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Characterization of ESI47, a salt stress regulated protein kinase from *Lophopyrum elongatum*

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ABSTRACT

Characterization of ESI47, a salt stress regulated protein kinase from *Lyphopyrum elongatum*

Demetra Elias

The ESI47 serine/threonine protein kinase from *Lophopyrum elongatum* was investigated to elucidate its potential role in salt tolerance and salt stress response. The yeast two-hybrid system was used to screen a cDNA *Arabidopsis* library and identify proteins that interact with the ESI47 kinase. Six partial DNA sequences encoding a 33 kDa oxygen-evolving enhancer precursor protein (OEE1) from the photosystem II oxygen-evolving complex were identified which showed an interaction with the ESI47 kinase. Sequence analysis showed that the partial length *OEE1* clones were missing the first 81 or 82 amino acids at the amino-terminus of the protein. This region serves as a transit peptide that is required for import of precursor proteins into the chloroplast and is later cleaved. The full length *OEE1* clones from *Triticum aestivum* and *Arabidopsis* were subcloned into the yeast two-hybrid library vector to determine whether the ESI47 kinase could interact with the OEE1 from either of these species. An interaction between the ESI47 kinase and the full length OEE1 precursor was not observed, which suggests that the kinase interacts with the mature OEE1 protein in the chloroplast. Northern analysis was used to compare the expression of *ESI47* homologues, in different species of wheat grasses, which have differing degrees of salt tolerance. *ESI47* expression was not observed with northerns prepared with total RNA nor with poly-$(A)^+$ RNA samples, and a correlation between the *ESI47* expression and salt tolerance was therefore not established in this work.

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ABA: Abscisic acid

ABRE: Abscisic acid responsive element

cfu: colony forming unit

dCTP: 2'-Deoxycytidine 5'-triphosphate

DRE: Dehydration-responsive element

EDTA: Ethylene diaminetetraacetic acid

EGTA: Ethylene glyco-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid

ESI: Early salt-stress induced

MOPS: 3-(N-morpholino) propanesulfonic acid

OEE: Oxygen-evolving enhancer protein

ORF: Open reading frame

PCR: Polymerase chain reaction

pfu: plaque forming unit

SDS: Sodium dodecyl sulphate

Tris: Tris (hydroxymethyl) aminomethane

UV: Ultraviolet light

CHAPTER 1. INTRODUCTION

1.1. Plants in saline environments

Agriculture in large areas of the world depends on extensive irrigation for crop production. Approximately 10 million hectares of irrigated lands are thought to be abandoned each year due to increased concentrations of salts in the soil (Barkla *et al.*, 1999). Improvements are needed in water management and in plant tolerance to stress, in order to maintain crop productivity while coping with the increasing salinity in soils and water (Epstein *et al.*, 1980). Potassium, a major component of fertilizers, plays a key role in controlling crop productivity and is required in large quantities for optimum plant nutrition (Liu and Zhu, 1997). The elevated concentrations of sodium ions that arise in saline soils become toxic to plants and disrupt potassium nutrition. The exposure of plants to salt stress results in changes in water potential, the synthesis of compatible solutes, the exclusion of sodium ions from the cytosol and the intracellular compartmentalization of sodium ions. The relative importance of each of these responses, varies depending on the particular plant species (Sanders, 2000). Salt stress can cause cell wall alterations and a decrease in photosynthesis (Zhu *et al.*, 1998). Many genes with altered expression involved in the physiological and metabolic changes associated with salt stress have been identified, however, only a few have been demonstrated to contribute to salt tolerance. The study of genes involved in the signal transduction pathways of the stress response would help to elucidate the mechanisms responsible for salt tolerance.

Plants can respond to environmental changes by transducing extracellular signals to produce a physiological response. There are growing efforts aimed at understanding

the signals that are propagated as a result of drought, cold, and salt stress, as well as by the stress related phytohormone, abscisic acid (ABA). Cold, drought and salt stress, all have the common component of osmotic stress. Salt stress contributes to osmotic stress by reducing water potential and cold by reducing the water supply from the roots to green tissues (Hong *et al.*, 1997). This introduction will focus on the effects of salt stress, although other stress stimuli will be mentioned, given the intertwined signaling pathways that often lead to the same gene response in plants.

1.2. Ion transport across the vacuolar and plasma membranes

The transport of inorganic ions is important in understanding the membrane-associated mechanisms involved in the stress response pathways that are present in plants. Salt stress causes a reduction in water potential and increases internal levels of sodium ions that can become toxic to plants (Epstein *et al.*, 1980). High concentrations of sodium ions affect potassium uptake across the plasma membrane because potassium and sodium cations compete for the same high affinity potassium transporters of the plasma membrane (Rubio *et al.*, 1999).

Different plant species, when exposed to salt stress, will employ different mechanisms to avoid high concentrations of sodium ions in their cytoplasm. Some plants avoid sodium ion toxicity by limiting sodium accumulation in the cytoplasm and by sequestering sodium ions into their vacuoles. In these plants the mechanisms involve the activation of H^+-ATPases, H^+-pyrophosphatases and Na^+/H^+ antiporters located in the vacuolar and plasma membranes. In yeast, the plasma membrane Na^+-ATPase and Na^+/H^+ antiporters actively exclude sodium ions from the cell (Bañuelos *et al.*, 1998;

Gaxiola *et al.*, 1999). In plants, however, plasma membrane Na^+-ATPases and Na^+/H^+ antiporters involved in sodium ion exclusion mechanisms have yet to be characterized. Other plant species accumulate sodium and avoid sodium ion toxicity by sequestering sodium ions in the vacuole. Proton translocating, H^+-ATPases, H^+-pyrophosphatases, and Na^+/H^+ antiporters present in the vacuolar membrane play important roles in reducing sodium ion levels in the cytoplasm by driving sodium ion uptake in the vacuole (Schroeder and Hedrich, 1989). Salt-tolerant species are known for their ability to accumulate sodium ion in their vacuoles, whereas plants unable to tolerate salt have been recognized as sodium ion excluders (Barkla *et al.*, 1999).

Potassium transport is of central significance in Na^+ stress tolerance because potassium ions accumulate in the cytoplasm to counter the low water potential in the vacuole and soil solution created by high sodium ion levels. In addition, there is direct competition for common transporters for the two ions in the plasma membrane. Potassium is a major mineral nutrient that is essential for plant growth and several plasma membrane potassium symporters and antiporters that mediate high and low affinity potassium transport have been identified (Chrispeels *et al.*, 1999). A Na^+/K^+ symporter, *HKT1* expressed in wheat roots and leaves has been isolated that shows similarity to yeast plasma membrane potassium transporters, *TRK1* and *TRK2* (Rubio *et al.*, 1999). HKT1 is mainly involved in potassium uptake across the plasma membrane but also plays a role in low-affinity Na^+ transport. The study of *HKT1* in yeast mutants which had genes encoding sodium and potassium transporters deleted revealed that in the presence of high Na^+ concentrations, potassium uptake by HKT1 is blocked and sodium ion uptake is reduced. The HKT1 potassium transporter could, therefore, be an important component

of mechanisms involved in salt tolerance employed by plants to avoid sodium ion toxicity by reducing sodium uptake in the roots and leaves. Potassium transport across the plasma membrane could be sustained during periods of salt stress by other potassium transporters such as K^+/H^+ antiporters (Rubio *et al.*, 1999).

