Kinase Open Reading Frame and a Short Upstream Open Reading Frame

Esi47 is a salt-stress-inducible gene from the salt stress tolerant wild wheatgrass *Lophopyrum elongatum*. The original *Esi47* cDNA clone isolated from the subtraction cDNA library has an insert size of 810 bp, whereas the northern hybridization analysis revealed an mRNA band of about 1.7 kb (Gulick and Dvořák, 1992). Since the partial cDNA sequence for *Esi47* showed similarity to the plant protein kinase genes from the GenBank database, it would be interesting to see if this gene is involved in stress signaling. Thus, my experimental research would focus on structural and functional characterization of this putative protein kinase gene. To start, the full-length cDNA for the *Esi47* gene should be obtained and the gene product identified.

A 5'-end 246-bp restriction fragment of the original cDNA clone, from the *Eco*RI site on the vector to the *Sal*I site in the cDNA insert, was labeled with ³²P. This probe was used to screen about 5×10^5 pfu of a previously constructed λ cDNA library derived from mRNAs of *L. elongatum* roots treated with 250 mM NaCl for 6 h. Eight positive clones were obtained and the plasmids were rescued from the λ Uni-ZAP XR phages (Stratagene). Restriction enzyme digestion of the plasmid DNA indicated that these clones contain four classes of cDNA inserts with sizes ranging from 1.0 kb to 1.8 kb. Sequences of the 3'-ends of the inserts match the sequence of the original cDNA clone.

All these newly isolated cDNAs have their 3'-ends upstream of that of the original cDNA. The longest cDNA insert is 1.8 kb in length and was completely sequenced. Figure 1 shows the combined sequences of this cDNA insert and the original cDNA (This cDNA sequence appears in the GenBank database as accession number AF131222). The additional sequence from the 3'-end of the original cDNA that was not present in the newly isolated clones was later confirmed by the genomic sequence of *Esi47*. The combined cDNA sequence is 1874 bp in length and contains a major long open reading frame (ORF) from positions 192 to 1490. This 1299-bp ORF translates into a protein of 433 amino acid residues in length and a predicted molecular mass of 49380 D. The predicted amino acid sequence of the *Esi47* gene product again shows similarity to plant protein Ser/Thr kinases. There is a short ORF of 51 bp, from positions 108 to 158, upstream of the protein kinase ORF. This small upstream ORF (uORF) can be translated into a sequence of 17 amino acid residues that has no similarity to any of the protein sequences in the GenBank database. The two ORFs are separated by 33 bp. As mentioned earlier, several *Esi47* cDNA clones have different polyadenylation sites. However, only the original cDNA contains a sequence of AATAAG 22-bp upstream of its polyadenylation site that has similarity to the common consensus sequence for a potential polyadenylation signal, AATAAA (Wahle and Keller, 1992).

1.2. *Esi47* Encodes a Plant NAK Group Protein Serine/Threonine Kinase

The deduced amino acid sequence of the gene product shows that *Esi47* contains a protein kinase catalytic domain of 281 amino acid residues. The amino terminal and carboxyl terminal non-catalytic domains have 89 and 63 amino acid residues.

TGTCTTTCAACTTCTCTTGGGTTGGTTTTGGAGAACAAGGTTGAGCAGTTGGTTGGTCGG 60
 TTGTTGGCTTGAAAGATCTTGGCGTTTTCTGTCTGGCTGATCGTCTCCATGGGTGTCTGTT 120
M G V C
 CTGGTCTGGTGGCTTCTTTTCGTCGACCGTCCTTGTTTAGATTGAGCAGGAGGGTCAAA 180
 S G P G G F L S S T V L V -
 GCAGAAGCGCGATGCAGTGCTTCCGGTTCGCGAGCTGGGAGAAGGAGCGGGAGGAGGC 240
M Q C F R F A S W E K E R E E E
 TGCAGGGGCTGCACGGTCTCAGTCGGCGCTTCCAACAGCAGCATGTCCACGGACCGTG 300
 L Q G P A R S Q S A L S N S S M S T D R 36
 ACGCGCGGGCGGTGGGCTCAGAGTGCTGCTCGCTCACCGTCTCATCGGAGATCAGCGTCG 360
 D A R R S G S E C C S L T V S S E I S V 56
 ACTCCTTCGGCCGGTACCGGCAGCTATCGCTGCCGCATCGGCCCAACAATGACCTCCGCA 420
 D S F G R Y R Q L S L P H R P N N D L R 76
 TCTTACCTTCCAGGAGCTCAAGAGCGCCACCCGAGCTTACGCCGTGCACTAATGATCG 480
 I F T F Q E L K S A T R S F S R A L M I 96
 GCGAGGGCGGTTTTGGCTGCGTCTACCGCGCACCATCCAGAGCACCTCGAGCCGCGCC 540
G E G G F G C V Y R G T I Q S T L E P R 116
 GAAGCCTCGATGTCGCCATCAAGCAGCTCGGACGGAAAGGCCTCCAAGGCATAAGGAAT 600
R S L D V A I K Q L G R K G L Q G H K E 136
 GGGTGACCGAGGTGAACTTTCTTGGGGTGGTGGATCATCCCAACCTGGTGAAGCTCATCG 660
W V T E V N F L G V V D H P N L V K L I 156
 GCTACTGCGCAGAGGACGACGAGAGGGGATTGAGTTGCTGCTCGTCTACGAGTTTATGC 720
G Y C A E D D E R G I Q L L L V Y E F M 176
 CTCATGGAAGCCTAGCTGATCACCTGTCAACAAGTACCAAAGCCCGCTTTCATGGGCAA 780
P H G S L A D H L S T R S P K P A S W A 196
 TGAGGCTGAGAGTAGCACTCGACACTGCTCGTGGTCTCAAGTACCTGCACGAAGATTCCG 840
M R L R V A L D T A R G L K Y L H E D S 216
 AATTCAAAGTAAATATTCCGTGATCTGAAGCCTTCAAACATCCTGCTCGACGAGAAGTGA 900
E F K I I F R D L K P S N I L L D E N W 236
 ATGCAAAACTGTGCGACTTCCGCTTGGCTAGACTGGGGCCGCAAGAGGGAAGCCATGTT 960
N A K L S D F G L A R L G P Q E G S H V 256
 CAACAGCGGTGGTGGTACTATAGGGTATGCAGCTCCTGAGTATATCCATACAGGACGCC 1020
S T A V V G T I G Y A A P E Y I H T G R 276
 TCAGCAGTAAGAATGACATATGGAGCTATGGAGTAGTTCTCTACGAGCTCCTCACAGGCC 1080
L S S K N D I W S Y G V V L Y E L L T G 296
 GGCGCCTCTGGACCGAACAGGCCGAGGGTGAACAGAACCTTGTGGAATGGGTGAAGC 1140
R R P E D R N R P R G E Q N L V E W V K 316
 CCTACTCCTCCGACACCAAGAAATTTGAGACCAATAATGGATCCAAGGCTCGAAGGGAAT 1200
P Y S S D T K K F E T I M D P R L E G N 336
 ACAACCTGAAGTCGGCAGCCAGGATTGCTTCCGCTGGCAAACAAGTGCCTGGTGGCCATG 1260
Y N L K S A A R I A S L A N K C E V R H 356
 CAAGGTATAGACCTAAGATGAGCGAGGTGCTGGAGATGGTGCAGAAGATTGTCGACAGCA 1320
A R Y R P K M S E V L E M V Q K I V D S 376
 GTGACCTTGGAAACACCAGAGCGTCCCCTGATAAGCCATTCAAAAAAAGTGGCTAGTGATG 1380
S D L G T P E R P L I S H S K K L A S D 396
 AGAAAAAAGGAAAGGCCTTAATCTGAAGAGGAGGATCGCAGATATTAAGCTGGAGATG 1440
E K K R K G L N L K R R I A D I K A G D 416
 GTAGATGGTTTATAGTGGCACAAGTGGACACCCAAGCTTGTGAGAACAACAATGACTCAAAA 1500
G R W F R W H K W T P K L V R T Q - 433
 TATGGATGAGATGTTTCTTTCATGACTTTAACATCCAGAGATATTAGCAAAAAACAGCAA 1560
 CGGCTGAAATGCCACTTGAGTTGCTGTTTACCGTACAATTCAGAGAACATGATAACTAAA 1620
 AACAAGCGATTTGGCAGGCCTAATGCTATCATCACTAAATAATGGCTATGGCTGGGTTTG 1680
 TGAGGTCACACGCAGGCAGTGTCCACTCGCCACATAGTTCGGCATTACATGTTTTTCATAA 1740
 GAGCTGAAAATCAGAACTGAAGCAGTCTTTTTGAAGTAGAAATATTGGTGTACAGATTT 1800
 AAATGGAATTCGATATTTTGGCTACTAATTTGAGTTGTTTCTAGTGCCAAATAAGAA 1860
 TTTAAGGAGGTG 1874

Figure 1. Nucleotide sequence of the cDNA for *Esi47* and amino acid sequence of the protein kinase encoded by *Esi47*. The amino acid sequence of the catalytic domain is underlined. All the amino acid residues conserved in the protein Ser/Thr kinases are shown in bold letters; highlighted amino acid residues indicate the positions at which *Esi47* has a residue other than the one conserved in protein Tyr kinases. The nucleotide sequence of the short upstream open reading frame (uORF) and the amino acid sequence it might code for are shown in italics. The 3'-ends of the cDNA inserts are shown as white letters with black background. A putative polyadenylation signal sequence, AATAAG, at position 1853 is double underlined. The four intron splicing sites are framed in the nucleotide sequence. This nucleotide sequence of the *Esi47* cDNA appears in the GenBank database as accession number AF131222.

respectively (Figure 1). The catalytic domain of Esi47 contains all of the invariant amino acid residues conserved in protein Ser/Thr kinases; in contrast, it does not have seven of the residues conserved in protein Tyr kinases (Figure 1; Hanks et al., 1988; Hanks and Quinn, 1991). By comparing to the amino acid sequences of the proteins in the protein databases, the Esi47 protein is most similar to three putative protein Ser/Thr kinases predicted from the *Arabidopsis thaliana* genome sequence. These *Arabidopsis* kinases, F8A24.12, F12E4.50 and T7F6.28, have 59%, 59% and 54%, respectively, overall amino acid sequence identity with Esi47, and have 70%, 70% and 68% identity, respectively, within the catalytic domain (Figure 2). The most similar functionally characterized plant protein kinase to Esi47 is the *Arabidopsis* protein APK1 (Hirayama and Oka, 1992), which shows 45% and 54% sequence identity in the whole protein and in the catalytic domain to Esi47, respectively (Figure 2). Furthermore, ten proteins most similar to Esi47 were retrieved from the protein databases by a BLASTP search. The amino acid sequences of the catalytic domains of these kinases and representatives from several major groups of plant protein kinases were used to build a phylogenetic tree (Figure 3). The tree clearly shows that Esi47, along with F8A24.12, F12E4.50, T7F6.28 and APK1, belong to the NAK group of plant protein Ser/Thr kinases (Hardie, 1999). This group acquired its name from the *Arabidopsis* gene *NAK* which is the first gene identified in this group (Moran and Walker, 1993). Like Esi47, the NAK group protein kinases all have a central catalytic domain flanked by short non-catalytic amino and carboxyl domains. None of the kinases in this group has been functionally characterized in any cellular processes. Esi47, F8A24.12, F12E4.50, T7F6.28 and another *Arabidopsis* putative protein kinase, T9I4.2, cluster in a clade other than the one that includes APK1

Esi47	MQ-CERFASWENEREELQGFARSOSALSNSSMSTDRPARRSSBCCSLT	49
F8A24.12	MK-CELESGGDRGRGQKTPISVSLTSIFS-----DREINSSGSEFNDRD	43
F12E4.50	MK-CELEPLGDHKKDQORSPKVSPSINFS-----DVKNSGSDFSPRD	41
T7F6.28	MK-CEYYSKDKTQDDAKTRKFGSATMARGG-----SSSEFNSDT	39
NAK	GGGG--SNRIETDIASSTWLSKFLSRDGSKGS-----STASFSYMP	41
APK1	GTGL--SAQVKAESSGASTKYDAKDIGSLGSKA-----SSVSVRPS	41
Esi47	VSEISVDSFGHYRQLSLPHRPNIDRITTEQHESSAASSSEHALMCEG	99
F8A24.12	VGTSTESSMERKNSYPPVSTRASNEEESITDSSGKNTGASVMDCEG	93
F12E4.50	VGTSTVSSSTGH--NSNTSMSARENNREETTIGDLSMARNSSSGMCEG	90
T7F6.28	STATSITSSLHVLE-----THSINNKVVLDDLETHKNSRSLMCEG	84
NAK	RTEGEILQN-----ANKNESLSLSSATRNERPDSVVCEG	77
APK1	RTEGEILQS-----PNEKSESABEKSATRNERPDSVLCG	77
Esi47	GGCVYRGTIQLSTLEPERS-----IDVAHFGGKRCLOSHENWTEVNLG	145
F8A24.12	GGCVFRGTVRNLEDSSVK-----IEVAVRDLGKRCLOSHENWTEVNLG	139
F12E4.50	GGCVFWGTHKRNLEDPSKK-----IEVAVRDLGKRCLOSHENWTEVNLG	136
T7F6.28	GGCVFHGVTONPQDSHK-----IDIAVHDSRRCLOSHENWTEVNLG	130
NAK	GGCVFKGWIDESSLAPSKPGTGIVIAVVRINQEGFOGHRNLABINYLEG	127
APK1	GGCVFKGWIDEKSLTASRPGTGVIAVVKINQDQWGHQENLABVYLEG	127
Esi47	VVDHENLVKLGICAEDDRRCSTOLELAVFERMHCSEADHLSSTRS--PKPA	193
F8A24.12	IVEBTNEVNLGICAEDDRRCSTORLEAVVEYENRSVEFHLSSTRS--LTVL	187
F12E4.50	VVEHSNEVNLGHCADDDRCSTORLEAVVEYENQSVVEFHLSSTRS--PTVL	184
T7F6.28	VVEHENLVKLGICAEDDRRCSTORLEAVVEYVQNRSVODHLSNRFI-VTFL	179
NAK	QLDHEENLVKRGYCLEBEEHR-----LEAVVEYENRTRGLENHFRFRGTFYQPL	173
APK1	QFSRHLEVNLGICLEDEHR-----LEVVEYENRTRGLENHFRFRGLYFQPL	173
Esi47	SWAMRERVADTARGCKYVHDSEHKLEHRLKPSLEALDENNARKLSDF	243
F8A24.12	TWDLNRRIAQDAARGCAYRHEMEMEQEERDFKSSNEHEDENTARKLSDF	237
F12E4.50	TWDLNRRIAQDAARGCAYRHEMEMEQEERDFKSSNEHEDENTARKLSDF	234
T7F6.28	PWSTREKIAQDAARGCAYRQGMEEQEEERDFKSSNEHEDENNARKLSDF	229
NAK	SWNTRVVMALGAARGCAFLH--NAQPQVTYRDFKASNLEDSNYNARKLSDF	222
APK1	SWKLRKVALGAARGCAFLH--SSSTRVTYRDFKSNLEDSSEYNARKLSDF	222
Esi47	GLARIGPQBG--SHVSTAVGIGIYAPPEMHTGREGSSKNBINSYGVVLYE	292
F8A24.12	GLAREGSPBGLTHVSTDVVGMGYAPPEMHTGRLTSKSDVNGYGVFLYE	287
F12E4.50	GLARIGSPGSSHVSTDVVGMGYAPPEMHTGRLTSKSDVNGYGVFLYE	284
T7F6.28	GLARIGSPDGIHVST--VAGIGIYAPPEMHTGRLTAKSDVNSYGVFLYE	278
NAK	GLARDGPMGDNSHVSTRVMTQGYAPPEMLAIGHESVSVSVYSGVWVLE	272
APK1	GLAKDGFIGDKSHVSTRVMTQGYAPPEMLAIGHETTESVYVSGVWVLE	272
Esi47	ELTGRREFDRNRPEHONILEIRPHLSDIENKMTIDENHONVYENSA	342
F8A24.12	ELTGRBEVDNRPKGEOKLEAVRELSDRRERKLLDEREGKYPIKSV	337
F12E4.50	ELTGRREFDRNRPKGEOKLEAVRELSDRRERKLLVDRREGKYMINKSV	334
T7F6.28	ELTGRREFDRNRPEHONILEIRPHLSDIENKMTIDENHONVYENSA	328
NAK	ELSGRRADKNQPVVEHADVAREBYLTNKRLLRVMDERTQOQYSETRA	322
APK1	ELSGRRAVDKNRPSGERNVEVAREBYLVNKRIFRVIDNREQDOYSMEEA	322
Esi47	ARLASLANKCLVRHARYRPMSEVHEMVOQLVD--SSDLGTFE-RPLISHS	390
F8A24.12	QKLVVAVNRGMNSKARPMSEVHEMNNVVEASS--GNGS--PQLVPL	383
F12E4.50	QKLVVAVNLCTRNKAKARPMSEVHEMNNVVEASS--PENGKKPQLVPL	383
T7F6.28	LKLVAVNRCMVKAKARPMSEVHEMNNVVEASS--GASGLPEMKSL	377
NAK	LKLVKALDGISIDAKSHETNIVKTMEELE-----HIQKEASKEQQNPQ	367
APK1	CKVATLSLRCETTEIKLEHNSVIVSHLEHQLSNAAIGNMDKTDRRMR	372
Esi47	KKLASDEKRRKGLNLKRIADIKAGDRWRERWHKWTPELWRTQ	433
F8A24.12	NSVKA---SHDARGKNNG---GGGEGGEG-KLNNERTIAC	418
F12E4.50	NSQET---SRVEEGKNKKVLD---GAESGLE-KLNNKNTIAC	420
T7F6.28	TPKDAFASHRERVKRK-FVELLIGENGCPNLPVSHRATSI	419
NAK	ISIDNIINSPQA-----VNYPRPSIM	389
APK1	RRSDSVVSEKVNAGFAPOTAVGSTVVAYPRPSASPLYV	410

Figure 2. Alignment of the amino acid sequences of Esi47 and its closely related plant protein kinases. The alignment is made by the CLUSTAL method (Higgins and Sharp, 1988) and modified by eye. Highlighted amino acid residues in the Esi47 sequence indicate the positions at which at least one of the other kinases has the identical residue: these are also highlighted in the other kinases as well. F8A24.12, F12E4.50 and T7F6.28 are the predicted gene products from the *Arabidopsis thaliana* genome sequence. The database accession numbers of their genes are AC015985, AL162751 and AC005770, respectively. The accession numbers of the genes for NAK (Moran and Walker, 1993) and APK1 (Hirayama and Oka, 1992) are L07248 and D12522, respectively. The overall sequence identities between Esi47 and F8A24.12, F12E4.50, T7F6.28, NAK and APK1 are 59%, 59%, 54%, 47% and 45%, respectively. The sequence identities in the catalytic domains (from positions 90 to 370 of the Esi47 sequence) between Esi47 and F8A24.12, F12E4.50, T7F6.28, NAK and APK1 are 70%, 70%, 68%, 54% and 54%, respectively.