Sodium ions are toxic for yeast, as they are for plants. In the yeast *Saccharomyces cerevisiae*, a plasma membrane Na^+-ATPase encoded by the *ENA1* gene actively extrudes sodium ions from the cell. *ENA1* is normally expressed at low levels in the cell and its expression rapidly increases in response to salt and osmotic stress (Bañuelos *et al.*, 1998). Disruption of the *ENA1* gene decreased Na^+ efflux from yeast cells and reduced salt tolerance. A yeast plasma membrane Na^+/H^+ antiporter, *NHA1* and its homologue in fission yeast, *SOD2*, are activated by a proton gradient (Gaxiola *et al.*, 1999). In *Arabidopsis* the SOS1 gene which shows higher sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria than to vacuolar transporters from plants, is thought to play a role in sodium ion export from the cell (Shi *et al.*, 2000). Na^+/H^+ antiporters in plants are thought to be activated by the proton gradient generated by H^+-ATPases in the plasma membrane (Blumwald, 2000).

Sodium ion transport into the vacuole is an important ion transport element for salt tolerance. Sodium ion sequestration into the vacuole removes the ions from the cytoplasm and benefits the plant by lowering the internal water potential relative to soil water potential. Low water potential facilitates water uptake that is essential for plant growth and survival, since most plants that are actively photosynthesizing have continual water loss through transpiration. In plants, ion transporters vary in structure, function and tissue distribution. The vacuole, for example, has three major ion transporters, Na^+/H^+

antiporters, H^+-ATPases and H^+-pyrophosphatases (Taiz, 1992). The H^+-ATPases and H^+-pyrophosphatases actively pump protons into the vacuole creating a pH gradient that can be used by Na^+/H^+ antiporters to take up sodium ions in exchange of protons. Many plant species when exposed to salt stress can accumulate sodium ions in the vacuole to avoid toxicity. The halophyte, *Mesembryanthemum crystallinum* which has been studied extensively to identify genes responsible for its salt-adaptive traits, can efficiently store sodium ions in its vacuole in response to salt stress (Barkla *et al.*, 1999). Furthermore, an induction of the ion transport activity of vacuolar H^+-ATPases and Na^+/H^+ antiporters has been observed in the halophyte in response to salt stress. The induction of H^+-ATPase and Na^+/H^+ antiporter activity was greater in 6 weeks old plants than in 3 weeks old plants. Recently, overexpression of the *Arabidopsis* vacuolar H^+-pyrophosphatase, *AVP1* in *ENA1* yeast mutants that lack the plasma membrane sodium efflux transporters, revealed that *AVP1* restores salt tolerance (Gaxiola *et al.*, 1999). However, a decrease in sodium ion content was observed in *ENA1* yeast mutants overexpressing *AVP1* in comparison to *ENA1* mutants that are deficient in Na^+-ATPase activity. Critics point out that if the AVP1 transporter was involved in the sequestration of sodium ions into the vacuole, an increase in sodium ion content in yeast *ENA1* mutants should have been observed (Blumwald *et al.*, 2000). Thus, there remain some questions on the function of *AVP1*. The *Arabidopsis* vacuolar Na^+/H^+ antiporter, *AtNHX1*, a homologue of the yeast *NHX1* gene, was identified (Gaxiola *et al.*, 1999). Overexpression of *AtNHX1* in yeast *NHX1* mutants suppressed some of the salt-sensitive phenotypes, indicating that sodium ion detoxification mechanisms in plants and yeast may be similar. Transgenic *Arabidopsis* plants overexpressing *AtNHX1*, were able to tolerate 200 mM NaCl

concentrations, a treatment which greatly inhibited the growth and development of wild type plants (Apse *et al.*, 1999). These findings suggest similar improvements in the salt tolerance of crop plants can be achieved with the help of genetic engineering. Further characterization of ion transporters that are involved in the complex mechanisms employed by plants to avoid sodium ion toxicity could reveal important information about the mechanisms underlying salt tolerance.

1.3. Osmolyte accumulation

Salt stress also causes plants to accumulate low molecular weight organic compounds such as glycine betaine, sugar alcohols and proline which balance the changes in water potential induced by a rise in salinity (Sugihara *et al.*, 2000). These compatible osmolytes, which accumulate in the cytoplasm when plants are exposed to osmotic stress, are non-toxic and do not interfere with normal biochemical reactions, are often referred to as osmoprotectants (Yoshiba *et al.*, 1997).

In *Arabidopsis* proline accumulates in response to osmotic stress initiated by drought, salt and cold stress. Under these stress conditions, proline has the role of a mediator of osmotic adjustment. It is thought to stabilize subcellular structures, scavenge free radicals and act as a stress-related signal (Nanjo *et al.*, 1999). Osmotic stress induces proline synthesis and inactivates its degradation processes. In transgenic tobacco plants, it was found that the overproduction of proline, resulted in increased tolerance to osmotic stress (Yoshiba *et al.*, 1997).

Studies in *Saccharomyces cerevisiae* have shown that sugar alcohols such as glycerol, also accumulate during osmotic stress and compensate for differences in water

potential. High osmolarity triggers a MAP-kinase "high osmolarity glycerol" (HOG) signaling cascade that involves phosphorylation of the HOG1 kinase, which enhances the accumulation of glycerol (Shen *et al.*, 1999). Glycerol accumulation is essential for salt tolerance, since mutants unable to accumulate glycerol are salt-sensitive. In the halophyte, *M. crystallinum*, proline and the sugar alcohol, pinitol, were found to accumulate in chloroplasts in response to salt stress (Barkla *et al.*, 1999). The enzyme, L-*myo*-inositol methyltransferase (IMT1) involved in pinitol biosynthesis, is induced by salt stress but not by drought, which suggests that different mechanisms exist in plants that respond specifically to different environmental stress factors.