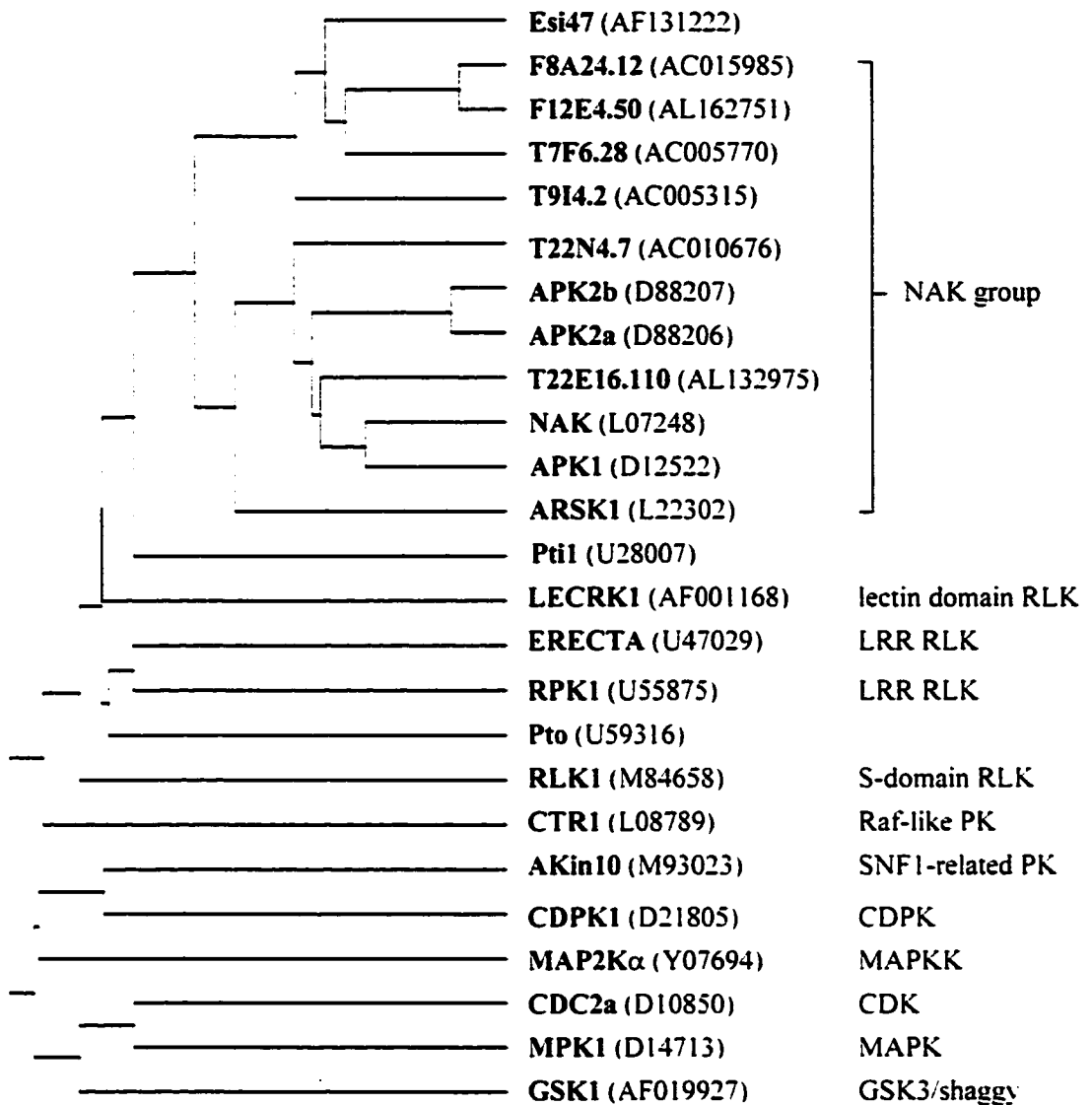


Figure 3. Esi47 belongs to a unique subgroup in the NAK group of plant protein Kinases.

The amino acid sequences of the catalytic domains of the protein kinases shown are compared by the CLUSTAL method (Higgins and Sharp, 1988). The kinase groups, according to Hardie (1999), are indicated in the right column. The kinases in the NAK group except ARSK1 are the most similar proteins to Esi47 revealed by a BLASTP search in the databases. The database accession numbers for the genes encoding the kinases are shown in brackets. RLK, receptor-like kinase; LRR, leucine-rich repeat; PK, protein kinase; CDPK, Ca²⁺/calmodulin-dependent protein kinase; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase. The two disease resistance kinases, Pto (Martin et al., 1993) and Pti1 (Zhou et al., 1995), are from tomato; the rest, except Esi47, are from *Arabidopsis thaliana*.

and NAK. Therefore, *Esi47* represents a novel subgroup in the NAK group of plant protein kinases.

2. GENOMIC STRUCTURE OF THE *ESI47* GENE

2.1. Linear Structure of the *Esi47* Gene

Since *cis*-regulatory sequences are normally located in the genomic sequence of a gene, in the flanking regions and sometimes in the 5'-untranslated region (UTR), isolation of a genomic clone of a gene is an important step in the study of regulation of gene expression. An *L. elongatum* genomic DNA library was constructed from the partially *Sau3A*-digested leaf DNA in the phage vector λ Fix II (Stratagene). This library was screened with the ^{32}P -labeled *Esi47* cDNA to isolate genomic DNA clones for *Esi47*. One of the positive clones contains an insert of 15 kb encompassing the entire coding region for *Esi47* and flanking DNA fragments of approximately 7 kb and 6 kb, respectively, at 5'- and 3'-ends. Two plasmid subclones of this *Esi47* genomic clone were made: One contains a 3-kb *XhoI* fragment corresponding to the 5'-portion of the gene and the other contains an overlapping 4-kb *KpnI* fragment for the 3'-portion of the gene. The DNA sequence was determined from 3-kb upstream, to 0.5-kb downstream of the coding region (Figure 4 and 5. Note the numbers for the genomic sequence, with indications of " + " and " - " relative to the transcription start, is different from the numbers for the cDNA sequence shown in Figure 1. In Figure 1, numbering starts at the first base of the longest cDNA clone; in Figure 4, base +1 is at the start of transcription as determined by primer extension).

-2489 CTCGAGTGTGCCGATCTAAGAACAAGGC
 -2460 GGGCTGGGCGTTAAGGACCTTCATAGACAAAATATTTCTCTCCTAGTGAAATGGTGGTGG
 -2400 AAGTTGGATACGGAGGAGGGTCTTTGGCAAGATATAGTGAGGGCGAAATACCTCAAAAAT
 -2340 AGCTCGGTGGCCACTGTAAAACCAAGTTCAATGACTCCCCCGTATGGAAAACCTTACTG
 -2280 AAAGTTAAGGAGGTCTATATGGCCGGGAGAAAAGTGGTTATTAAGTCTGGCAATGTAGCT
 -2220 CGACTGTGGTATGACCCATTGGCTCTCATCCACCTCTGTGTGATACTTATCCAGAGCTG
 -2160 TTTAGCGTCGCTAATTATCCGAATGATACTATTGCCCAATTTAGGGATCGTGTGGTGGG
 -2100 GGAGTGTTCAGACGCAGATTAACGTCGACTTGACACACCAGCTGGAGGGGGTGCAGGGC
 -2040 GTCGTTGATAGCCTTGGACCTTCTGATGTTTCGCGATGGGATTGAATGGGGTTTCGGTCCC
 -1980 AAAGGCAAAATACACAATAAATCTGTGTATGCTTACTTAGAAAAAGACCTTGCTGGGTGT
 -1920 GATTACAGATGGATTTGGAAGGCTAAGCTACCTCTCAAAATTCAGATTTTCTATGGCAA
 -1860 CTCCTTCAGGATGCTGTCTCACCAGAGATGTTATGAAACGCAGGAATTGGGCTGGCAAC
 -1800 CCGCAATGTCTTCTGTGATTGCAGAGAAACTTATCTGCACTTGTTTTTCTTTGCCCT
 -1740 GTCGCAAGGGTAGCTTGGCGCACTTGGGGTGATGCTTGGTACTGATTTATGCCCTAAC
 -1680 AATCTTTGTCAGTTTTACTCCTGGTGTATGCTTTTTCTTCCCACGGGAAACGATATTTTC
 -1620 ACTATGGGCTAGCGGCCATTTGCTGGGCCATCTGGAATCGCAGAAATCAGGCCACCTTT
 -1560 GAATTTAAGAAATGTCATCTCCTTTTGGAGATTGTCTTCACTGCTTGTGTACATTTATCC
 -1500 TATTGGGCAGGTCTACTGAAGGGCGAGAACGAACGGAGCTGGAGCATGGCGCGTCTGCTTC
 -1440 TGAAGAAGAACTAAGGAGATGATGAGACTCTGTGCAACTGCGTACGCGGGCTTCGGCA
 -1380 TGGGGTGAATCTGGCGCTGTGCTTCTTAAAGCCCTTGAAGCTGTTGGTCTGTGTCTG
 -1320 GTGGATCCCTGTAGAATAATAGCCGTTTATGTTTGCTCTATGCTCCTGCTGTATCGCCT
 -1260 CTGCTAGGCTGCGGTGTTTTCTGAACCTTTGCTACTTTGTTGCGTGTGTGATAGTGTCTCC
 -1200 AGGGTTTTTGCCTCGTAGTGGGTGTTTCGACTGCGAGCGCCACAGCGGGTTTTCCCTGTA
 -1140 GTGCTTGAACCTCGCTTTTTCCCTTTTCTTTTGAACCTCGGATATGGCTGTATTTCC
 -1080 GTTATTTCTATAATGGAAAGGGGATTAGCCCGGTTCAAAAAAAAAAATGTTCCATGATGAT
 -1020 TGCTATGGGCATTTGTGTGATATTTATCCAGTTTTCCGCTAATTAAGTGCATCATTA
MYE
 -960 TTCTTATTTAATAAGACGAGGCAAGCTGGTGCCTCCCTTTCAGATTTTTTTTTTACTAA
DRE
 -900 AATGAAATCCGCAGGTCAACGAAGAAGAGTGAAAACACAGGTGCAAAAAGTCCGCTGAAACC
MYC
 -840 AGATGTGAAAGAGAGCTATATTTGACTTGGTTGCCACCACCCAGCAGTGAAAATCGTTGT
DRE
 -780 CCTGACTCGTGTGCAACTTCCAGTAGTTAGCCAAGTCAGACCAGACCATTGTGCGGATC
 -720 TTTTCAAAGTTTTCGTGTTTGCCACCAATAAGTGTGTGATTTCTAGTTTTTTTTTTTCAA
DRE
 -660 AACGTTCCGACCATAATGTTTGTATTTTTTTTTTCTCCAGAGTACTTTGACCAAGTTGAGCA
G-box
 -600 AGATTTCTAGCCATGTGTTTCTTCAAACCTACCGTCCATCAAGATGAAAACGACTGAATGG
MYC
 -540 AGAAAATCACTATGTTACTTCGGGAAACATATACAAAAGTTGCTTTCTGAGAGATCTGTA
 -480 CAGCAAGTACATCATGTACGGCAACCATACGCTCCACTGGTGATTACTCTTTTAGTAGTT
 -420 TTCTTCTTTGAACTTTGATTACTCTTTTAGTTTTATTTACTACCCACACCCTGATGA
 -360 AAGTCTTGTTCCTGCGATTTGTCAAGTTGCCATTTCCCTCTCCTCGCCGTGCTTATTTCA
MYB, MYC Motif IIb
 -300 ACACCTGCACCCTATCACCATACTCGTCTCCCTCTCGCCATCCAATCCCTCACGTTTCT
MYC CE3 G-box
 -240 CTCGTGCCCCCTCGCCCCAGCTCCCAAAGCAAGCAAGCGAGCAGCGAGCGACCAAGCAAG
CE1, DRE
 -180 CAGAAAGAGACAGCCCGAAGCAGGGGCCCGCCATCCATTGACCTACTAATACTACGCAATCC
CDeT27-45 element DRE
 -120 AATATCCAAATCCCGTACCCTGAACCAGAAGCAGCCGCGTCTTCTTCCCTCCCAACCCCAT
DREx2, Motif IIa MYC
 -60 GTGGGGGAGGCTCAGCTAGGTGGCTTTGCCCAGGTGAGCAGGAAACGACCCCGCCCGTGA
MYC DRE
 -1 **AGAGAGCTTCGTGCTTTCAACTTCTTTGGGTTGGTTTTGGAGAACAAAGGTTGAGCAGT**

+3241 GGGTGGTCGAATTTGCCTGTGTATATCCATAAAAAATTAGAATCAATGTGTTATAACTTTA
+3301 AAAAAATTAAATGGATAATGTGAAGAGAGGTATTATA

Figure 4. Nucleotide sequence of the genomic DNA for the *Esi47* gene. The numbers here are different from those for the cDNA sequence shown in Figure 1. Here, the +1 position represents the transcription initiation site measured by primer extension, the primer sequence for which is underlined. The regions corresponding to the mature mRNA are indicated by bold letters. The upstream open reading frame (uORF) is highlighted as well as the codons for the translation start and stop for the protein kinase open reading frame (ORF). The putative *cis*-regulatory elements in the promoter region are also underlined. DRE, dehydration responsive element; CE, coupling element.

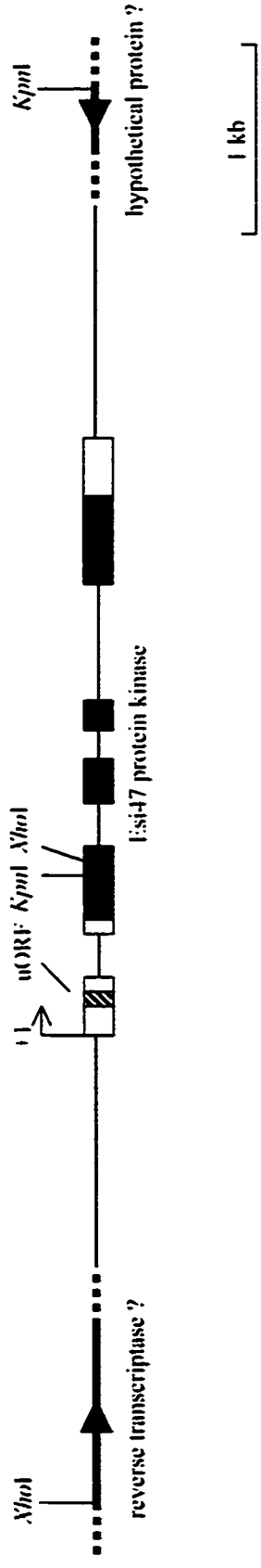


Figure 5. A schematic illustration of the gene structure and the flanking regions of *Esf47*. Closed boxes, coding regions of the *Esf47* gene; open boxes, transcribed, non-coding regions; hatched box, upstream open reading frame (uORF); thin lines, introns and flanking sequences; thick lines with arrows, probable gene loci and their orientations. +1 indicates the transcription initiation site of *Esf47*. The restriction enzyme sites used for subcloning the genomic DNA are indicated. The distances are roughly proportional.