Glycine betaine is another compatible solute that functions as an osmoprotectant, and is found to accumulate in plants exposed to low-temperature, drought and salt stress (Holmström *et al.*, 2000). In plants, glycine betaine biosynthesis consists of two steps; the oxidation of choline by a ferredoxin-dependent choline monooxygenase (CMO) into betaine aldehyde, followed by its conversion into betaine by a NAD^+-dependent betaine aldehyde dehydrogenase (BADH). Holmström *et al.* (2000) introduced genes from the *E.coli* betaine biosynthesis pathway into tobacco, which does not normally synthesize betaine, in order to produce transgenic tobacco plants that accumulate glycine betaine. The transgenic tobacco plants showed improved tolerance to low-temperature and salt stress. Furthermore, the transgenic plants showed enhanced recovery of photoinhibition caused by high light intensity and salt stress, which suggests that osmolytes such as glycine betaine posses diverse physiological functions.

1.4. Effects of salt stress on photosynthesis

The effects of salt stress on plant growth are often associated with decreases in photosynthetic activities (Greenway and Munns, 1980), although the mechanisms responsible for decreased photosynthetic activity remain to be elucidated. In plants, photosynthetic reactions occur in the chloroplast. The study of chloroplast structure and function is, therefore, important in understanding the inhibitory effects of salt stress on photosynthesis. In the cyanobacteria *Synechococcus*, salt stress has been shown to inactivate photosystems I and II, which are involved in the light reactions of photosynthesis (Allakhverdiev *et al.*, 2000). The studies in *Synechococcus* also showed that the major site of photoinhibition by salt stress is the oxygen-evolving activity of photosystem II.

Photosystem II is a multisubunit protein complex composed of more then 25 different proteins. It uses light energy to split water into oxygen consequently releasing protons and electrons (Nield *et al.*, 2000). At the core of the photosystem II complex there is a reaction center composed of D1 and D2 proteins, involved in light absorption and electron transfer. Photoinhibition caused by elevated temperature and high light intensity has been shown to damage the D1 and D2 proteins in the reaction center (Rokka *et al.*, 2000). Phosphorylation /dephosphorylation cycles regulate DI and D2 protein turnover and are thought to play important roles in the repair process of stress-induced damage. Analysis of the three-dimensional structure of the *Synechococcus elongatus* photosystem II complex revealed that the manganese cluster involved in the water splitting reaction appears to be associated with the reaction center (Nield *et al.*, 2000). The manganese cluster is maintained by three extrinsic proteins that are induced by NaCl

treatment (Sugihara *et al.*, 2000). These extrinsic proteins are oxygen-evolving enhancer proteins (OEEs) which are nuclear-encoded chloroplast proteins that are peripherally bound to photosystem II (PSII) on the lumenal side of the thylakoid membrane (Görlach *et al.*, 1993). Three OEEs have been identified in plants, OEE1 (33 kDa), OEE2 (23 kDa) and OEE3 (16 kDa), which are initially synthesized as precursor proteins. The precursor proteins contain a transit signal peptide consisting of two domains, one responsible for the localization of OEEs in the chloroplasts, and the other to the thylakoid membrane (Waegemann and Soll, 1996). Phosphorylation and proteolytic cleavage of the signal peptide of the precursor proteins is required for import into the chloroplast. Alkaline- and salt-washing of thylakoid membranes liberates the OEE1, OEE2, and OEE3 proteins from the oxygen-evolving complex and results in a significant loss in the oxygen-evolving activity of photosystem II (Ono and Inoue, 1983; Ädelroth *et al.*, 1995). The OEE1 protein is thought to play an important role in stabilizing the photosystem II complex, and it has been suggested that the OEE1 protein may be involved in the regulation of D1 protein turnover, to help maintain the structural integrity of photosystem II during photoinhibition (Yamamoto *et al.*, 1998). Studies aimed at understanding the regulation of components of the oxygen-evolving complex could reveal the mechanisms underlying photoinhibition induced by different stress factors.

1.5. Salt stress and gene regulation

Plants are commonly exposed to drought, salinity and low-temperature stresses, which are associated with osmotic stress. These stress factors often lead to similar physiological responses in plants, which suggests the existence of similar signal

transduction mechanisms (Ishitani *et al.*, 1997). The differential expression of genes in response to osmotic stress has been studied extensively, with the intent of identifying the genes responsible for stress tolerance (Busk and Pagès, 1998; Xiong *et al.*, 1999). The plant hormone ABA, whose levels increase when plants are exposed to drought and low-temperature stress, enhances the ability of plants to tolerate these stresses (Liu *et al.*, 1998). Similarly, several genes have been identified that exhibit differential expression in response to more than one of the osmotic stresses, which suggests that there is cross-talk between signaling pathways initiated from the various stress stimuli (Moons *et al.*, 1997, Piao *et al.*, 1999; Mizoguchi *et al.*, 1996). The study of the regulation of genes whose expression is altered by the different osmotic stress factors, could aid in the understanding of mechanisms underlying salt tolerance. The characterization of transcription factors that control the transcriptional activation of genes induced by osmotic stress, is crucial in elucidating the mechanisms involved in the salt stress response.

The plant hormone ABA, can mimic the effects of salt stress by producing similar physiological responses in plants. Endogeneous levels of ABA increase when plant tissues become dehydrated from water-limiting stresses, such as salt stress, desiccation, and cold (Moons *et al.*, 1997). The increase in ABA synthesis is required for stomatal regulation to help prevent extensive water loss (Busk and Pagès, 1998). Genes such as group 3 *LEA*, *salT*, and *AtGSK1* show transcriptional regulation in response to ABA and salt stress (Moons *et al.*, 1997; Piao *et al.*, 1999), which suggests there is cross-talk between the ABA- and salt stress-initiated signaling pathways. The expression of many genes, however, is altered by only one of these stress factors, which indicates that the

signaling pathways initiated by salt and ABA are also independent. Other gene transcripts such as those for mitogen-activated protein kinases (*ATMEKK1*, *ATMPK3*) increase in response to both cold and salt stress (Mizoguchi *et al.*, 1996), whereas the receptor-like kinase gene (*RPK1*) is rapidly induced by ABA, drought, salt and cold stress. Thus, there appears to be multiple overlapping and parallel pathways, initiated from different stimuli that lead to a similar response (Hong *et al.*, 1997).

1.6. Transcription factors and the stress response

The investigation of transcriptional regulators and proteins that interact with them in response to different stress factors, could lead to a better understanding of signal transduction pathways, which regulate the stress response. The analysis of promoter sequences of ABA-, and drought-inducible genes, has led to the identification of DNA sequence elements required for the transcriptional activation of the stress-inducible genes (Nakashima *et al.*, 2000; Busk and Pagès, 1998).

The characterization of promoters of the ABA regulated *Em* gene from wheat and the *rab16A* gene from rice revealed an ABA-responsive element (ABRE) that is important for transcription (Busk and Pagès, 1998). The dissection of barley gene promoters, *HVA22* and *HVA1*, led to the identification of cis-elements, termed "coupling elements" CE1 and CE3, which together with an ABRE are required for activation of transcription by ABA. In *Arabidopsis*, ABA-induced Myb and drought-induced Myc transcription factors that interact with an ABRE sequence in the promoter region of the *rd22* gene have been identified (Iwasaki *et al.*, 1995). Transcription factors DREB1 and DREB2 are involved in drought and low-temperature signal transduction pathways.