Aligning the cDNA and the genomic sequences of the *Esi47* gene reveals that the gene has four introns (Figures 4 and 5). The first intron is 312 bp in length and is located in the 5'-UTR, between the uORF and the protein kinase ORF. The other three introns are in the coding region and their lengths are 171 bp, 142 bp and 602 bp, respectively. The DNA sequence in the exons is identical to the cDNA sequence.

The transcription initiation site was determined by primer extension with an antisense primer 64-bp downstream of the first nucleotide of the longest cDNA insert. The extension product is 75 bp. Therefore, the longest cDNA insert obtained only lacks 11 bp of the transcribed sequence at the 5'-end (Figure 4).

2.2. Putative *Cis*-Regulatory Elements in the Promoter Region of the *Esi47* Gene

The region around the -35 position of the *Esi47* genomic DNA does not have a TATA-box core sequence TATA. Putative *cis*-regulatory elements that have been implicated in environmental stress regulation were searched for in the 1-kb DNA fragment upstream of the transcription start site. The G-box core sequence ACGT occurs twice at -659 and -248. The context of the -248 G-box-like sequence, CACGTTTC, shows weak similarity to the ABRE consensus (C/T)ACGTGGC (Figure 4). A CE1-like sequence, TACCGCCGT, is located 12 bp downstream of the -248 G-box; and a CE3-like sequence, ACTCGTCTCCCC, is found 31bp upstream of that G-box. These CE-like elements are possibly functional since their locations relative to the G-box elements are similar to those found in the *Hva1* and *Hva22* genes (Shen et al., 1996). Seven DRE-like sequences with a consensus of GCCGCC, one MYB-recognition sequences and seven MYC-recognition sequences are also found in that region (Figure 4). In addition, the

sequences CAGCCGCGTCTT at –89 and TCGCCGTGCTT at –317 are similar to Motif Ila and Motif I Ib, respectively, which are found in the rice abscisic acid (ABA) responsive gene *rab16A* (Mundy et al., 1990). Finally, the sequence AGCCCGA at –169 matches the (A/G)(A/G)CCC(A/G)(A/G) consensus found in the ABA inducible promoter of the resurrection plant *Craterostigma plantagineum* gene *CDeT27-45* (Nelson et al., 1994).

2.3. Genomic Organization of the Region Surrounding the *Esi47* Locus

By a TBLASTX search the genomic sequence shows that the region about 1.5 kb to 2.5 kb upstream of the transcription initiation site of *Esi47* has similarity to putative non-long-terminal-repeat retroelement reverse transcriptase genes. Two examples are the rice gene with GenBank accession number AP002816 (33% deduced amino acid sequence identity in a DNA fragment corresponding to 248 amino acid residues) and the *Arabidopsis* gene *F13B15.21* (26% amino acid identity in a fragment of 362 residues) (Figure 4 and 5). A 0.25-kb single-pass sequence located 1.7 kb downstream of the transcription termination site of *Esi47* is similar to the gene for the *Arabidopsis* hypothetical protein F14M13.19 as revealed by a TBLASTX search (55% amino acid identity in a fragment of 40 residues; Figure 5).

3. PROTEIN KINASE ACTIVITY OF THE GENE PRODUCT OF *ESI47*

In order to demonstrate the protein kinase activity of the *Esi47* gene product, the *Esi47* protein kinase ORF was fused to the bacterial gene for glutathione-S-transferase (GST) in the vector pGEX-2T (Pharmacia). The expression of the fusion protein GST-

Esi47 in the *Escherichia coli* host is under the regulation of the *tac* promoter and the *lac* operator, and thus could be induced by addition of IPTG to the culture medium. The soluble fraction of the lysate of IPTG-induced *E. coli* expressing GST-Esi47 contained largely degraded forms of the fusion protein since no proteins of proper size could be purified from glutathione-Sepharose affinity chromatography as revealed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 6). The GST fusion protein with the K124Q mutant form of Esi47 (GST-Esi47^{K124Q}) also was not stable in the soluble fraction of the bacterial extract and had a similar pattern of degradation as shown in SDS-PAGE of the proteins eluted from the glutathione-Sepharose affinity column (Figure 6). When the total bacterial lysates were analyzed, IPTG induced *E. coli* expressing GST-Esi47 exhibited a slightly enhanced band at 68 kD in comparison to the bacteria not treated with IPTG (Figure 7A). The size of this band is close to the predicted molecular mass of the GST-Esi47 fusion protein, that is, 76 kD. This reflects a possibility that part of the intact GST-Esi47 fusion protein was sequestered in an insoluble form and was thus protected from degradation.

The catalytic domain of the Esi47 protein kinase, from amino acid residues Gln-81 to Gly-380, was also fused with GST. This fusion protein, GST-Esi47CD, was stable in *E. coli* and could be purified with glutathione-Sepharose resins. However, in the *in vitro* assays with [γ -³²P]ATP as phosphate donor, GST-Esi47CD failed to autophosphorylate or to phosphorylate protein substrates such as histone and casein regardless of the presence of Ca²⁺ (data not shown).

Nevertheless, other data that suggest Esi47 is a functional protein kinase came from the *in vivo* assays of *E. coli* cells containing the plasmid DNA construct for

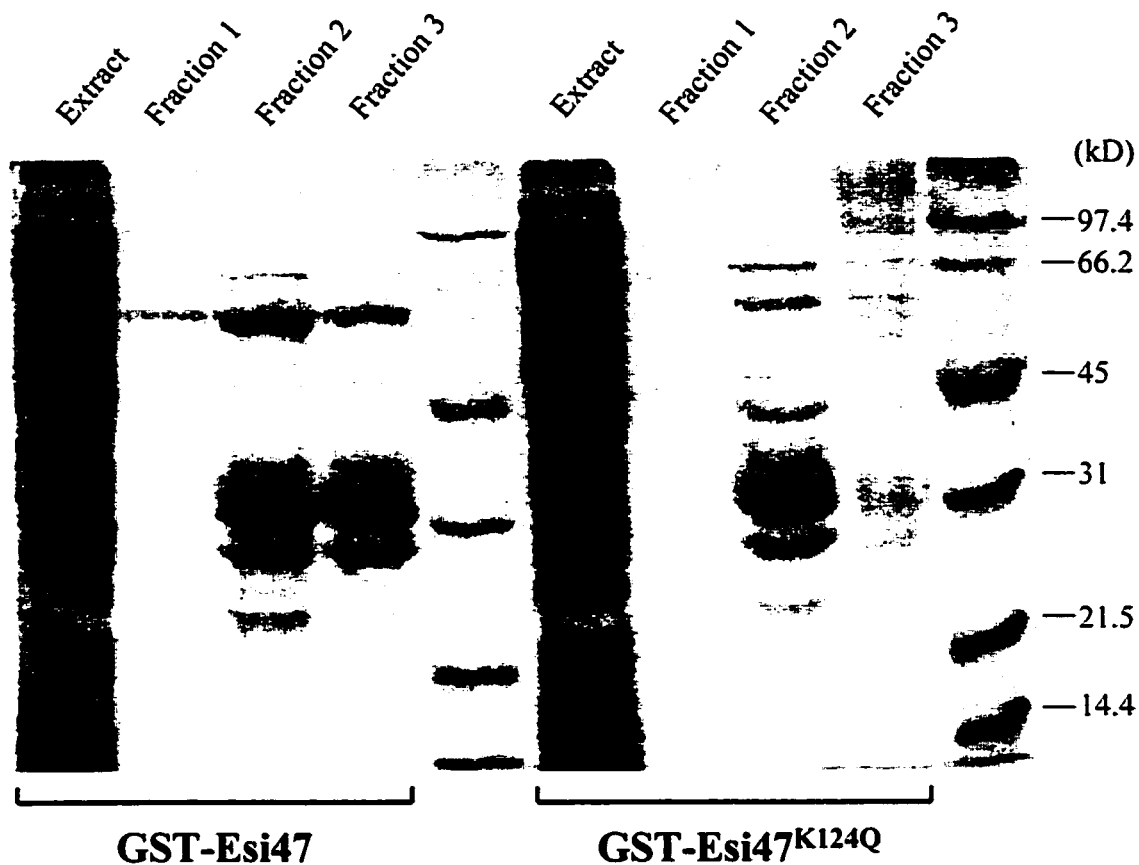


Figure 6. Affinity purification of the GST-Esi47 and GST-Esi47^{K124Q} fusion proteins from soluble bacterial protein extracts. The extracts were obtained by disrupting cells from the IPTG-induced *Escherichia coli* strains expressing the fusion proteins by sonication. Glutathione-*S*-transferase (GST) fusion proteins were purified by glutathione-Sepharose affinity chromatography. Proteins were eluted with glutathione. Aliquots of the extracts and the elution fractions were subjected to SDS polyacrylamide gel electrophoresis and proteins were stained with Coomassie Blue. The protein profiles of the first three elution fractions (Fractions 1 to 3) are shown.

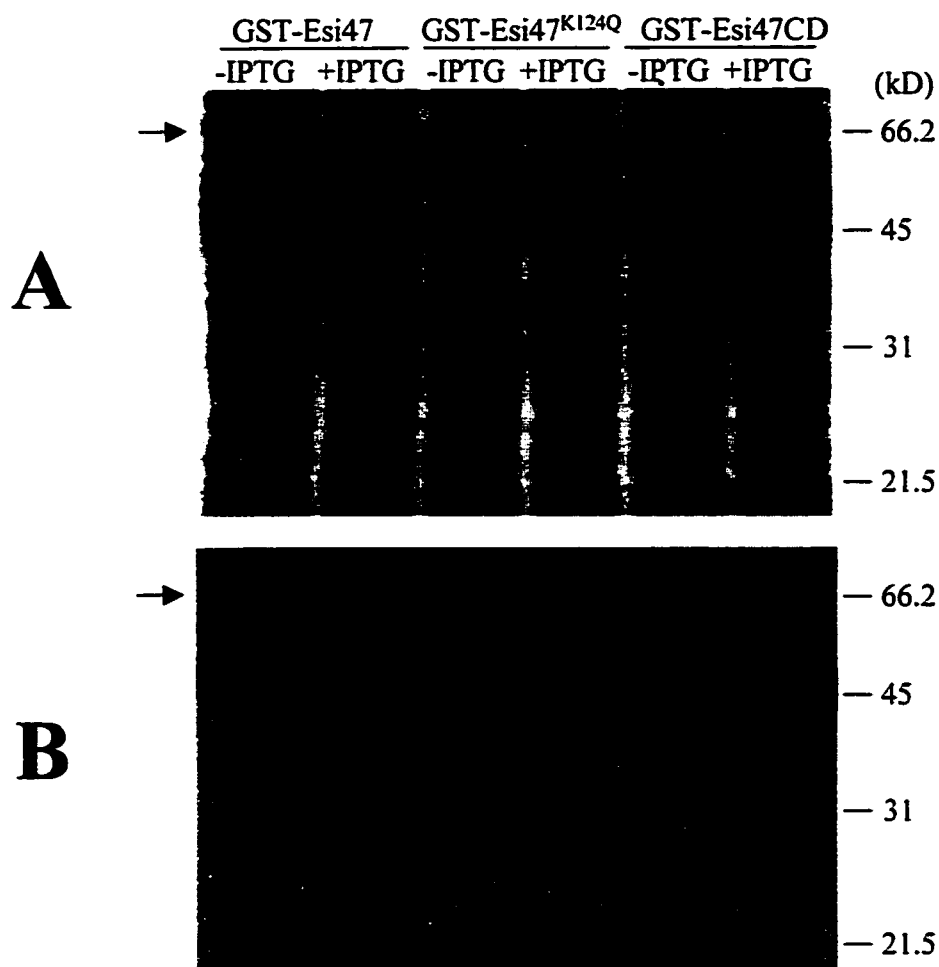


Figure 7. Protein kinase activity of Esi47. *Escherichia coli* cells containing plasmid DNA constructs for expressing the glutathione-S-transferase (GST) fusion proteins with various forms of Esi47 were either treated with IPTG or untreated, as indicated, and incubated with [³²P]-orthophosphate. Total bacterial lysates were then subjected to SDS polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue (A) and subjected to autoradiography (B). GST-Esi47 is the fusion of GST with the wild-type Esi47 protein kinase; GST-Esi47^{K124Q} is the fusion of GST with Esi47 containing the K124Q mutation; GST-Esi47CD is the fusion of GST with the catalytic domain of Esi47. The arrows indicate the 68-kD protein band from the IPTG-treated bacteria expressing GST-Esi47, which was uniquely phosphorylated .

expressing the GST-Esi47 fusion protein. In the cultures of these bacteria, IPTG was added to induce the production of the fusion protein. [³²P]orthophosphate was then included for tracing phosphorylation reactions. The profiles of phosphorylated proteins from the total bacterial lysates were analyzed by SDS-PAGE and autoradiography. An extra phosphorylated protein band of approximately 68 kD was present in the total lysate of IPTG-induced bacteria expressing the GST-Esi47 fusion protein (Figure 7B). The size of this band is the same as the one revealed by the Coomassie Blue stained gel of the total lysate (figure 7A). This ³²P-labeled band was not present in the lysate of uninduced bacteria, or, in the lysate of induced bacteria harboring the plasmid DNA construct for expressing GST-Esi47^{K124Q} (Figure 7B). In the mutant form of Esi47, the Lys-124 residue was changed to Gln (K124Q) to abolish the kinase activity since the lysine residue at this position is critical for the phosphate transfer by protein kinase (Hanks et al., 1988). The size of the unique phosphorylated protein (68 kD) from bacteria expressing GST-Esi47 is close to the predicted size of 76 kD for the GST-Esi47 fusion protein. Therefore, the ³²P-labeled 68-kD band is likely the product of autophosphorylation of GST-Esi47. Bacteria containing the plasmid DNA construct for expressing GST-Esi47CD did not display any changes in protein phosphorylation profile after IPTG induction (Figure 7B). These results serve to suggest that Esi47 is an active protein kinase since the GST-Esi47 fusion protein may be able to autophosphorylate before being degraded or trapped in the insoluble protein fraction in *E. coli*.

4. INVOLVEMENT OF ESI47 IN STRESS AND ABSCISIC ACID SIGNALING

4.1. No Altered Phenotypes Were Observed in Transgenic *Arabidopsis thaliana* Plants Expressing *Esi47*

Arabidopsis is an excellent plant species for functional study of plant genes due to its rapid development as a model genetic system and its ease of transformation as compared to monocot species. It is believed that the stress and ABA signaling in monocot and dicot plants are conserved and are controlled by similar sets of genes. Therefore, *Esi47*, a gene from the monocot *L. elongatum*, was introduced to *Arabidopsis* to investigate what phenotypic changes the *Esi47* transgene could cause in transgenic plants.

Transgenic *Arabidopsis* plants were obtained in which the *Esi47* gene was expressed under the control of the strong and constitutively active cauliflower mosaic virus (CaMV) 35S promoter. Three homozygous lines, B22, B23 and B42, with single insertions of the 35S-*Esi47* transgene were selected on the basis of a 3:1 segregation for kanamycin-resistance and used for characterization. Transcripts of the *Esi47* transgene were detected by northern hybridization assay in RNAs from all of the transformed lines but not in the wild-type parent strain Col-0 (Figure 8A).

Homozygous transgenic *Arabidopsis* expressing the K124Q mutant form of *Esi47* (35S-*Esi47*^{K124Q}) could not be obtained from nine lines of initial transformants in this study. All the lines carrying the 35S-*Esi47*^{K124Q} transgene continued to segregate for kanamycin-resistance in the third generation. This suggests that high dosage of the 35S-*Esi47*^{K124Q} transgene might be lethal to *Arabidopsis*.