These transcription factors interact with the dehydration-responsive element (DRE) that regulates the expression of dehydration-response genes (Liu *et al.*, 1998).

1.7. Protein kinases and signal transduction pathways

Protein kinases involved in the salt stress pathway in plants have not yet been extensively characterized, however, they are believed to play important roles in signal transduction mechanisms mediated by salt stress. In disease resistance signaling pathways, protein kinases have been identified as components of signaling cascades that interact with transcription factors to activate transcription of disease-resistance genes (Wilson *et al.*, 1997). Several plant receptor-like kinases and MAP kinases that are induced by ABA, salt, drought, low-temperature stress and which may possess important functions in signal-transduction pathways have been discovered in plants (Hong *et al.*, 1997; Hwang and Goodman, 1995; Mizoguchi *et al.*, 1996).

Eukaryotic protein kinases are commonly classified as serine/threonine or tyrosine kinases, even though some protein kinases have been identified that display both tyrosine and serine/threonine specificity. The plant receptor-like kinases (RLKs) are structurally similar to animal growth factor receptor protein kinases and almost all plant RLKs appear to be serine/threonine specific (Walker, 1994). Even though plant RLK's have diverse structures and substrate specificities, their catalytic domains contain eleven subdomains of conserved amino acid sequences found in most eukaryotic protein kinases. A receptor-like kinase, *RPK1* that is rapidly induced by salt, ABA, drought and cold treatments was isolated in *Arabidopsis*, which suggests that the ABA-dependent osmotic response in plants could involve RPK1 in transmembrane signaling mechanisms (Hong *et*

al., 1997). In *Arabidopsis*, a root-specific kinase, *ARSK1* was also identified which shows structural similarities to ser/thr protein kinases. *ARSK1* gene expression is induced with both ABA and NaCl treatments, which indicates that the kinase might play a role in the cellular response to water deficit (Hwang and Goodman, 1995).

Mitogen-activated protein kinases (MAPKs) are also known to play important roles in signal transduction pathways in higher plants (Jonak *et al.*, 1996). MAP kinases which are serine/threonine kinases, are often involved in phosphorylation cascades. Three protein kinases, ATMEKK1, ATMPK3 and ATPK19 that had elevated levels of mRNA when plants were treated with low temperature and salt stress and are structurally related to MAP kinases (Mizoguchi *et al.*, 1993; Mizoguchi *et al.*, 1996). Other MAPK's that are activated by ABA treatment have also been reported (Knetsch *et al.*, 1996).

1.8. ESI47 and *Lophopyrum elongatum*

The present study, is a further attempt to characterize the function of the ESI47 protein kinase isolated from the highly salt-tolerant species *Lophopyrum elongatum*. The ESI47 protein kinase was isolated on the basis of salt-inducibility (Gulick and Dvrák, 1990), and is thought to play a role in hormone and salt stress signaling (Shen *et al.*, 2001). Comparison of the level of salt stress induction of the gene in different genotypes showed a correlation between gene induction levels and salt tolerance (Galvez *et al.*, 1993).

Lophopyrum elongatum (Host) Love (2n = 2x = 14) [syn. *Elytrigia elongata* (Host) Nevski, *Agropyron elongatum* Host] is a diploid species that has a close phylogenic relationship to bread wheat, *Triticum aestivum* (McGuire and Dvrák, 1981).

This wild wheat grass is a highly salt-tolerant species found in salt marshes in the Mediterranean region. A cross between *Lophopyrum elongatum* and salt-sensitive *Triticum aestivum* produced an octoploid amphiploid, which was salt-tolerant. The amphiploid demonstrated high levels of ESI gene expression when subjected to salt stress and could tolerate 250 mM NaCl stress (Galvez *et al.*, 1993). The genetic loci responsible for salt tolerance in *L. elongatum* are at least partially dominant to the salt sensitive loci found in the wheat species.

Eleven genes, known as early salt stress induced (ESI) genes that show elevated levels of mRNA in the root tissues, when plants were stressed with 250 mM NaCl, were identified in *L. elongatum* (Gulick and Dv·rák, 1990). The identification of genes that are responsible for salt adaptive traits in diploid species related to wheat, such as *L. elongatum*, could elucidate mechanisms underlying salt tolerance.

One of the ESI genes, ESI47, encodes a serine/threonine protein kinase. The ESI47 protein kinase is involved in plant hormone signaling pathways; its expression inhibits gibberellin activation of the K-amylase gene promoter in barley aleurone (Shen *et al.*, 2001). The comparison of the amino acid sequence of ESI47 to the *Arabidopsis* genomic sequence database demonstrated a high similarity to serine/threonine kinases known as "novel *Arabidopsis* protein kinases" (NAKS). These kinases were also shown to be salt stress or ABA induced (Shen *et al.*, 2001) but their function has yet to be determined (Hardie, 1999; Moran and Walker, 1993). It is possible that the ESI47 protein kinase plays a role in the signal transduction pathways of the salt stress response. The study of ESI47 and proteins that interact with this kinase could contribute to the

characterization of signal transduction pathways regulating multigenic responses to salt stress.

In this study, the yeast two-hybrid system was used to screen an *Arabidopsis* cDNA library and identify proteins that interact with ESI47. The role of ESI47 in salt tolerance was also investigated in wheat grass species using northern analysis to determine if there is a correlation between the expression of ESI47 and salt-tolerance.

CHAPTER 2. MATERIALS AND METHODS

2.1. Plant materials

Eight genotypic accessions from five species of wheat grasses (Table 1) including *Elytrigia caespitosa*, *E. turcica*, three *E. intermedia*, and three *E. pontica* were grown hydroponically. The accessions were chosen for their varying degree of salt tolerance (McGuire and Dv·rák, 1981). Seeds were obtained from the USDA's National Small Grains Collection. The species names assigned by the USDA are used in this work (Table 1) though alternative species classifications were given by McGuire and Dv·rák (1981). Seeds were surface-sterilized with 5% sodium hypochloride, for 15 min. and rinsed 3 times with sterile water. The seeds were germinated on filter papers supported by near-vertical slant boards and pre-wetted with sterile water. Once seedling roots obtained a length of 5-10 cm, seedlings were transferred to 15 l culture tanks which contained modified Hoagland solution containing 3 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 5.5 mM $Ca(NO_3)$, 50 mg / l FeEDTA (10% iron), 25 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, and 2 μM H_2MoO_4; pH was adjusted to 6.5 with KOH (Gulick and Dv·rák, 1987). The plants were grown for 5 months in the 15 l tanks, then transferred to 150 l tanks containing Hoagland solution, for 2 months. The wheat grasses were then transferred to modified Hoagland solution containing 150 mM NaCl for treatments of 6, 12, or 24 hrs. Control plants were transferred to modified Hoagland solution without NaCl for 6 hrs. The roots of the wheat grasses were harvested, frozen in liquid nitrogen and stored at –80 °C.

2.2. RNA extraction from roots of wheat grasses and poly-$(A)^+$ isolation

Frozen root tissues were ground in a mortar to a fine powder with liquid nitrogen. To approximately 1 g of tissue, 5 ml of lysis buffer (0.2 M Sodium Borate, 30 mM EGTA, 2% SDS, 1% Sodium Sarkosyl) and 5 ml of (1:1, v/v) phenol/chloroform solution saturated with lysis buffer were added and mixed thoroughly. Samples were incubated at room temperature for 15 min., then centrifuged at 10 000 x g for 20 min. The aqueous phase was recovered and extracted once with 5 ml of (1:1, v/v) phenol/chloroform solution saturated with lysis buffer, and twice with 5 ml of chloroform. The aqueous phase was recovered and RNA was precipitated with an equal volume of 4 M LiCl at 4°C for 48 hrs. The samples were centrifuged and the pellet was washed by resuspending it in 1 ml of 2 M LiCl. The samples were re-centrifuged and the pellet was dissolved in 1 ml of 10 mM Tris-HCl (pH 7.5) and re-centrifuged. The supernatant was collected and the RNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol, and stored overnight at –20°C. Samples were centrifuged and the pellet was dissolved in 400 :l of RNase-free water. All samples were quantified by measuring OD_{260} with a spectrophotometer.

To isolate poly-$(A)^+$ RNA, samples containing approximately 0.5 mg of total RNA were heated to 70°C for 1 min. and then chilled on ice. The samples were removed from the ice and one volume of 2 X RNA binding buffer (1M NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA and 1% SDS) at 37°C was added to the RNA samples. The samples were applied to a column containing 70 mg of oligo (dT) cellulose pre-equilibrated with RNA binding buffer. The fraction that passed through the oligo (dT) cellulose column was applied a second and third time to the column to ensure that all of the poly-$(A)^+$ RNA

was bound. The oligo (dT) cellulose column was then washed twice with 30 ml of binding buffer. The RNA bound to the column was then eluted with elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.2% SDS) and collected in 500 :l aliquots. To identify which aliquots contained poly-$(A)^+$ RNA, 1 µl of each aliquot was spotted on a 1% agarose gel, containing 0.5 µg/ml ethidium bromide along with 1 µl aliquots of RNA standards and viewed under UV. The poly-$(A)^+$ RNA was precipitated by adding 0.1 volume of 4 M NaCl and 2 volumes of 95% ethanol, and stored overnight at -20°C. The poly-$(A)^+$ RNA was then collected by centrifugation and dissolved in RNase-free water. The poly-$(A)^+$ RNA containing fractions were pooled and quantified by measuring the OD_{260} with a spectrophotometer.

2.3. Northern hybridization

Northern blot analysis was carried out with 2 µg of poly-$(A)^+$ RNA or 20 µg total RNA according to Sambrook *et al.* (1989) with some modifications. The RNA samples were dried in a lyophilizer and resuspended in 5 µl of RNase-free water. An equal volume of 2 X loading buffer (0.04 M 3-(N-morpholino) propanesulfonic acid (MOPS) (pH 7.2), 0.02 M sodium acetate, 1 mM EDTA), 12.3 M formaldehyde, 50% (v/v) formamide, 0.004% bromophenol blue buffer, 0.5% glycerol, 0.5 mg/ml ethidium bromide) was added to the samples. The samples were then heated at 70°C for 10 min. and chilled on ice. The RNA samples were separated by electrophoresis on a 1.2 % agarose gel containing 0.04 M MOPS buffer and 2.3 M formaldehyde with a running buffer consisting of 0.04 M MOPS and 0.2 M formaldehyde. Electrophoresis was carried out at 80V until the dye front migrated a total distance of 8 cm. The agarose gel was then soaked in RNase-free

water for 10 min. and the RNA was transferred to a Hybond-N nylon filter (Amersham) by capillary blotting with 20 X SSC (3 M NaCl, 0.3 M sodium citrate) for 16 hrs. The RNA was UV cross-linked to the nylon membrane and membranes were stored at -20°C.

Northern blots were hybridized with either *ESI*47 or *ESI*3 gene-specific probes prepared from corresponding cDNA clones using a multiprime DNA labeling kit (Amersham) and [K-^{32}P]dCTP (3000 Ci/mmole, ICN), according to the supplier's protocol. The northern blots were prehybridized at 65°C for 1 hr in hybridization solution containing 5 X SSC (0.75 M NaCl and 75 mM sodium citrate), 0.02 M Tris (pH 7.6), 5 X Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polivinylpyrrolidone), 0.5% SDS, and 100 :g /ml sonicated denatured salmon sperm DNA. The radio-labeled probe was heat denatured at 95°C for 1 min., and added to the hybridization solution. The northern blots were hybridized at 65°C for 16 hrs and then were washed with 1 X SSC and 1% SDS at 22°C for 15 min. and twice with 1 X SSC and 0.1% SDS at 55°C for 15 min. and 30 min., respectively. Phosphoimage technology (BIO-RAD) was used to observe the level of RNA transcripts.

2.4. Yeast two-hybrid system screening

2.4.1. *ESI47* two-hybrid plasmid construct

The *ESI47* cDNA clone was amplified by polymerase chain reaction (PCR) with *Pfu* DNA polymerase using an *ESI*47-specific 5'-end primer (ESI47*Nco*I) and a 3'-end vector specific primer (ST-3, Table 3). The *ESI47*-specific 5' end primer was designed for amplification of the long open reading frame (ORF) of *ESI47*, which encodes the protein kinase. The primer begins at the ATG of the protein kinase ORF. The PCR product was

extracted with one volume of chloroform, centrifuged, and the aqueous phase was recovered. The aqueous phase was then extracted with one volume of (1:1, v/v) phenol/chloroform and one volume of chloroform. The PCR product recovered in the aqueous phase was precipitated by addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol and chilling on ice for 10 min. The DNA was precipitated by centrifugation, dried and re-dissolved in water.