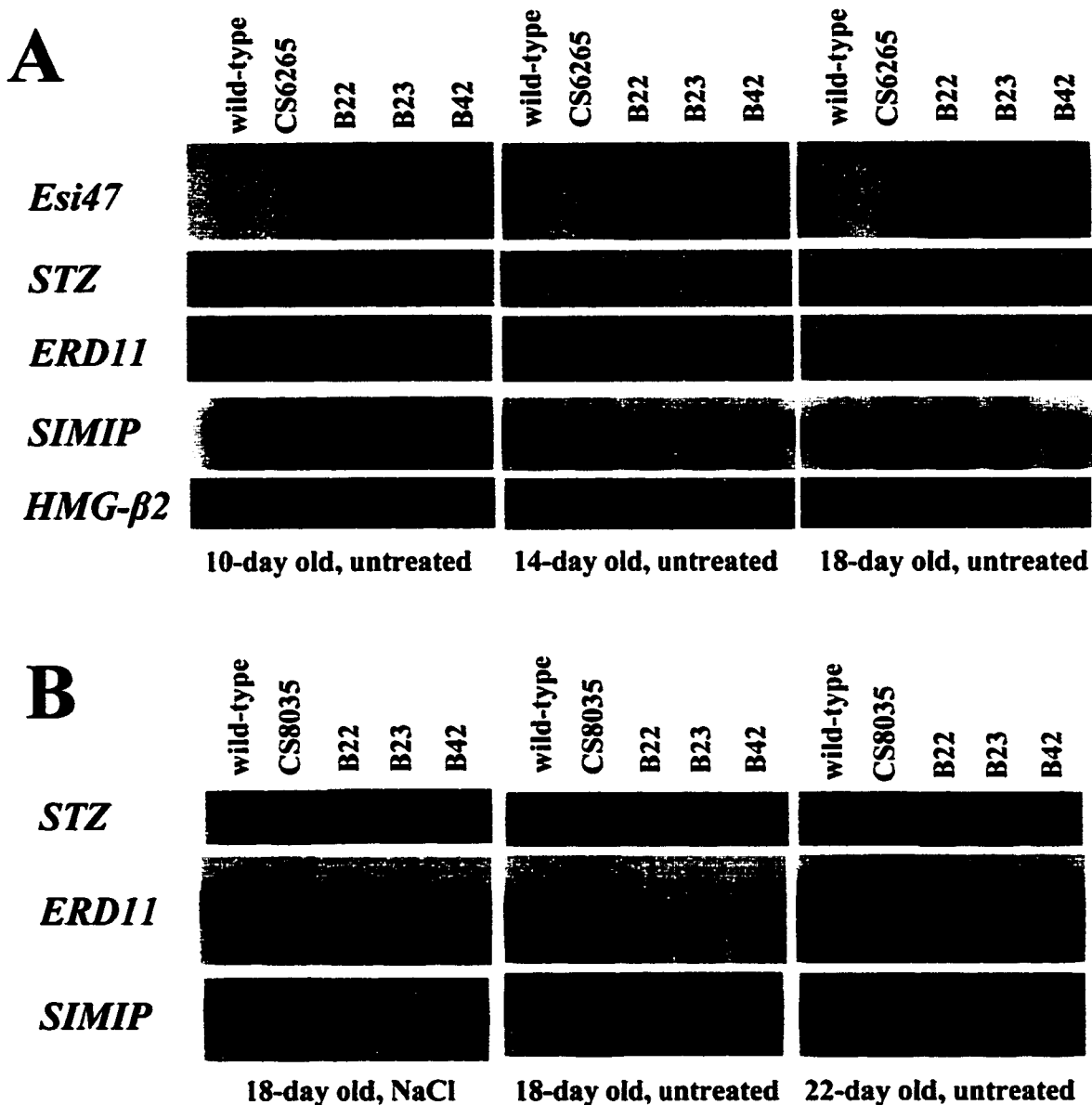


Figure 8. Northern hybridization analysis of the influence of the *Esi47* transgene on the expression of *Arabidopsis thaliana* stress and ABA regulated genes. (A) and (B) are the results from two independent experiments with different sets of plants. Total RNA samples from transgenic *Arabidopsis* lines B22, B23 and B42 expressing *Esi47*, the wild-type *Arabidopsis* strain Col-0 and the control transgenic line CS6265 (expressing the *Agrobacterium tumefaciens tms2* gene) or CS8035 (expressing the *Brassica napus FAD3* gene) were probed with radio-labeled cDNAs of *Esi47* or the *Arabidopsis* genes *STZ*, *ERD11*, *SIMIP* and *HMG-β2* (Table 2). The RNAs were from whole plants of various ages as indicated. Plants were untreated or treated with NaCl (250 mM, 6 h; the first panel of B only).

Transgenic *Arabidopsis* expressing the wild-type *Esi47* gene showed no morphological changes or growth variations in comparison to wild-type *Arabidopsis* plants. In addition, the transgenic plants demonstrated no enhanced stress-tolerance. The observations included: 1) root elongation of young seedlings of lines B22, B23, and B42 on agar medium containing 50 to 200 mM NaCl, as compared to the wild-type *Arabidopsis* and a control transgenic *Arabidopsis* line, CS6265, in which an unrelated gene (the *Agrobacterium tumefaciens tms2* gene for amidohydrolase) is overexpressed (Figure 9A); 2) growth and survival of mature flowering plants grown in soil and watered with solutions containing up to 200 mM NaCl, either applied in a single dose or the NaCl concentrations were increased stepwise (not shown); 3) water loss of detached leaves (figure 9B).

Northern hybridizations were carried out to determine whether the *Esi47* transgene could mimic the effect of salt stress in regulating the expression of any of the previously characterized salt stress or ABA regulated genes in the transgenic *Arabidopsis*. Probes derived from the expressed sequence tag (EST) clones for approximately 60 such genes (see Table 2 in Part II) were used in this study. No apparent effect on the expression of these genes by *Esi47* overexpression was confirmed, though initial observation had been promising. The initial screening with whole plants grown in liquid medium for 14 days showed that genes *AtCPI1*, *AtMPK3*, *ATsEH*, *Dr4*, *ERD1*, *ERD6*, *ERD11*, *ERD13*, *ERD15*, *RD21A*, *SIMIP* and *STZ* had apparent altered mRNA levels only in a single transgenic line (not shown). Subsequently, the expression of these genes, except *ERD15*, was verified in plants grown for 10, 14 and 18 days, respectively. In this experiment, only three of these genes, *ERD11*, *SIMIP* and *STZ*, showed higher

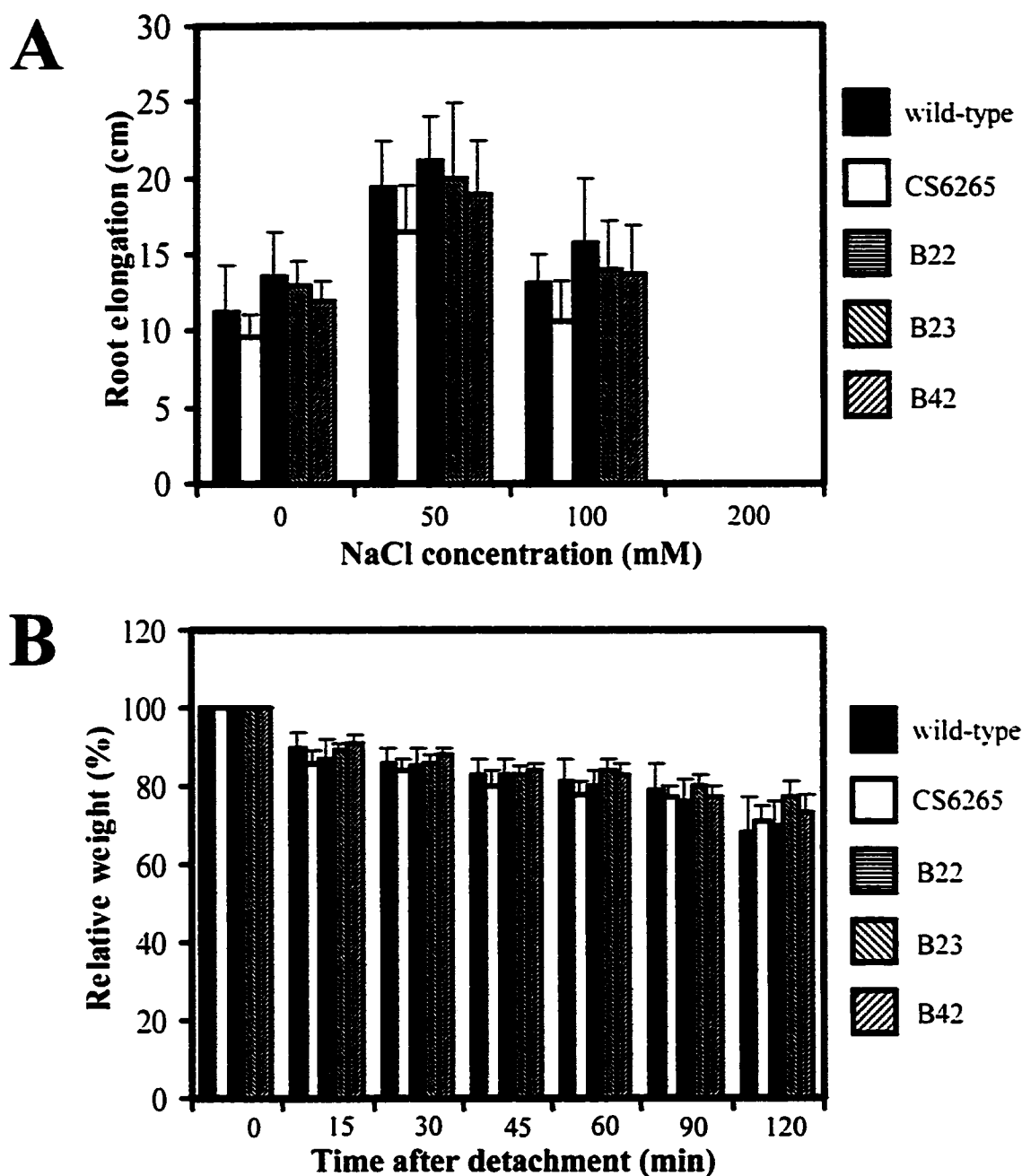


Figure 9. Stress tolerance of transgenic *Arabidopsis thaliana* expressing *Esi47*. Three lines of *Esi47*-expressing *Arabidopsis* (B22, B23 and B42), the wild-type *Arabidopsis* strain col-0 and a control transgenic line, CS6265, expressing the *Agrobacterium tumefaciens tms2* gene, were compared. (A) Root elongation in the presence of NaCl. Young seedlings grown on agar medium were transferred to the same medium containing various concentrations of NaCl as indicated. Root growth over an 8-day period was measured. The values shown are the averages from six to nine seedlings and their standard deviations. (B) Water loss in leaves within 2 h after detachment. The weights of the leaves were compared to those at time of detachment. The values shown are the average relative weights from the measurements of four leaves and their standard deviations. For B22, only three leaves were measured.

mRNA levels in 18-day old whole plants. However, the induction was not apparent in all the transgenic lines and was not observed at all in 10- and 14-day old plants (Figure 8A). The control transgenic *Arabidopsis* line CS6265 also had higher mRNA levels of the *ERD11* and *SIMIP* genes (Figure 8A). Northern analysis of 18-day old plants was repeated, together with 22-day old plants and also with 18-day old plants treated with 250 mM NaCl for 6 h. This time none of the transgenic *Arabidopsis* plants expressing *Esi47* demonstrated any elevation of mRNA levels of those three genes as compared to the wild-type plants (Figure 8B). Thus, the results from the previous experiment could not be repeated in this experiment. In addition, the last experiment also showed that *ERD11*, *SIMIP* and *STZ* were not superinduced by NaCl in *Arabidopsis* expressing *Esi47* (Figure 8B). Therefore, these experiments did not provide conclusive evidence that *Esi47* could regulate gene expression in transgenic *Arabidopsis*.

4.2. *Esi47* Suppresses the Gibberellin Response in Barley Aleurone

To assess if *Esi47* has any effect on plant hormone signaling, barley aleurone layer tissues were used as host for transient gene expression assay with particle bombardment for DNA delivery. The *Esi47* protein kinase ORF or its K124Q mutant form was placed under the control of the strong and constitutively active promoter and 5'-UTR intron of the rice *Act1* gene to form the effector gene constructs *Act1-Esi47* or *Act1-Esi47^{K124Q}*. The effects of *Esi47* on the activities of the promoters of hormone-regulated genes fused to the reporter gene for β -glucuronidase (GUS) could thus be measured. The barley ABA-inducible *Hval* gene promoter (Straub et al., 1994) and the barley gibberellin (GA)-inducible acidic α -amylase gene promoter (Lanahan et al., 1992) were

the two promoters tested in the forms of DNA constructs *Hval-GUS* and *Amy-GUS*, respectively.

The *Hval-GUS* chimeric gene was responsive to ABA in barley aleurone layers since the GUS activity was induced 27 fold by treatment with 20 μ M ABA compared to the untreated tissues (Figure 10A). Co-bombardment of *Hval-GUS* with *Act1-Esi47* did not show any significant effects on the *Hval* promoter when the aleurone tissue was not treated with ABA. Therefore, *Esi47* had no influence on the expression of the *Hval* gene in barley aleurone tissues. Moreover, in the presence of *Act1-Esi47* the induction of *Hval-GUS* by ABA was 28 fold, very similar to the induction rate in the absence of *Act1-Esi47* (Figure 10A). However, when the GUS values are considered, *Act1-Esi47* caused a minor, but nevertheless statistically significant ($p = 0.014$), 24% reduction of the GUS activity in the ABA-treated aleurone. *Act1-Esi47^{K124Q}* had no effect on the *Hval* promoter activity or the ABA induction of the *Hval* gene (Figure 10A). This mutant form of *Esi47* also showed no significant difference from the wild-type *Esi47* gene in the GUS activities controlled by the *Hval* promoter regardless of being treated with ABA.

The GUS activity controlled by the barley α -amylase gene promoter could be induced 55 fold by treatment of 1 μ M of GA when the barley aleurone tissues were bombarded with the DNA construct *Amy-GUS* (Figure 10B). Co-bombardment with the DNA construct *Act1-Esi47* did not affect the basal level activity of the α -amylase gene promoter, but *Act1-Esi47* caused the GA-induction of the gene promoter to be reduced to only 16 fold. That was a significant and dramatic 77% decrease of the GUS activity controlled by the α -amylase gene promoter in GA-treated aleurone compared to the assay without *Act1-Esi47* (Figure 10B). Such inhibition by *Esi47* on the GA-induction of the

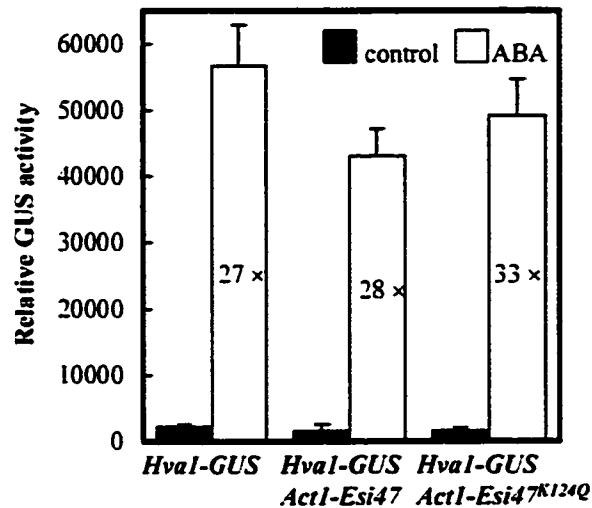
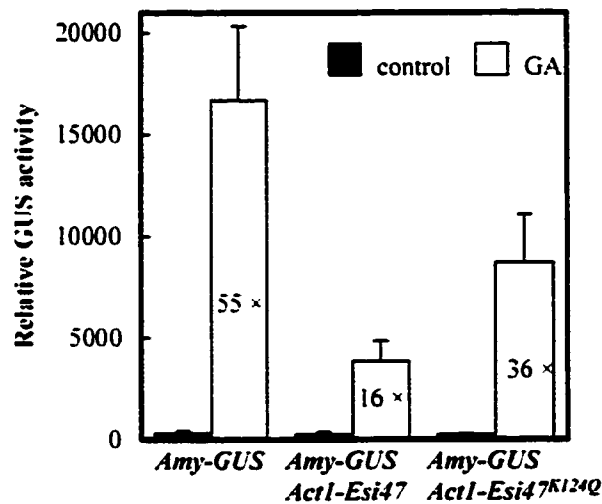
A**B**

Figure 10. Influence of *Esi47* on the promoter activities of the plant-hormone-inducible genes in barley aleurone. Barley aleurone layers were bombarded with the gene constructs indicated. The effector constructs are *Act1-Esi47* (the *Esi47* protein kinase coding region controlled by the promoter and 5'-UTR intron of the rice actin-1 gene *Act1*) and *Act1-Esi47^{K124Q}* (same as *Act1-Esi47* but *Esi47* contains the K124Q mutation). (A) The barley ABA-inducible *Hval* gene promoter was fused to the reporter gene for GUS to form the reporter construct *Hval-GUS* and the bombarded tissues were treated with 20 μ M ABA for 24 h as indicated. (B) The barley GA-inducible α -amylase gene promoter was fused to the reporter gene for GUS to form the reporter construct *Amy-GUS* and the bombarded tissues were treated with 1 μ M GA for 24 h as indicated. In both experiments, the controls were not treated with ABA or GA. In all assays, GUS activities were measured and normalized against the luciferase activities co-bombarded into the barley aleurone. The values are averages of four independent shootings and their standard deviations are shown as error bars. The induction rates by ABA or GA are also shown.

barley α -amylase gene promoter could be partially relieved when the K124Q mutant form of the *Esi47* gene replaced the wild-type *Esi47* in the assays. In the presence of the DNA construct *Act1-Esi47^{K124Q}*, the GA-induction of the α -amylase gene promoter was 36 fold. *Act1-Esi47^{K124Q}* caused a 48% decrease of the GUS activity controlled by the α -amylase gene promoter compared to the assay without *Act1-Esi47^{K124Q}* in the GA-treated barley aleurone (Figure 10B).