The PCR product was digested with *ApaI*, treated with T4 DNA polymerase to generate blunt-ends, re-purified with phenol/chloroform, and digested with *Nco*I. The PCR product was electrophoresed on a 0.8% agarose gel and gel-purified using a QIAEX II Agarose Gel Extraction kit. The yeast two-hybrid cloning vector pAS2 (Figure 1) which contains the *GAL4* DNA-binding domain was digested with the restriction enzymes *Nco*I and *Sma*I, dephosphorylated with calf intestine alkaline phosphatase (CIP), and purified by (1:1, v/v) phenol/chloroform extraction and ethanol precipitation. The *ESI*47 coding region was ligated to the pAS2 two-hybrid system cloning vector with T4 DNA ligase, to produce a *GAL4* DNA-binding domain fusion construct. The pAS2-*ESI*47 plasmid construct was transformed into XL1Blue cells by electroporation and ampicilin resistant colonies were selected. The pAS2-*ESI*47 plasmid construct was then verified by restriction digestion and agarose gel electrophoresis. The pAS2-*ESI47* plasmid construct digested with *Nco*I and *Sal*I produced three expected fragments of approximately 8400, 900, and 200 bp. The construct was verified by restriction digest with *Sac*I, which also showed three fragments of approximately 6600, 3000, and 600 bp, which correspond to the expected restriction fragments.

The pAS2-*ESI47* plasmid construct was transformed into the yeast *S. cerevisiae* strains HF7C and Y190 according to the protocol of Gietz and Woods (1994). The pAS2 vector contains a tryptophan selection marker, which allowed the selection of transformants on growth media without tryptophan. An X-Gal colony filter assay was used to verify that the pAS2-*ESI47* plasmid construct alone did not activate the *GAL4* promoter (Bai and Elledge, 1997). The amount of X-Gal (100 :g/ml) for the colony filter assay, described in the protocol by Bai and Elledge (1997) was increased to 200 :g/ml.

2.4.2. Determination of *Arabidopsis* cDNA library titer

The *Arabidopsis* 9ACT library, CD4-10, constructed with mRNA isolated from leaf and root tissues, was obtained from the Arabidopsis Biological Resource Center (Ohio State University) The *Arabidopsis* library was made from cDNA synthesized with random primers for reverse transcription and the original cDNA library contained $1.9x10^6$ independent clones. The library titer was determined according to the protocol described by Elledge (1993). LE392 *E.coli* cells were grown to mid-logarithmic phase in LB medium supplemented with 0.2 % maltose and 10 mM $MgSO_4$. The cells were collected by centrifugation (3 000 x g for 5 min.), and resuspended in 10 mM $MgSO_4$. Serial dilutions (10^{-1}-10^{-8}) of the *Arabidopsis* 9ACT library were prepared in SM buffer (0.01% gelatin, 0.05 M Tris-pH 7.5, 0.1 M NaCl, 8 mM $MgSO_4$). The resuspended LE392 cells were divided into 100 :l aliquots and infected with 10 :l of each 9ACT-library dilution. The cells were incubated at 37°C for 25 min. To plate the cells, 3.5 ml of warm (47°C) NZY top agar (1% N-Z-amine, 0.1 M NaCl, 0.5% yeast extract, 8 mM $MgSO_4$, 0.8% agar) was added. The cells were rapidly vortexed, then plated on NZY plates (150 mm)

containing 1.5% agar and incubated at 37°C for 8 hrs. Plaques were counted and the library titer was determined to be $6.7x10^{10}$ pfu/ml.

The library titer was also determined in BNN132 *E. coli* cells, which mediate *in vivo* excision of the pACT plasmid and are recovered as colonies rather than plaques. According to the protocol described by Elledge (1993), BNN132 cells were grown overnight in LB media, centrifuged at 3 000 x g for 5 min., and resuspended in 1 ml of 10 mM $MgCl_2$. After 5 :l of 9ACT library was added to the cells, they were incubated at 30°C for 30 min. without shaking. The cells were removed from the incubator, 2 ml of LB was added and the cells were incubated at 30°C for 1 hr. Dilutions (10^{-1}-10^{-5}) of the cell culture were prepared and 200 :l of each dilution was plated on 150 mm LB plates supplemented with 50 :g/ml ampicilin and 0.2% glucose. The library titer was determined by counting colonies and found to be $1.3x10^9$ cfu/ml.

2.4.3. Amplification of *Arabidopsis* cDNA library for yeast two-hybrid screen

The library was amplified and recovered as plasmid DNA, according to the modified protocol in Current Protocols in Molecular Biology (1996). The BNN132 cells were grown to mid-logarithmic phase, centrifuged at 3 000 x g for 5 min., and resuspended in 10 mM $MgCl_2$ to OD_{600} of 0.5. The BNN132 cells were then infected with 100 :l of library (~$1x10^8$ pfu/ml), and plated on 20 LB plates (150 mm) supplemented with 50 µg/ml ampicilin and 0.2% glucose, and incubated at 37°C overnight. The resulting colonies were collected from the plates by scraping with a glass rod spreader using 10 ml of LB media per plate, and pooled to a 2 l solution of Terrific broth (1.2% bacto-tryptone, 2.4% yeast extract, 0.4% glycerol, 17.0 mM KH_2PO_4, 72.0 mM K_2HPO_4, 50 µg/ml

ampicilin) and grown overnight. The plasmid library DNA was then isolated using a QIAGEN plasmid Maxi kit.

2.4.4. Yeast two-hybrid library screen

The Y190 yeast strain containing the pAS2-*ESI47* open reading frame was used to screen the *Arabidopsis* cDNA library cloned in the yeast two-hybrid plasmid pACT-1, as described by Bai and Elledge (1997). The cDNA library clones are fused to the open reading frame of the *GAL4* DNA-activating domain in the pACT vector containing a leucine selection marker (Figure 1). A positive interaction between ESI47-fusion protein and a fusion-protein from the cDNA library produces a functional *GAL4* transcription factor, which can activate transcription of the reporter genes, *lacZ* and *HIS3*, in yeast. Yeast transformants that displayed a positive interaction between two hybrid proteins were selected on minimal media SC/-leu/-trp/-his supplemented with 3AT (3-amino-1, 2, 4-triazole). 3AT was added to eliminate basal levels of *HIS3* expression from the reporter construct which could otherwise interfere with the selection of transformants bearing a positive interaction. The X-Gal colony filter assay was then used to detect 2-galactosidase activity, which results from transcriptional activation of the *lacZ* gene (Bai and Elledge, 1997).

2.4.5. Specificity test for false positive interactions

Five unrelated cDNA clones fused to the *GAL4* DNA-binding domain in the yeast two-hybrid vectors pGBT9 or pAS2 were obtained from Dr. Tsang's and Dr. Joyce's laboratories (Table 2). These plasmids were transformed into the yeast strains (*S.*

cerevisiae), Y187 and PCY3, to test the specificity of the putative positives, isolated in the two-hybrid screen. The yeast strains containing the test "baits" were mated to the Y190 yeast strain containing the library plasmid clones that had been selected by positive interaction with ESI47, according to Bai and Elledge (1997). Transformants containing the library and bait clones were selected on SC/-leu/-trp growth media, and the X-Gal colony filter assay was used to detect a positive interaction.