These data indicate that the *Esi47* gene suppresses the GA action in barley aleurone as far as the α -amylase gene is concerned. Such regulatory function of the *Esi47* gene may be partially dependent on the protein kinase activity of its gene product. On the other hand, the data do not indicate that *Esi47* has any definite influence on the ABA induction of certain genes such as *Hval*.

5. CHARACTERIZATION OF THE *ARABIDOPSIS THALIANA* HOMOLOGS OF THE *ESI47* GENE

5.1. The *Arabidopsis thaliana* *Esi47*-Homologs Are Structurally Similar to *Esi47*

Identification of the *Arabidopsis* homologs of the *L. elongatum* *Esi47* gene would provide a basis for manipulating the expression of the endogenous genes in *Arabidopsis* to assess their functions. This strategy was feasible since the *Arabidopsis* genome sequencing project was nearly completed at the time of the experiment and thousands of EST cDNA clones are also available.

The gene sequences with the highest similarity to *ESI47* that are currently available in the GeneBank non-redundant database are from *Arabidopsis*. In the

sequence similarity tree shown in Figure 3, *Esi47* clusters with *F8A24.12*, *F12E4.50* and *T7F6.28* in a minimal clade. *F8A24.12*, *F12E4.50* and *T7F6.28* are located in the *Arabidopsis* chromosomes 3, 5 and 2, respectively. *F8A24.12* and *F12E4.50* are derived from a duplication of segments in chromosomes 3 and 5 as indicated by the profound similarity of the BAC clone (*F8A24* and *F12E4* or *MOK16*, respectively) sequences encompassing the two genes (Blanc et al., 2000). Since the genome sequence of *Arabidopsis* has been completely determined, it can be concluded that these three genes are the *Esi47*-homologs in *Arabidopsis*. Coding sequence prediction indicates that all the three genes contain three introns in their coding regions at exactly the same positions as in the *Esi47* gene (Figure 11). ESTs exist in the GenBank database for these *Arabidopsis* protein kinase genes. One of the ESTs for *F8A24.12* (GenBank accession number A1993614, derived from the clone 701496785), includes the 5'-UTR of the gene. Comparing the 5'-UTR sequence and the corresponding genomic sequence of the *F8A24.12* gene reveals that this gene, like *Esi47*, also has a small uORF and a 5'-UTR intron that separates the uORF and the protein kinase ORF (Figures 11 and 12). The length of the *F8A24.12* uORF (27 bp), its sequence and its distance to the kinase ORF in the mRNA (125 nucleotides) are different from those of the *Esi47* gene (compare Figures 4 and 12). The 338-bp 5'-UTR intron of *F8A24.12* does not show any sequence similarity to that of the *Esi47* gene (Figures 4 and 12). However, the order of these elements is conserved between the *L. elongatum* gene *Esi47* and the *Arabidopsis* gene *F8A24.12*. The EST clones available for *F12E4.50* and *T7F6.28* are partial length cDNAs and are too short to determine the presence of any uORFs or introns in their 5'-UTRs (Figure 11).

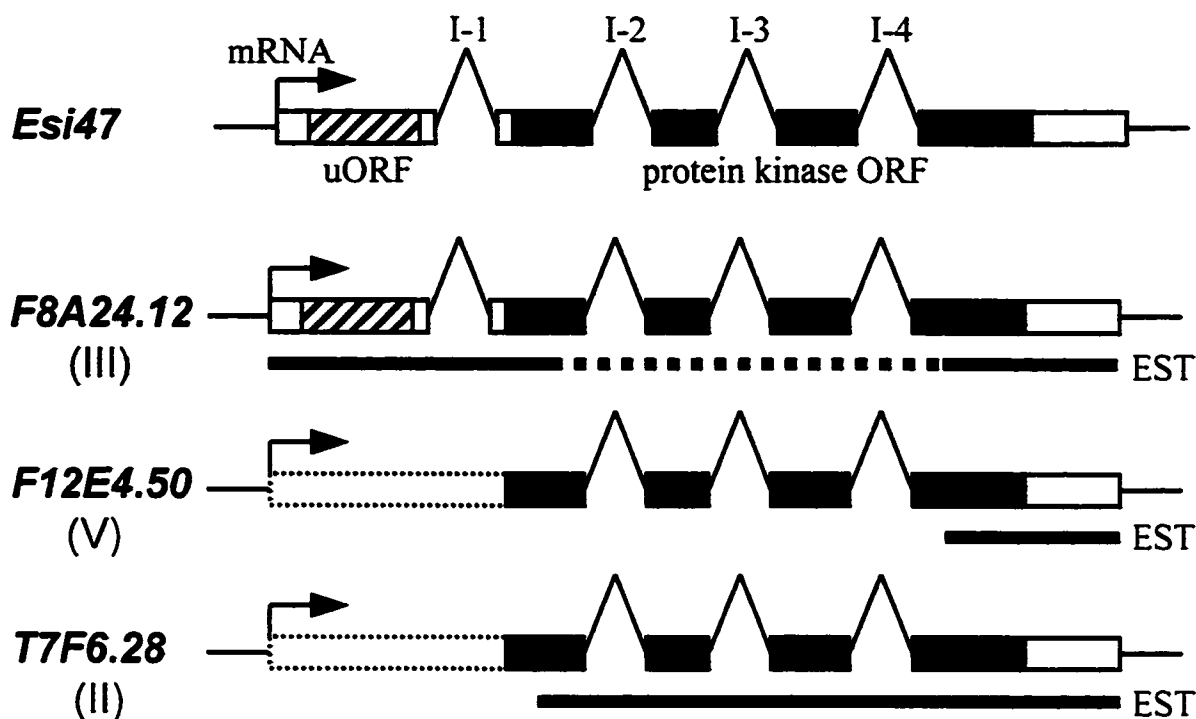


Figure 11. Schematic diagrams of the structures of *Esi47* and its *Arabidopsis thaliana* homologous genes. Open boxes represent untranslated exons; filled boxes represent protein kinase ORFs; hatched boxes represent uORFs; boxes with dotted lines are hypothetical 5'-UTRs for which no cDNA sequence is available; inverted "Vs" represent introns. For the EST clone representation, solid thick lines represent sequenced parts of cDNA insert of EST clone; dashed thick lines represent unsequenced parts of cDNA. The chromosomes on which the *Arabidopsis* genes are located are indicated in brackets. The introns of *Esi47* are numbered as I-1, I-2, I-3 and I-4. Introns of the *Arabidopsis* genes shown in the region not confirmed by cDNA sequences are predicted by gene annotation as provided in the GenBank entries. The regions are not drawn to scale.

1 TTCTACTTCTTATCTTCTACGTATACTAATCAAACGAATTTGTGCATAAATTGGCATGG
61 GAAAGGCCACGAGTCAAATATGATTTGATCCATTTTAGTTTTAACATCTATTGGTACGTA
121 CGTGAAGTTACGAGATTTGAGTGAAGGCCAATAATATCAATACGAAAAGTACAAAACGGG
181 GATCAAACCGCAAATTGTAACACAGCAAGCGTAAGCAACACGACAAAGTGGTATGGATTA
241 GTTTAACATAATCGTATAAATAGGTTAAGTTACCAAGGTTAAGGTATCTCAACAAGTGG
301 TACAAATTACCAACAAGATTTAAGAAACATATAGATCAATCGGGATTCCGAAACCAAT
361 ACCGAGGATTTCTTAAATCATTATTTTTGTGTGAAAATGCTAAACTTTGTCGTTAAAT
421 TTATGTACTCTAGGTTCTAGGAAGATTTAGGAAATCTCCTAGGTGACCCAAGTGAATAT
481 CCCTTCCACTTCCCATTGCAAATCTCATGATTACCATAAAACAAATAAGATTAGTAGTAG
541 AGAACTATGCTTGTTAACATAAAACATTAATGTAATTATGTAAGATTATATTTTTGAACCAC
601 TTTGGAAAGTGGAAACCATTTTGGCATCATGGGCCGACTCACCCACCTAAGAATAAAAC
661 TTCGTATTGTTATAGATTTTCAATCACTGATATTTAACAATGGTTTTTTTTTTTTGCAA
721 CAGATGGTTGATGTTAGATGATAGTAAGTTAGTACCATTAATATAAAAACAACATTAATG
781 AATACTTGAAAATTATAAACTGTGACTATATTATCTATACATAATCTATAAAAAATATT
841 TATTTTTCAAAGCTCAAACATTGGTTGAGTTGCTATCATAAATGCATAATCATATACA
901 TCTTGAGTCACTCACCGTCGTTTATTGTAGACTTTGTAGATAGTTTTATTTTGTATGTTA
961 AAACGTTTCACCATCGTTTATTTCGCATTCCATTAATTTGAAGATTTATTCTGTCATAGTA
1021 AAATCCCTAAACAATAGAAAATGATGTTGTTGAAATAAGTAAGATTTTATTACTAAATA
1081 AGACCATAACTCATAAGTGATTTAGATACACTGATCGATCTGTTGGCTACTTTTCTAAGC
1141 ACCTCACCGTTGAACAAGTCAAAGGAAAAAGAAATGAAAACGTTGTTTTATGTGTGATT
1201 GTGTGTCTAATAAAGAATATTAATTTTGGAAACATCTTTAATAGTAACAATAATTCA
1261 TTTGTTTTGTCCAATTTAAATCTCAGTTCTTCGTTCTTTTCGCTGACTTTAGCTATCTCT
1321 CTCTGTAAAGACTTCGTTTTTTTTTGTGGCCGTGTGACGACTTCTTCTCTCTCTCTC
1381 TTCGACCTCTTCTCTCGGAAGCTTAGCTTCTTAAATCCATTTCTCTCGGAAGCTTAGC
1441 **TTCTCAAATCCTCGATCTCTTTGTATCGTGATTCAATTAACCACTTCATGATATTTATA**
M I I I
<uORF>

1501 **TTCTCCTTCTCTCTCGATCGATTGAATTACATCTTCTTCTGATAGCTGTTCTGATTTTTTT**
I F L L F -
uORF>

1561 **CTGTATACAAAGTAAATTCAAAGGTGAGAGTTGTGTCATCTTTTGCTTTTTATATACTTG**
<5'-UTR intron>

1621 TTCAATAAGTCAAAGTCTTTATGTTGCTGTTGGGTTTTAGTTTATTGGAATCAACTTGC
1681 GAAATTTGATTACTTTTAGCTTCGAGATCCCTTCTATTACTACTGGAACCTTAGGGTTG
1741 TGTTACTTGGAAAAGAATTTGGATCTGTTGGGTCTAAAGGCCCTCCTCTGTTAAGTGG
1801 TTTTGTACTTGGGGGAAGCCTTAACCTTTGAAAATGCTTGTACTTTGCAGATTAGTTGTT
1861 GATAATATGCTTCAATGTTTTGATTTGTGGTTCGGTATATGTTATATGATCATTATCA
5'-UTR intron

1921 **GTGTTTTATTGGTGTCTCAGTGGACAAACATCGGTATTTTAACTCCTCTCGATAATG**
> <

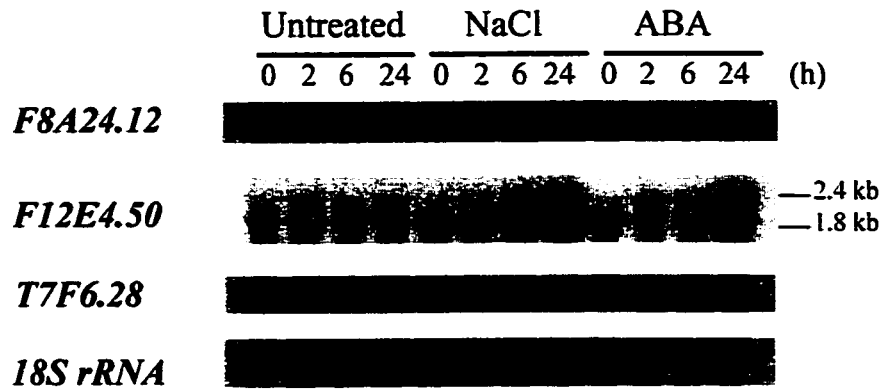
1981 **AAGTGTCTTCTGTTCTCTGGTGGGGATAAGAGAGGGGAACAGAAGACCCCTATTTCCGGTT**
protein kinase ORF

Figure 12. Promoter and 5'-untranslated region of the *Arabidopsis thaliana* F8A24.12 gene. The transcribed sequences confirmed by the expressed sequence tag 701496785 are shown in bold letters. The small upstream open reading frame (uORF) is highlighted and its translated amino acid sequence is shown. The 5'-untranslated region (UTR) intron is indicated. The start codon for the protein kinase open reading frame (ORF) is also highlighted. Note the transcription initiation site is not determined.

5.2. The *Arabidopsis thaliana* *Esi47*-Homologs Are Differentially Regulated by NaCl and Abscisic Acid

Regulation of the expression of these *Arabidopsis Esi47*-homologous genes was analyzed by northern-blot hybridization with RNA samples from hydroponically grown *Arabidopsis* plants. In leaves the expression of the three genes was very weak when the plants were not treated, and only *F8A24.12* was induced by treatment of 250 mM NaCl (Figure 13). In roots, all three genes were slightly expressed in control untreated plants and the gene expression was differentially regulated by NaCl and ABA. *F8A24.12* was induced by treatment of 250 mM NaCl within 6 h but not by treatment of 100 μ M ABA in roots (Figure 13). Though *F8A24.12* was induced in both roots and shoots by NaCl treatment, the patterns of expression were different in the two tissues. Induction in roots was high after 6 h of treatment but had declined by 24 h, whereas the pattern of expression in the leaves was biphasic with gene induction apparent at 2 and 24 h but not at 6h. The reliability of the latter result was confirmed by the control probe, *AtP5CS1* for Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) (Yoshida et al., 1995), a salt-stress-inducible gene which showed a normal linear pattern of induction when used on the same blot (Figure 13). In contrast, *T7F6.28* was not induced in roots by NaCl treatment but could be induced by ABA treatment as early as 2 h (Figure 13). For *F12E4.50* two transcript bands of 1.8 kb and 2.4 kb were detected (Figure 13). This is not likely due to cross-hybridization with a transcript from a related gene since the probe was derived from the EST clone 311H3T7 which corresponds to the 400-bp at the 3'-end of the transcribed sequence of *F12E4.50* and has only up to 61% sequence identity to the other *Arabidopsis* homologs. The levels of the two transcripts were not elevated within 6 h

Root



Leaf

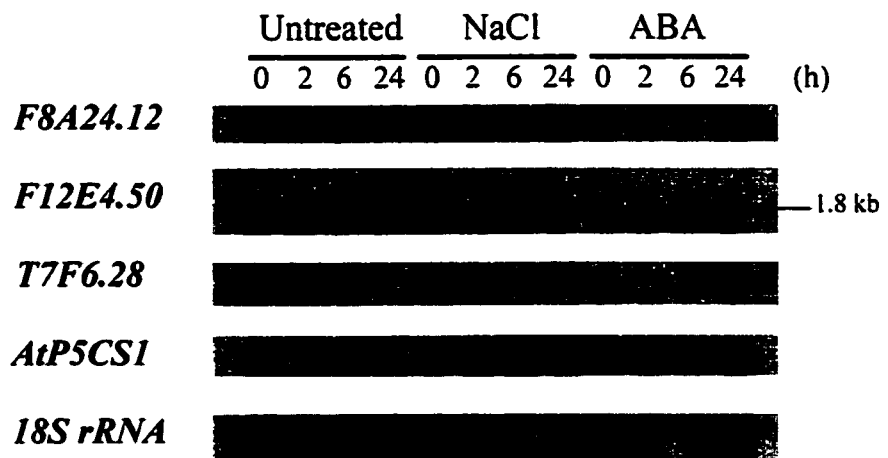


Figure 13. Northern blot analysis of the expression of the *Arabidopsis thaliana* genes homologous to *Esi47*. Total RNA samples from roots or leaves of hydroponically grown *Arabidopsis* plants were analyzed. The plants were either untreated, or treated with 250 mM NaCl or 100 μ M ABA for various durations as indicated. The cDNA inserts from the EST clones for the *Arabidopsis Esi47*-homologous genes *F8A24.12*, *F12E4.50* and *T7F6.28* and for the *18S rRNA* and *AtP5CSI* genes were isolated and labeled with 32 P to be used as probes. The control probe, *AtP5CSI*, was used as an example of a previously characterized NaCl and ABA inducible gene.

after the onset of any treatment, but they showed elevated levels 24 h after the start of both NaCl and ABA treatments (Figure 13). The fact that the three genes did not show overlapping expression patterns and that these genes are the most related members in a cluster in the phylogenetic tree based on the completed sequence of the *Arabidopsis* genome (Figure 3) indicate that the expression patterns revealed by the northern analysis are specific to each gene.

PART IV. DISCUSSION

In this study, I obtained the full-length cDNA and genomic DNA clones for the salt stress induced salt-tolerant wheatgrass *Lophopyrum elongatum* gene *Esi47*. The nucleotide sequences of both cDNA and genomic DNA of the *Esi47* gene were determined. The sequenced genomic DNA includes the entire transcribed region and the 2.5-kb 5'-upstream region. *Esi47* was defined as a protein Ser/Thr kinase gene due to the high amino acid sequence similarity of its product to plant protein Ser/Thr kinases. The protein kinase activity of *Esi47* was indirectly demonstrated in an *Escherichia coli* strain containing the plasmid for expressing the gene. Transgenic *Arabidopsis thaliana* lines expressing *Esi47* were obtained but they did show any altered phenotypes of stress tolerance or gene regulation. However, *Esi47* was shown to suppress the induction of the barley α -amylase gene by gibberellin (GA) in barley aleurone, possibly through the protein kinase activity of its gene product, although no effect of *Esi47* could be clearly demonstrated on the abscisic acid (ABA) induced gene *Hva1* in the same tissue. In addition, the *Esi47* homologs in *Arabidopsis* were identified and they showed differential regulation patterns by salt stress and ABA in roots and leaves.

1. *ESI47* IS A PROTEIN KINASE GENE

The amino acid sequence of the *Esi47* gene product can be deduced from the nucleotide sequence of its cDNA (Figure 1). By comparing this sequence of 433 amino acid residues to the amino acid sequences of the proteins in the GenBank and other databases, it is apparent that *Esi47* belongs to the protein kinase family since it shows

very high scores of similarity to many plant protein kinase sequences. Some of the sequences showing similarity to *Esi47* are themselves predicted to be protein kinases from their DNA sequences, mostly from the DNA sequence of the *Arabidopsis* genome, whereas many others have already been shown to be functional protein kinases. The *Arabidopsis* protein kinase APK1 is most similar to *Esi47* among the protein kinases whose catalytic activities have been demonstrated (Hirayama and Oka, 1992).

Protein Ser/Thr kinases and protein Tyr kinases have similar amino acid sequences in their catalytic domains, which can be divided into 11 subdomains (Hanks et al., 1988; Hanks and Quinn, 1991). *Esi47* shows sequence similarity to APK1 and other closely related plant protein kinases throughout all the 11 subdomains (Figure 2). A number of the amino acid residues at certain positions in these subdomains are conserved in all of the protein Ser/Thr kinases and protein Tyr kinases (Hanks et al., 1988; Hanks and Quinn, 1991). There are additional amino acid residues conserved in all of the protein Tyr kinases. *Esi47* possesses all of the residues that are conserved in all of the protein kinases but not all of the additional invariant residues conserved particularly in protein Tyr kinases (Figure 1). On the phylogenetic tree (Figure 3) *Esi47* does not cluster with the mitogen activated protein kinase kinases (MAPKK), which are able to phosphorylate both threonine and tyrosine residues in their substrates. Therefore, *Esi47* is almost certainly a protein Ser/Thr kinase gene. However, final classification is possible only when *Esi47* is shown to phosphorylate specific substrates and the phosphorylated amino acids can be detected in thin layer chromatography assay.

It should be noted that the *Arabidopsis* protein Ser/Thr kinase APK1 was also shown to have an activity of phosphorylating tyrosine residues in proteins as assayed by

immunodetection of phosphotyrosine in the phosphorylated protein substrates with antibodies against phosphotyrosine (Hirayama and Oka, 1992). However, since the data were published, there never has been any confirmation of such activity reported. Neither have there been any reports of similar activity from a related plant protein kinase. The real amino acid residue specificity of APK1 awaits assays with its natural protein substrate. The current state of characterization of APK1 does raise the possibility that Esi47 may have tyrosine phosphorylation activity.

2. IS ESI47 AN ACTIVE PROTEIN KINASE?

2.1. *In Vitro* Assay

The classical way of assaying protein phosphorylation is to use [γ - 32 P]ATP as labeled phosphate donor in a reaction mixture containing a protein kinase and its protein substrate, and subsequently detecting the phosphorylated substrate by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Histone, casein and some other proteins are commonly used as substrates in such assays. Many protein kinases can also autophosphorylate. Purified protein kinase is necessary for this method of protein phosphorylation assay. In an effort to obtain purified Esi47 protein in quantity, the *Esi47* protein kinase open reading frame (ORF) was fused to either the (His)₆ tag or glutathione-S-transferase (GST) for expression in the bacterium *E. coli* in this study. When it was fused to the (His)₆ tag, no *E. coli* transformants containing the plasmid DNA construct for expressing the fusion protein were obtained (data not shown). This indicates that the fusion protein might be toxic to *E. coli* cells and could serve as indirect

evidence that the *Esi47* gene product functions in *E. coli*. When GST was used in fusion with *Esi47*, the fusion protein in the *E. coli* soluble protein fraction was not stable and showed evident degradation as demonstrated by the protein profiles in the elution fractions from glutathione-Sepharose affinity chromatography (Figure 6).

Subsequently, the protein kinase catalytic domain of *Esi47* (Figure 1) was used to make a fusion protein with GST. This time the fusion protein was stable in the soluble fraction of bacterial extract. But it did not show any autophosphorylation activity or phosphorylation activities with histone or casein as substrates, even when different combinations of Mg^{2-} , Mn^{2-} and Ca^{2-} in the reactions were tried (not shown). It is possible that *Esi47* has a very narrow spectrum of substrates and the proteins used in the assays were not suitable substrates, or the protein phosphorylation activity of *Esi47* is dependent on either or both of the amino and carboxyl terminal non-catalytic domains. The latter reason was only a speculation since for some protein kinases, such as plant Ca^{2-} /calmodulin-dependent protein kinases (CDPKs), a region in the non-catalytic domains inhibits the kinase activity of the catalytic domain (Goldberg et al., 1996).

2.2. *In Vivo* Assay

Finally, I designed an assay in which carrier-free [^{32}P]orthophosphate was used to trace phosphorylation activity in *E. coli* cells. The above-mentioned GST fusion constructs were used in such assays. The fusion genes were under the control of the bacterial *lac* operator so that they could be induced by IPTG. The IPTG-treated bacteria were incubated with [^{32}P]orthophosphate and cells were disrupted by boiling in the presence of SDS. The protein profiles of the total bacterial protein extracts were

determined by SDS-PAGE and the phosphorylated proteins were shown by autoradiography. A unique labeled protein band of approximately 68 kD was shown to be present in the extract of the IPTG-treated *E. coli* containing the GST-Esi47 construct (Figure 7B). The autoradiography showed that this band was missing from the control *E. coli* cells that were not treated with IPTG. Moreover, the DNA construct for the fusion of GST with the K124Q mutant form of Esi47, GST-Esi47^{K124Q}, did not give rise to such a band (Figure 7B). This band was at the same position as the one showing slight overproduction in the Coomassie Blue-stained gel of the total protein extract (Figure 7A). Therefore, it is highly likely that this band was the autophosphorylation product of the GST-Esi47 fusion protein expressed from the plasmid DNA construct. This is a clear, although not direct, indication that Esi47 is an active protein kinase. The slight difference between the measured molecular mass of the phosphorylated protein band (68 kD) and the predicted molecular mass of the GST-Esi47 fusion protein (76 kD) might be due to either the inaccuracy of the SDS-PAGE for measuring protein molecular mass, or more likely, this band was the degradation product of the full-length GST-Esi47 protein and the loss of a few kD at either terminus did not affect the kinase activity of the protein. The presence of this band in the total bacterial extract but not in the soluble fraction of the protein preparation indicates that overproduction of the GST-Esi47 protein led to the formation of insoluble inclusion bodies. Such inclusion bodies must contain protein molecules that were phosphorylated immediately after being synthesized.

When *E. coli* harbouring the plasmid DNA construct for the fusion of GST with the protein kinase catalytic domain of Esi47 was used in such *in vivo* protein phosphorylation assay, no distinct labeled protein band was found in the IPTG-treated

cells compared to the bacterial extract from untreated cells (Figure 7B). This again demonstrates that the protein kinase catalytic domain of *Esi47* by itself may not have any protein phosphorylation activity.

However, the assays with carrier-free [³²P]orthophosphate in *E. coli in vivo* only provided indirect evidence for the protein kinase activity of *Esi47*. The phosphorylated band of 68 kD may be from an internal bacterial protein phosphorylated due to the presence of the *GST-Esi47* fusion gene through either the kinase activity of its gene product or an unknown function that led to the phosphorylation of the protein. Direct demonstration of protein kinase activity of *Esi47* would require a purified and catalytically active protein. *E. coli* is notorious for the instability of overexpressed proteins from exogenous genes. Other protein production systems could be tried in the future. For example, the Baculovirus gene expression system has been used successfully in many cases for expressing eukaryotic genes and quantitatively producing the proteins. Protein kinase activity assays with the gene product of *Esi47* would have higher chance of success if the natural protein substrate for *Esi47* were identified and available for assay.

3. INVOLVEMENT OF *ESI47* IN PLANT STRESS AND HORMONE SIGNALING

3.1. Does *Esi47* Have a Role in Mediating Signal Transduction in Vegetative Plants?

The rationale for beginning this study was that since *Esi47* is a salt stress induced protein kinase gene, it might play a role in stress or hormone signaling. Functional

characterization of this gene may contribute to our understanding of how plants respond and adapt to salt and other water deficit stresses. As mentioned in Part I of this thesis, protein kinases play key roles in detecting and relaying developmental and environmental signals for the regulation of specific genes and thus mediate cellular responses to those signals. None of the genes in the NAK group of plant protein kinases, to which *Esi47* belongs, has yet been investigated for a role in signal transduction.

One of the approaches to investigating the role of such a gene in signal transduction is to alter the expression level of the gene and then observe what phenotypes it might cause. The *Esi47* null mutant of *L. elongatum* or a genetically closely related monocot species is not available. On the other hand, overexpression of the *Esi47* gene in monocot species by stable transformation is beyond the available resources supporting this study. Thus, I chose *Arabidopsis* as host for constitutively expressing *Esi47* using the *Agrobacterium tumefaciens*-mediated DNA delivery technique. *Arabidopsis* is easy to transform. Moreover, the *Arabidopsis* genetic data and resources are widely available and would assist functional analysis of any genes being transferred. Therefore, *Arabidopsis* was transformed with the *Esi47* gene under the control of the constitutively active cauliflower mosaic virus (CaMV) 35S promoter (*35S-Esi47*). Homozygous transgenic lines expressing *Esi47* as assayed by northern analysis (Figure 8A) were obtained. However, these transgenic *Arabidopsis* plants showed no phenotypic changes in morphology, growth and stress tolerance (Figure 9). In addition, northern blot hybridization analysis of about 60 *Arabidopsis* genes known to be regulated by salt stress or ABA in the *Esi47*-expressing transgenic *Arabidopsis* lines does not indicate a possibility that *Esi47* could mimic stress or ABA signals in regulating some of the genes

tested (Figure 8). Any positive results from this experiment would have proved that *Esi47* is involved in signal transduction. The higher mRNA levels of the three *Arabidopsis* genes, *STZ*, *ERD11* and *SIMIP*, found in 18-day old whole plants of *Esi47*-expressing transgenic *Arabidopsis* than those in the wild-type plants could not be reproduced and could not be found in younger or older plants (Figure 8). These three genes themselves showed changing expression levels at different ages (Figure 8). Therefore, it is likely that slight differences in physiological age or in culture conditions would affect the expression levels of these genes.

The absence of any apparent effects of the *35S-Esi47* transgene in *Arabidopsis* may reflect a fact that *Esi47* is an exogenous gene and its gene product did not recognize the *Arabidopsis* target proteins to render its regulatory function. Or, the activity of *Esi47* might be too weak to cause any effect even though the signaling pathways are widely considered conserved between the monocots and dicots. It is also possible that *Esi47* alone might not be enough to show any detectable phenotypic changes in the transgenic *Arabidopsis* plants if the gene product is merely one of the components of a multi-protein complex controlling a specific signaling pathway. However, one indication that *Esi47* may have a critical cellular function came from the results of transforming *Arabidopsis* with the K124Q mutant form of *Esi47* (*35S-Esi47^{K124Q}*) together with the kanamycin-resistance gene as selection marker. None of a total of nine transgenic lines obtained could segregate to produce any homozygotic progenies by self-crossing (data not shown). It seems that the embryos homozygous for *35S-Esi47^{K124Q}* were not viable. It could be that the high dosage of the mutant form of the *Esi47* gene was lethal to the embryos by interfering with some important cellular process. The cellular process concerned might

be dependent on the protein kinase activity of the *Esi47* gene product since the altered Lys residue at the position is considered important for the phosphorylation activity of protein kinases (Hanks et al., 1988).

Another possibility for *35S-Esi47* having no detectable phenotypes in transgenic *Arabidopsis* plants is that the *Esi47* protein did not accumulate to a proper level that would be sufficient for exerting its effects. The transcript levels of the *Esi47* gene were high in all of the transgenic *Arabidopsis* lines tested (Figure 8A). However, the presence of the *Esi47* protein could not be detected by immunoblotting with polyclonal antibodies raised in rabbit against the fusion protein of GST and the amino terminal non-catalytic region of *Esi47* (not shown). It is possible that the *Esi47* protein was degraded immediately after being translated. Moreover, when the effect of *Esi47* on the expression of stress or ABA regulated genes is concerned, it is possible that overexpression of *Esi47* led to a certain degree of negative feedback in a loop of a pathway in the early stages of plant development. That would consequently result in desensitizing those genes in later growth.

Alternatively, *Esi47* may have no regulatory activity in gene expression and it may not play a critical role in mediating salt stress tolerance in plants. However, before reaching such a conclusion, a broader spectrum of investigations is necessary. This is possible because the technologies developed recently in genomics studies may help to solve this puzzle. For example, by using the DNA microarray technique, thousands of genes, instead of a merely 60, can be screened to see if any of them are regulated by *Esi47* in transgenic *Arabidopsis* plants. Moreover, the yeast two-hybrid system might be used for searching for any *L. elongatum* or *Arabidopsis* proteins interacting with *Esi47*

and such proteins may give suggestive clues to the function of *Esi47*. On the other hand, identification of the *Arabidopsis* counterparts of *Esi47* would greatly help to decipher the cellular functions of the genes since it would be possible to manipulate the expression of the *Arabidopsis* genes, especially to disrupt their expression by, for example, T-DNA insertional mutation, co-suppression, or dsRNA-mediated posttranscriptional gene silencing (PTGS). Searching for and characterizing the *Arabidopsis* homologs of the *L. elongatum Esi47* gene is part of this thesis.

3.2. *Esi47* Suppresses the Gibberellin Action in Barley Aleurone Tissues

Another way to study the cellular function of a gene is to look at any effect it has in plant cells or tissues by transient expression of the gene. To do this, plasmid DNA constructs can be delivered by particle bombardment of plant tissues or by electroporation of protoplasts. In this study the barley aleurone layer tissues were chosen for transiently expressing the *Esi47* gene. Barley is a monocot species and is genetically close to wheat and its wild relatives including *L. elongatum*. Species in the Gramineae, including barley, wheat and *L. elongatum*, possess the special aleurone layer tissues in their seeds. During seed germination, aleurone cells synthesize and secrete a variety of hydrolytic enzymes for breaking down and mobilizing nutrients stored in endosperm starch, protein and lipid. For germination GA in the aleurone cells promotes the production of the hydrolytic enzymes by upregulating the genes for these enzymes, whereas ABA prevents germination in part by inhibiting the GA action in the aleurone tissues. The ease of obtaining the tissues and the availability of a number of well-characterized ABA or GA regulated genes from these tissues made the barley aleurone a

model system for studying the ABA and GA signaling in plants (Lovegrove and Hooley, 2000). Since *Esi47* is also induced by ABA in *L. elongatum* roots (Galvez et al., 1993), looking at potential involvement of this gene in plant hormone signaling would shed light on its role in salt and water deficit stress signaling.