2.4.5. Sequencing of positive clones

The library plasmids (pACT) that contained cDNA clones that interact with ESI47 were sequenced using specific vector primers: "library A" for 5' end and "library B" for 3'end amplification, (Table 3), and the CEQ 2000 Dye Terminator Cycle Sequencing kit. The sequence reactions were run on the Beckman CEQ2000 automated sequencer at the Center for Structural and Functional Genomics (Concordia University).

2.5. Sub-cloning of full length oxygen-evolving enhancer precursor (*OEE1*)

The full-length cDNA clone for the *OEE1* gene from *Arabidopsis* was obtained from the Arabidopsis Biological Resource Center. The full open reading frame (ORF) of *OEE1* was amplified by PCR using *OEE1* specific primers, 5'OEEP and 3'OEEP, (Table 3) each of which contains a *Bgl*II site. The PCR product was digested with *Bgl*II, and inserted in the multiple cloning site of pACT yeast two-hybrid library vector to create an *OEE1-GAL4* DNA-activating domain fusion protein. The *OEE1*-fusion construct was verified by restriction digestion with *Bgl*II which showed a fragment of approximately 1000 bp that corresponds to the expected size of the *Arabidopsis OEE1*. The *Arabidopsis*

OEE1 gene contains a *Bam*HI site, and to verify its orientation, the *OEE1*-fusion construct was digested with *Bam*HI and *Eco*RI, which produced fragments of approximately 3900, 3200 and 1750 bp (Figure 2). The resulting fragments were compared to the fragments obtained by restriction digestion with *Eco*RI and *Bam*HI, of the pACT-VI-partial length *OEE1* plasmid isolated in the two-hybrid screen, which showed fragments of approximately 3200, 3200, 1750 and 500 bp. One of the fragments of approximately 3200 bp increased to about 3900 bp when the full length *OEE1* clone was digested. The fragment of approximately 1750 bp observed with restriction digestion of the partial and full length *OEE1* clones, contains the 3' end of the *Arabidopsis OEE1* gene. This indicates that the full length clone is in the correct orientation for expression.

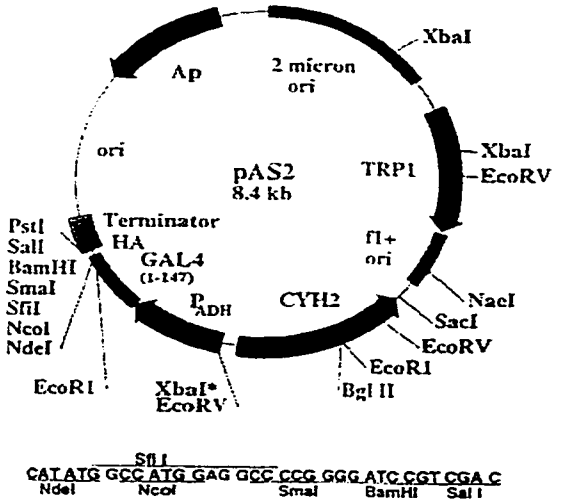
The full-length clone *OEE1* from wheat was obtained from Dr. Sarhan's laboratory at UQAM. The ORF of the wheat *OEE1*, was PCR amplified with specific primers, 5'OEEPWheat and 3'OEEPWheat (Table 3), both of which contain *Bgl*II sites. The fragment was digested with *Bgl*II and cloned into the *Bgl*II site of the pACT vector to create a fusion protein similar to that for the *Arabidopsis OEE1*. The *OEE1*-fusion construct was verified by restriction digestion with *Bgl*II, which showed a fragment of approximately 1000 bp that corresponds to the expected size of the wheat *OEE1*. Both the wheat *OEE1* gene and the vector pACT1 contain *Pst*I sites. To verify the orientation of the insert, the *OEE1*-fusion construct was digested with *Pst*I and *Eco*RI, which produced fragments of approximately 2600, 2300 (presumably 2 fragments), 1300, and 750 bp. Comparison to the fragments of approximately 2500, 2300, 1750, 1300, 750, and 500 bp, obtained with restriction digestion of the pACT-VI-partial length *OEE1* plasmid

isolated in the two-hybrid screen (Figure 2) with *Pst*I, *Eco*RI, and *Bam*HI indicates that the wheat OEE1 is in the correct orientation. The fragments of approximately 2500 bp and 1750 bp observed with digestion of the partial OEE1 clone increased to approximately 2600 bp and 2300 bp, respectively, in the wheat clone.

Species name: USDA current Classification	Species name as classified by McGuire and Dvõrák	Source	Accession #	Salt tolerance
Elytrigia elongata	Elytrigia intermedia	Belgium	PI238222	High
Elytrigia caespitosa	Elytrigia caespitosa	Iran	PI229914	Low
Elytrigia elongata	Elytrigia pontica	Germany	PI276399	High
Elytrigia elongata	Elytrigia pontica	Former Soviet Union	PI142012	High
Elytrigia elongata	Elytrigia turcica.	Turkey	PI179162	Intermediate
Elytrigia elongata	Elytrigia pontica	Turkey	PI206624	Intermediate
Elytrigia juncea subsp. Juncea	Elytrigia intermedia	Portugal	PI297873	Low
Elytrigia varnensis	Elytrigia intermedia	Germany	PI281863	Low

Table 1. Wild wheat grass accessions. Eight accessions of wheat grasses were selected for their varying degrees of salt tolerance as classified by McGuire and Dvõrák (1981). The seeds were obtained from the National Small Grains Collection (NSGC) of the United States Department of Agriculture. The wheat grass species are listed as classified by NSGC and McGuire and Dvõrák (1981) along with their USDA accession numbers and degree of salt tolerance. Note the source species of ESI47, *Lophopyrum elongatum* is synonymous with the older designations of *Elytrigia elongata*.