In collaboration with Dr. Tuan-Hua David Ho, in whose laboratory the transient gene expression system in barley aleurone has been established for routine experimentation, we measured the effect of the *Esi47* gene on the regulation of expression of ABA or GA induced genes. *Esi47* was placed under the control of the rice actin-1 gene (*Act1*) promoter and 5'-untranslated region (UTR) intron as the effector gene *Act1-Esi47*. The K124Q mutant form of the *Esi47* gene was also placed in the control DNA construct *Act1-Esi47^{K124Q}*. The reporter gene constructs were the β -glucuronidase gene (*GUS*) under the control of the promoters of either the barley ABA induced gene *Hval* (*Hval-GUS*) or the barley GA induced gene for acidic α -amylase (*Amy-GUS*). The experiments included bombardment of the aleurone tissues with various combination of the effector and reporter DNA constructs, subsequently treating the tissues with ABA or GA before measuring GUS activity in the tissues. The results showed that *Act1-Esi47* or *Act1-Esi47^{K124Q}* had no apparent effects on the basal level expression of the *Hval* gene promoter or on the ABA induction of this promoter (Figure 10A). *Act1-Esi47* also had no influence on the activity of the acidic α -amylase gene promoter when the tissues were not treated with GA. However, *Act1-Esi47* inhibited the GA induction of the α -amylase gene promoter by 77% (Figure 10B). Moreover, such inhibition on the GA action might be partially dependent on the protein kinase activity of the *Esi47* gene product since the

K124Q mutant form of the *Esi47* gene had a much less degree of inhibition of 48% on the GA induction of the α -amylase gene promoter (Figure 10B).

Thus, the effects of *Esi47* in barley aleurone tissues are very similar to those of the wheat protein kinase gene *PKAB1*. *PKAB1* had been shown to have only a small, irrelevant enhancing effect on the promoter activity of *Hva1* but to be able to inhibit the GA induction of a number of barley genes for hydrolytic enzymes, including both acidic and alkaline α -amylases and a cysteine proteinase (Gómez-Cadenas et al., 1999). Like *Esi47*, *PKAB1* is induced by ABA, salt and water deficit stresses (Anderberg and Walker-Simmons, 1992; Holappa and Walker-Simmons, 1995). The amino acid sequences of the two protein kinases are relatively different since *PKAB1* is more similar to the members in the SNF1-related protein kinase (SNRK) subfamily (Holappa and Walker-Simmons, 1995, 1997). Nevertheless, the two kinase genes both might be involved in the suppression of the GA induction of the genes for hydrolytic enzymes.

These results are in agreement with the model that the pathway with which ABA induces the expression of the *Hva1* gene is independent of that for the ABA-mediated suppression of the GA induction of the genes for the hydrolytic enzymes (Lovegrove and Hooley, 2000). The magnitude of the effect of *PKAB1* on the basal level expression of the *Hva1* gene observed by Gómez-Cadenas et al. (1999) was much lower than the degree of gene induction by ABA. Since such an effect was dependent on the dosage of the effector DNA, a satisfying explanation could not be obtained, as the authors did not do control assays in which the vector would be used as the effector DNA. In contrast, our results showed that *Esi47* had no effect on the basal level expression of *Hva1* but a minor, nevertheless statistically significant ($p = 0.014$), 24% reduction of the *Hva1*

promoter activity in the ABA-treated aleurone when the values of the GUS activities are compared (Figure 10A). However, when the ABA induction rates are compared, there is almost no difference between the assays with and without the *Act1-Esi47* effector DNA. Moreover, there is no significant difference between the effects of the *Esi47* gene and its K124Q mutant form on the GUS activities controlled by the *Hva1* promoter in the ABA treated aleurone tissues. There is also no difference between the assays with *Act1-Esi47^{K124Q}* and the assays without effector DNA. Therefore, the true nature of the relationship between *Esi47* and the ABA induction of *Hva1* needs to be investigated in another independent way. An explanation for our observations so far could be that a certain negative feedback mechanism activated by *Esi47* caused slight inhibition on ABA production or perception in the aleurone tissues.

How do the two protein kinase genes suppress the GA induction of the genes for hydrolytic enzymes in barley aleurone? In grass seeds GA is synthesized *de novo* in the embryo and induces the expression of the genes for hydrolytic enzymes in the aleurone cells when the ABA level is diminishing during seed germination. Exogenously applied ABA can suppress the GA action on those genes (reviewed by Lovegrove and Hooley, 2000). Both *Esi47* and *PKABA1* are induced by ABA and these experiments demonstrated that they could suppress the GA action in the aleurone cells. Thus, the two genes can mimic the effects of ABA (Figure 14). The transcripts for the *PKABA1*-like gene in barley aleurone could be detected and the levels could be elevated by ABA treatment (Gómez-Cadenas et al., 1999). *Esi47* is only known to be induced by ABA or stress in vegetative tissues. In this study confirmation of whether the barley homologous gene of *Esi47* is induced by ABA in aleurone was not attempted. Since the *Esi47* gene

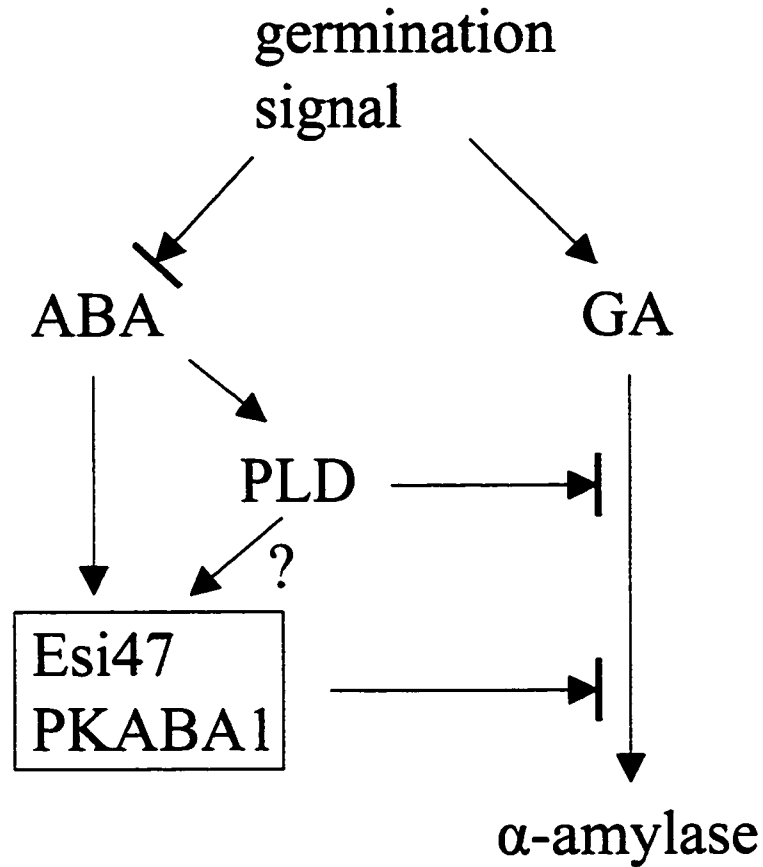


Figure 14. A schematic diagram of the involvement of the *Esi47* gene in stress and hormone signaling. Abscisic acid (ABA) increases the phospholipase D (PLD) activity which in turn represses the gibberellin (GA) induction of the genes for hydrolytic enzymes including α -amylase. ABA induces the expression of *Esi47* and *PKABA1* probably through the PLD activity (?). *Esi47* and *PKABA1* both suppress the GA induction of the gene for α -amylase.

showed effects on gene expression in aleurone, it could be assumed that at least the pathway downstream of the *Esi47*-like gene is present in seed aleurone and is ready to be activated.

Another signaling component implicated in mediating the ABA-promoted suppression of the GA action in aleurone tissues of grass seeds is phospholipase D (PLD) (Ritchie and Gilroy, 1998). In barley aleurone protoplasts the PLD activity was induced within 10 min after exogenous ABA was applied, and at the same time the product of PLD activity, phosphatidic acid (PA), also increased transiently. Treatment of aleurone protoplasts with PA caused inhibition of the GA promoted production of α -amylase as demonstrated by the immunoblotting assays. The rapid induction of the PLD activity by ABA indicates that PLD might be an early component in the ABA signaling pathway leading to the suppression of GA action. Therefore, there is a possibility that PLD acts upstream of *Esi47* and *PKABA1* (Figure 14).

Although *Esi47* and *PKABA1* may likely perform different functions in vegetative tissues from those in aleurone, which is a specialized type of tissue, segments of the signaling pathways may be well conserved. If so, in vegetative tissues *Esi47* or *PKABA1* may be regulated by ABA, probably through PLD, to regulate the GA actions in response to environmental stresses. Since GA is well known for its role in promotion of cell growth, there could be an intriguing possibility that activation of *Esi47* or *PKABA1* may cause transient cell growth arrest by suppressing the GA-mediated functions upon salt and water deficit stresses so as to allow cells to have enough time to adjust to the new, unfavourable environmental conditions. Our characterizations on the *Esi47*-transformed *Arabidopsis* did not show any growth retardation of such plants, probably due to the

inactivation of the transgene in these plants. Manipulating the expression of the *Arabidopsis* homologs of *Esi47* or *PKAB1* may offer further insight into the mechanism.

4. REGULATION OF THE *ESI47* GENE EXPRESSION

Previous data concerning the regulation of expression of *Esi47* in *L. elongatum* came from the northern analyses. The expression of *Esi47* was upregulated by salt or ABA treatment in root tissues but not in shoots (Galvez et al., 1993; Gulick and Dvořák, 1992). The mRNA level of *Esi47* could be elevated by about 4 to 5 fold within 6 h of salt treatment, would stay at similar elevation for 3 days and then decline gradually (Gulick and Dvořák, 1992). If we hypothesize that the *Esi47*-like genes contribute to stress tolerance in plants, then the pattern of the expression of the genes as well as the strength of the expression would be relevant to stress tolerance. One way to demonstrate the possible contribution of a gene to stress tolerance is to compare the expression pattern of the gene among different species or genotypes whose degrees of stress tolerance are different. A positive correlation of gene induction and stress tolerance in different genotypes was observed for *Esi47*. The degree of salt induction of *Esi47* in the salt-tolerant *L. elongatum* is higher than that in the salt-sensitive Chinese Spring wheat (Galvez et al., 1993). Therefore, it is important to know how the expression of the *Esi47*-like genes is regulated.

The most well characterized *cis*-determinants for regulating the expression of genes reside in the 5'-flanking region of the gene. Dissecting this region and analyzing the elements with potential regulatory functions would help to understand how the gene

expression is regulated. The nucleotide sequence of the 3-kb genomic DNA region upstream of the translation initiation site of the *Esi47* protein kinase ORF was determined in this study (Figure 4). Sequences similar to the *cis*-elements reported to mediate transcriptional control of gene expression in response to stress and ABA were searched for in the 1-kb region immediately upstream of the transcriptional start. A number of sequences similar to these elements have been located in this region (Figure 4). It contains several ACGT sequences that are the core of the G-box-like ABA responsive elements (ABRE) (Busk and Pagès, 1998; Shinozaki and Yamaguchi-Shinozaki, 1997). Sequences similar to the ABRE coupling elements (CEs) (Shen and Ho, 1995; Shen et al., 1996) CE1 and CE3 are found located around one of these putative ABREs. The distances of these putative CEs to the putative ABRE are similar to those of the functional CEs in the barley *Hva1* and *Hva22* genes. Other *cis*-element-like sequences appearing in this region of the *Esi47* promoter include the dehydration responsive element (DRE) (Yamaguchi-Shinozaki and Shinozaki, 1994), the MYB and MYC recognition sequences (Abe et al., 1997; Urao et al., 1993), Motif IIa and Motif IIb originally found in the rice ABA-responsive gene *rab16A* (Mundy et al., 1990), and a motif found in the ABA-inducible promoter of the *Craterostigma plantagineum* gene *CDeT27-45* (Nelson et al., 1994). However, it should not be concluded that these sequences mediate the regulation of the *Esi47* gene by salt stress or ABA unless each of them are proven necessary for the responsiveness in transient assay systems or in stable transgenic plants. Not all of the sequences located in the promoter region of a gene that resemble the *cis*-elements mediate the responses of the gene to the corresponding signals (Busk and Pagès, 1998). Therefore, a prediction is that only a small number of the

putative elements found in the promoter region of *Esi47* are functional for the regulation of *Esi47* by salt stress and ABA.

Unique structural features of *Esi47* reside in the 5'-UTR of this gene, namely, an intron and a short ORF upstream of the major protein kinase ORF (Figures 4 and 5). Many 5'-UTR introns especially in monocot plant genes have been shown to enhance gene expression levels (Simpson and Filipowicz, 1996). In contrast, upstream ORFs (uORFs) in plants have been reported to be able to reduce the translation efficiency of downstream major ORFs (for reviews, see Fütterer and Hohn, 1996; Gallie, 1996). To test what kind of regulatory activities the 5'-UTR intron and the uORF in *Esi47* have on the expression of the gene, the 3-kb *Esi47* genomic DNA fragment upstream of the protein kinase ORF start codon containing the 5'-UTR intron and the uORF was fused with the *GUS* reporter gene (*Esi47-GUS*) for assay in transgenic *Arabidopsis* plants. Modifications of the *Esi47* sequence included: 1) the 5'-UTR intron was removed (*Esi47^{ΔI}-GUS*), 2) the uORF was corrupted by removing the A and T nucleotides of the ATG start codon (*Esi47^{ΔuORF}-GUS*), and 3) both the 5'-UTR intron was removed and the uORF ATG codon was deleted as in *Esi47^{ΔI,ΔuORF}-GUS* (*Esi47^{ΔI-ΔuORF}-GUS*). In this attempt, no GUS staining was observed in any tissues from the transgenic *Arabidopsis* plants containing any of the DNA constructs, neither in untreated control plants nor when the plants were treated with salt or ABA (data not shown). It seemed that the promoter activity of the *Esi47* gene was too weak in *Arabidopsis* to drive the transcription of the *GUS* reporter gene, or the induction of the *Esi47* promoter by salt or ABA was not strong enough. A certain enhancer element, for example, the CaMV 35S minimal promoter,

might be necessary to be included in the gene constructs to elevate the basal level of expression.

In a parallel study, maize callus was used as host for transient expression assays through biolistic DNA delivery. In the constructs for bombardment of maize callus the 5'-UTR intron from the rice actin-1 gene *Act1* was included in the chimeric gene constructs mentioned previously, placed between the *Esi47* upstream sequence and the *GUS* reporter gene (*Esi47-Act1-GUS*, *Esi47^{Δ1-1}-Act1-GUS*, *Esi47^{ΔuORF}-Act1-GUS*, and *Esi47^{Δ1-1-ΔuORF}-Act1-GUS*). The rice *Act1* gene 5'-UTR intron is used to increase the basal level expression of the gene in which it resides (McElroy et al., 1990). Moreover, maize, like *L. elongatum*, is a monocot plant and could have a genetic background more similar to *L. elongatum* than that of *Arabidopsis*. The experiment showed that the unmodified upstream sequence of *Esi47* was able to respond to ABA treatment with a 2.6-fold increase of the GUS activity, although it was not induced by salt treatment (E. Routly, W. Shen and P. J. Gulick, unpublished data). The 5'-UTR intron of *Esi47* showed no effects on the GUS activities in both control and ABA-treated maize calli. However, disruption of the *Esi47* uORF increased the basal level of the GUS activity also by 2.6 fold (E. Routly, W. Shen and P. J. Gulick, unpublished data). Moreover, in the absence of the uORF, the *Esi47* gene promoter lost its ABA inducibility.

These data confirmed that the *Esi47* gene is responsive to ABA. More interestingly, this experiment showed that corrupting the ATG codon of the *Esi47* uORF elevated the basal gene expression to a level similar to the one induced by ABA. Modification of the uORF ATG codon also caused loss of ABA inducibility of the gene. Therefore, it is likely that ABA induces the expression of the gene through the relief of

gene repression mediated by the sequence related to the uORF. But this experiment did not distinguish whether the uORF sequence mediated gene repression was at the transcriptional or at the translational level. This puzzle might be solved by comparing the transcript levels of the *GUS* reporter gene to the measured GUS activities. It is possible that the RNA sequence surrounding and including the AUG start codon for the uORF represents a repressor element, for example, forming a secondary structure that prevents the moving of the ribosomal subunit. A more plausible explanation is that translation of the uORF mediates the repression of the gene and the modification of the AUG codon of the uORF alleviated such repression. Upstream ORFs occur in 7% to 10% of plant genes and have been implicated in reducing the translation efficiency of the downstream major ORF (Fütterer and Hohn, 1996; Gallie, 1996). The scanning model for translation of plant mRNAs says that the ribosome subunit binds to the 5'-end of the mRNA, moves along downstream and initiates translation once it meets the first AUG codon. Thus reinitiation at the AUG codon for the downstream major ORF is partially or completely inhibited and consequently the translation of the major ORF is repressed. Translation of the uORF seems necessary for efficient inhibition of the downstream major ORF (Wang and Wessler, 1998). In some genes, such as the maize *Lc* gene, the nucleotide sequence of the uORF is important, probably due to the rare codons present in the uORF that prevent rapid movement of the ribosome (Damiani and Wessler, 1993). Sometimes the intercistronic nucleotide sequence has an effect on reinitiation (Wang and Wessler, 1998). Reinitiation in the yeast *GCN4* gene, which contains four uORFs, is dependent on the degree of phosphorylation of the translation factor eIF2 α that is promoted by amino acid deprivation in culture medium (reviewed by Hinnebusch, 1996). Such translational

control by environmental conditions may suggest a mechanism for the probable uORF-mediated repression of the *Esi47* gene since it is also regulated by environmental stresses. Repression of the *Esi47* gene would be necessary if the presence of a certain amount of the gene product in the cell is deleterious and this gene is needed only when the plant is under salt or water deficit conditions.

Inserting the *Esi47* uORF into the 5'-UTR of an unrelated gene in a gain-of-function assay may help to demonstrate that this uORF does mediate translational repression. The occurrence of a uORF in one the *Arabidopsis* homologs of the *Esi47* gene, *F8A24.12*, supports the significance of the uORF in the probable translational control of this type of genes. Since it is easy to obtain stable *Arabidopsis* transformants, analyzing the expression of the *F8A24.12* gene in transgenic *Arabidopsis* would reveal the role of such uORFs in the control of gene expression in different environmental conditions.

5. PHYLOGENY OF THE *ESI47* PROTEIN KINASE GENE

5.1. *Esi47* Belongs to the NAK Group of Plant Protein Serine/Threonine Kinases

There are 860 protein kinase genes in the *Arabidopsis* genome which is 125 Mbp in size and whose gene content has been predicted to be 25498 (The Arabidopsis Genome Initiative, 2000). There could be more such genes in larger genome species such as those in the Gramineae, including wheat and *L. elongatum*. The large number of protein kinase genes indicates that each of the genes may have a very specific cellular function. Within a large gene family, genes with more similar primary structures, including the amino acid

sequences of the gene products, may have similar cellular functions, or, at least be involved in similar cellular processes. Moreover, if we assume that the genes and their functions may be well conserved among different species, then differences in phenotypes of the species may be largely explained by the differences in the expression patterns of some of the genes among the different species. Based on these assumptions, the phylogeny of the *Esi47* gene was studied by comparing the Esi47 protein kinase to the plant protein kinases already identified or even functionally characterized in order to draw any functional implications for the *Esi47* gene.

The amino acid sequence of Esi47 was compared to the sequences in the protein databases. The most similar proteins to Esi47 are almost all from *Arabidopsis* due to the completed genome sequencing of this species and thus the availability of a very large number (860) of protein kinase sequences (The Arabidopsis Genome Initiative, 2000). Hardie (1999) constructed a phylogenetic tree of 89 *Arabidopsis* protein kinases available in August 1998 and divided those kinases into as many as 12 subfamilies according to their positions in the tree. That tree was constructed based on the comparisons of the amino acid sequences of the catalytic domains of the kinases and the grouping from the tree agreed well with the biochemical and cellular properties of the kinases. In this study, a phylogenetic tree was also built with the amino acid sequences of the catalytic domains of Esi47 and its similar kinases (Figure 3).

In this tree the protein kinases that are most similar to Esi47 all belong to the NAK subfamily of plant protein kinases. This group acquired its name from the *Arabidopsis* gene *NAK* (for novel *Arabidopsis* protein kinase) which is the first gene identified in this group (Moran and Walker, 1993). Like Esi47, the NAK group protein

kinases all have a central catalytic domain flanked by short amino and carboxyl non-catalytic domains. *Esi47* clusters with four *Arabidopsis* kinases that form a distinct clade different from the one to which NAK and APK1 belong. These four *Arabidopsis* protein kinases are F8A24.12, F12E4.50, T7F6.28 and T9I4.2, and their genes are on chromosomes 3, 5, 2 and 2, respectively. All these kinases are predicted from the genome sequence, but an expressed sequence tag (EST) clone containing full-length cDNA for *F8A24.12* is available (Figure 11). For *F12E4.50* there are several short EST cDNA clones; and for *T7F6.28*, one EST cDNA clone containing most of the coding region is available (Figure 11). Thus, the genes for F8A24.12, F12E4.50 and T7F6.28 are expressed in *Arabidopsis* plants. Predictions on intron splicing sites revealed that the four genes all have three introns in the coding regions at exactly the same positions as the introns determined for *Esi47* (Figure 11; comparisons not shown). In contrast, the protein kinase genes in the adjacent clade that includes *APK1* and *NAK* in the phylogenetic tree have four introns in the coding regions, all at different positions from those of *Esi47* (not shown). Therefore, it can be concluded that *Esi47* and its four similar *Arabidopsis* genes are descendants from a common ancestor. That ancestor might have already separated from the common ancestor of *APK1* and *NAK* before the separation of monocot and dicot plants in the course of evolution.

However, none of these four *Arabidopsis* genes, *F8A24.12*, *F12E4.50*, *T7F6.28* and *T9I4.2*, or the other protein kinase genes in the NAK group, has been investigated for roles in signaling, except for the *Arabidopsis* gene *ARSK1*, which was only shown to be upregulated in roots by salt stress and ABA treatment (Hwang and Goodman, 1995).

This study is the first to demonstrate any cellular functions for a member of this group of

plant protein kinase genes by showing that *Esi47* is involved in the ABA-promoted suppression of GA activation of genes for hydrolytic enzymes in barley aleurone.

The phylogenetic trees built in this study (Figure 3) and by Hardie (1999) show that the NAK group protein kinases are closely related to the receptor-like kinases (RLKs). Since all the RLKs cluster together in the similarity trees, it is likely that the RLK genes and the genes in the NAK group have evolved separately from their common ancestor. Did this ancestor and the kinases in the NAK group also function similarly to RLKs in the capacity to perceive signals? The NAK group protein kinases, including *Esi47*, do not have membrane-spanning domains. However, it is still possible that these kinases could bind to membrane proteins that are able to perceive extracellular signals and thus transduce them into the cytoplasm. This speculation needs to be proved experimentally by determining if membrane proteins physically interact with any protein kinases in the NAK group. The yeast two-hybrid system could be the method of choice for such a screening although it is not the optimal method when hydrophobic proteins are concerned.

It is interesting to note that the two tomato disease resistance protein kinases, *Pto* and *Pti1*, are also closely related to the RLKs and the NAK group protein kinases in the phylogenetic tree derived from comparisons of sequence similarities (Figure 3). The genes for the two kinases confer plant resistance to bacterial speck diseases caused by *Pseudomonas syringae* pv. *tomato* (Martin et al., 1993; Zhou et al., 1995). Both kinases have similar linear structures to those of the NAK group kinases in that their catalytic domains are flanked by short amino and carboxyl terminal non-catalytic domains. *Pti1* is so close to the NAK group protein kinases as shown in the phylogenetic tree that it might

be one of the members of this subfamily (Figure 3), or the Pti1-like kinases and the NAK subfamily of protein kinases could form a larger group. However, it is not known what cellular processes Pti1 is involved in conferring disease resistance except that it was shown to be phosphorylated by Pto (Zhou et al., 1995). On the other hand, Pto is closer to the Leu-rich repeat (LRR) RLKs than other types of LRKs (Figure 3). It is possible that during recent evolution the precursor of Pto lost its receptor domain and acquired the property, as observed experimentally, of intracellular binding to the bacterial avirulent signal protein AvrPto (Scofield et al., 1996; Tang et al., 1996). The structural similarity between these disease resistance genes and the NAK group protein kinase genes suggest that the latter might also have roles in signal perception or transduction.

5.2. Structure and Regulation of the *Esi47*-Homologs in *Arabidopsis thaliana*

The necessity of identifying the *Arabidopsis* homologs of the *L. elongatum Esi47* gene is due to the limitation of the available means for manipulating gene expression in grass species, or more broadly, in monocot species. In addition, there is no convenient way to obtain targeted mutants in any monocot species, nor is there a large pool of mutants for screening. In contrast, *Arabidopsis* as a model plant species for genetic studies is easy to transform through *Agrobacterium*-mediated DNA delivery. Moreover, the nucleotide sequence of the whole genome of *Arabidopsis* is completely determined (The Arabidopsis Genome Initiative, 2000). There is also a large collection of ESTs and T-DNA insertional mutants from *Arabidopsis*. Therefore, functional characterization of the *Esi47*-homologs in *Arabidopsis* would help to understand the role of the *Esi47*-like genes in plant stress response and tolerance.

From the phylogenetic tree it is clear that the *Esi47*-homologs in *Arabidopsis* should be the three genes, *F8A24.12*, *F12E4.50* and *T7F6.28*, since all of these genes are in a common minimal clade (Figure 3). The amino acid sequence identities between the gene products of *Esi47* and each of the three *Arabidopsis* kinases are similar: they are 59%, 59% and 54% for *F8A24.12*, *F12E4.50* and *T7F6.28*, respectively. In addition, *F8A24.12*, like *Esi47*, has a uORF and an intron in the 5'-UTR (Figures 11 and 12). Such similarity in gene structure implies that the expression of *Esi47* and *F8A24.12* may be regulated in similar fashions. However, the other two genes may have similar structures, which could be determined once the corresponding cDNA sequences for their 5'-UTRs are available.

Analysis of the regulation patterns of these *Arabidopsis Esi47*-homologs would reveal clues to the evolution of the plant *Esi47*-like protein kinase genes and the conservation of gene functions among different species. On the other hand, different species may have similar genes but their unique characteristics may be due to the unique regulation pattern of gene expression. While the *L. elongatum Esi47* gene is inducible by both salt stress and ABA in roots, its three *Arabidopsis* homologous genes have evolved specific salt and ABA regulation patterns. *F8A24.12* can be induced by salt but not by ABA, whereas *T7F6.28* is ABA-inducible but not salt-inducible (Figure 13). Only *F12E4.50* is upregulated by both salt and ABA but the increase in *F12E4.50* transcripts is much slower than those of *F8A24.12* and *T7F6.28* (Figure 13). In contrast, the expression of *F8A24.12* in leaves is induced by NaCl, but not by ABA; neither of the other homologs is upregulated in leaves in response to either stress. It is likely that the ancestral *Esi47*-like gene that existed before the separation of monocot and dicot plants

was regulated by both salt stress and ABA in roots. The regulation patterns of the three *Arabidopsis Esi47*-homologs indicate the separation of the salt and ABA signaling pathways in dicot plants. Such separation was facilitated by the gene duplication that gave rise to *F8A24.12* and *F12E4.50*. It is not clear how the *T7F6.28* gene evolved. It is also interesting that there are two transcripts with different sizes detected for *F12E4.50* in *Arabidopsis* roots but not in leaves (Figure 13). Therefore, a mechanism of alternative transcription or RNA processing might exist for *F12E4.50* as an additional level of gene regulation. Moreover, the biphasic pattern of salt induction of *F8A24.12* in leaf tissue suggests that it is regulated by multiple signaling pathways.

The regulation patterns of the three *Arabidopsis Esi47*-homologs by salt stress and ABA suggest that the gene regulation mechanisms of the *Esi47*-like genes are only partially conserved during evolution. Therefore, all the three *Arabidopsis Esi47*-homologs should be included in future functional investigation of such genes. Further investigation of the regulation of the *Arabidopsis Esi47*-homologs and the comparison to that of the *Esi47* gene and its possible emerging paralogs in grass species would result in much information not only about the mechanisms of gene response to environmental stresses and plant hormones but also about the evolution of gene regulation. The partial sequence of an EST clone from bread wheat which shows 94% nucleotide sequence identity to *Esi47*, and is an apparent homolog of *Esi47*, has recently been deposited in GenBank's EST database (accession number BE518403). The accumulation of further DNA sequences in the ongoing genomic programs in the grass species is likely to significantly expand our current view of this protein kinase gene family in monocot species.

A number of experiments can be done with the *Arabidopsis Esi47*-homologs to alter their expression in plants for studying the cellular functions of these genes. Their coding regions could be placed under the control of the CaMV 35S promoter and such DNA constructs could be used to transform *Arabidopsis*. This would be a better strategy than overexpressing the exogenous *L. elongatum Esi47* gene in *Arabidopsis* since they are *Arabidopsis* genes and we might expect certain phenotypes from transgenic plants. Also, because in such transformants the transgenes would be identical to the endogenous copies, gene co-suppression might occur when these genes are overexpressed and such transgenic lines can be studied as null mutants of the *Arabidopsis Esi47*-homologs. Alternatively, the genes can be expressed in antisense in transgenic *Arabidopsis* plants. The T-DNA insertional mutants of these genes might also exist in the available pools. Proteins in *Arabidopsis* that might physically interact with the gene products of the *Arabidopsis Esi47*-homologs can be screened for with the yeast two-hybrid system and any resulting proteins can be confirmed in *L. elongatum* with its *Esi47* protein if the similar genes have been isolated from the wheatgrass. Of course, these *Arabidopsis Esi47*-homologs can be tested in barley aleurone for their potential involvement in the GA signaling so that the function of *Esi47* could be confirmed. Any potential data may help not only to understand the stress and hormone responses in plants, but also to decipher the cellular functions of the NAK group plant protein kinases which are so far still largely uncharacterized.

6. CONCLUDING REMARKS AND PERSPECTIVES

In this study, I characterized the structure of the *L. elongatum* salt stress and ABA inducible gene *Esi47*, which encodes a protein Ser/Thr kinase. Functional analysis of the gene revealed that *Esi47* is involved in the ABA promoted suppression of the GA response in barley aleurone. A similar function has been suggested for *Esi47* in vegetative tissues to suppress GA action in order to transiently arrest cell growth in response to stress. Structural characterization and gene regulation analysis of the *Arabidopsis Esi47*-homologs pointed to a way for further investigation of this type of plant protein kinase genes in a model plant species.

It is still unknown what the *Esi47* protein kinase does in plant cells, namely, what its protein substrates are. Identification of the substrates for *Esi47* or the gene products of the *Esi47*-homologs in *Arabidopsis* would add components to the signaling pathway in which these genes are involved. A protein physically interacting with *Esi47* may also be the candidate for a regulator of *Esi47* and could thus be an immediate upstream component in the signaling pathway involving *Esi47*. Of course, it would still be interesting to know what genes *Esi47* regulates. The availability of such data would make it possible to define a signaling pathway that may more clearly reveal the role of *Esi47* in stress and hormone response and in stress tolerance in plants. So far only the wheat stress and ABA regulated protein kinase gene *PKABAI* shows similar behaviour to *Esi47*.

The signal transduction pathways for the salt stress response are complex and may be composed of parallel pathways with points of cross-talk and feedback loops. Identification of a single component may not reveal any groundbreaking insight into the

puzzle but is definitely a necessary step towards further revelations. This work is in the early phase of the characterization of a single gene, *Esi47*, directed towards understanding plant stress signaling. Traditional approaches in molecular biology have been used throughout this study and may still be the important tools in follow-up studies. This study has also benefited from the accumulating data from the genomics studies, especially in *Arabidopsis*. In the future it will certainly rely more and more on both the data and tools from the plant genomics, for example, the pools of EST clones and T-DNA insertional mutants from *Arabidopsis* and other species.

The availability of mutants is extremely important for studying the function of a gene as has been proved by a century of progress in modern genetics. To decipher the cellular function of the *Esi47*-like genes, therefore, immediate efforts should be focused on making or searching for *Arabidopsis* null mutants of the three *Esi47*-homologous genes. This can be achieved from the T-DNA insertion lines, by expressing the antisense mRNA in transgenic plants, or, by screening for transgenic lines in which gene silencing happens. If we can obtain mutants more sensitive to salt stress than wild-type plants, it will be certain that we could know more about the salt stress tolerance in plant.

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