A



B

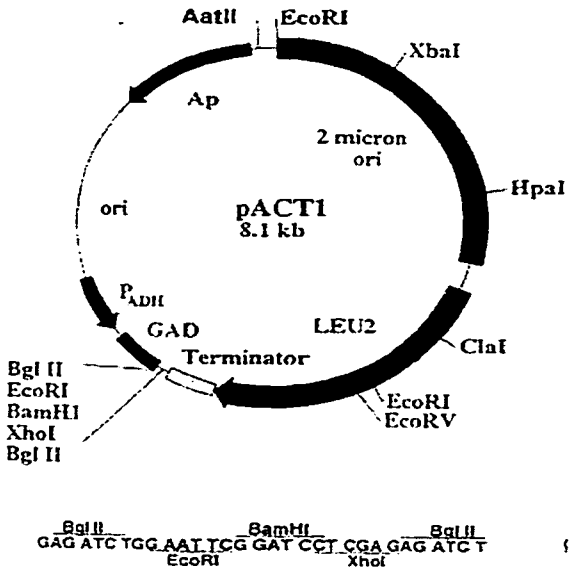
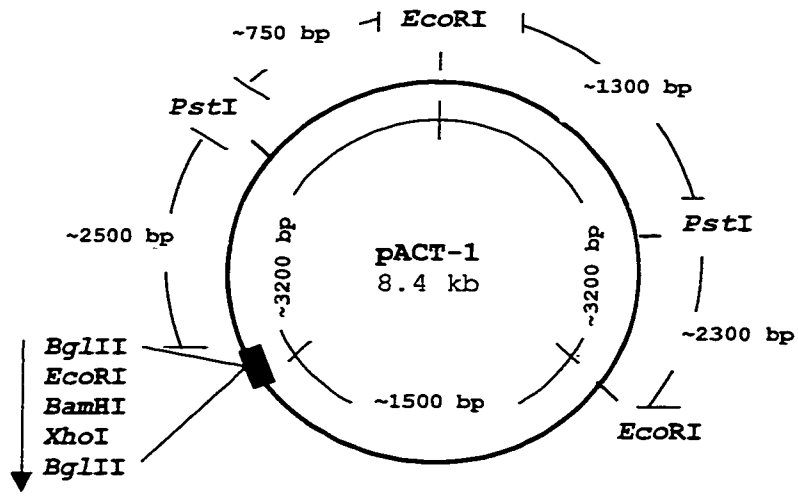


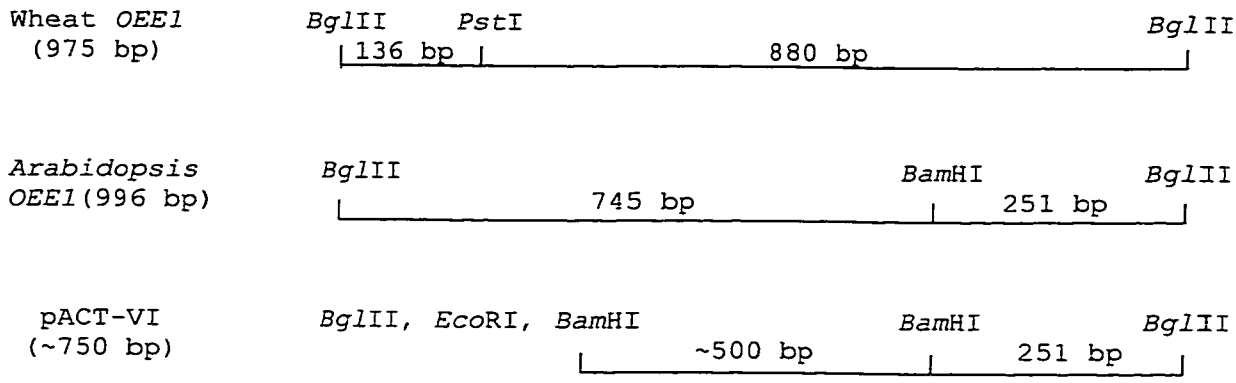
Figure 1. Vectors used with the two-hybrid system. (A) pAS2 vector which contains the *GAL4* DNA-binding domain and tryptophan marker, was used for subcloning the *ESI47* coding region. (B) pACT-1 vector which contains the *GAL4* DNA-activating domain and leucine marker, was used for the cDNA library from *Arabidopsis*.

Unrelated cDNA clones in "bait" vectors	Source	Lab
pGBT9-mousep21	*Mus musculus*	Dr. Tsang
pGBT9-lamin	*Dictyostelium discoideum*	Dr. Tsang
pGBT9-actin	*Dictyostelium discoideum*	Dr. Tsang
pGBT9-ribonucleotide reductase small subunit	*Dictyostelium discoideum*	Dr. Tsang
pAS2-tRNA nucleotidyltransferase	*Arabidopsis*	Dr. Joyce

Table 2. "Baits" used in the specificity test. "Baits" for five proteins were obtained from Dr. Tsang's and Dr. Joyce's laboratories (Concordia University). All clones produce a fusion of the protein to the *GAL4* DNA-activating domain in the pGBT9 or pAS2 vectors.

A





B

cDNA clone	Restriction enzymes	Approximate size of digested fragments (bp)
pACT-VI	*Eco*RI, *Bgl*II	**750**, 1500, 3200, 3200
pACT-VI	*Eco*RI, *Bam*HI	**500**, **1750**, 3200, 3200
pACT-VI	*Eco*RI, *Bam*HI, *Pst*I	**500**, 750, 1300, **1750**, 2300, 2500
Arabidopsis OEE1	*Bgl*II	**1000**, 8400
Arabidopsis OEE1	*Eco*RI, *Bam*HI	**1750**, **3900**, 3200
Wheat *OEE1*	*Bgl*II	**1000**, 8400
Wheat *OEE1*	*Eco*RI, *Pst*I	750, 1300, 2300, **2300**, **2600**

Figure 2. Restriction maps for pACT-1 vector and *OEE1* inserts. **(A)** The pACT-VI clone, isolated in the yeast two-hybrid screen was cloned into the *Xho*I site and encodes a partial OEE1 protein from *Arabidopsis*. The full length *OEE1* cDNA clones from wheat and *Arabidopsis* were subcloned into the *Bgl*II sites of the pACT-1 vector. **(B)** Restriction digestion of the partial and full length *OEE1* clones. Fragments shown in bold contain the *OEE1* inserts.

Primer	Sequence	Application
ESI47 *NcoI*	5'-CAACATGCCATGGCGATGCAGTGC TTCCGG-3'	5' primer including the start codon of the *ESI47* ORF
ST-3	5'-TAGGGCGAATTGGGTACCG-3'	pBluescript SK$^-$ vector primer for 3' end of *ESI47*
5'OEEPWheat	5'-GGCAGATCTCCATGGCAGCGTCTC TCCAAG-3'	5' primer for start of *OEE1* ORF from wheat
3'OEEPWheat	5'-GGCAGATCTCTAGTTAGACTCGAG CTGCGCGT-3'	3' primer for *OEE1* from wheat
5'OEEP	5'-CGGAGATCTCCATGGCAGCCTCTC TCCAATCC-3'	5' primer for *OEE1* from *Arabidopsis*
3'OEEP	5'-GCGAGATCTCTAGTGATGGTGATG GTGATGCTCAAGTTGACCATACCA-3'	3' primer for *OEE1* from *Arabidopsis*
Library A	5'-GCGTTTGGAATCACTACAGG-3'	pACT forward 5' end vector primer
Library B	5'-GCACGATGCACAGTTGAAGT-3'	pACT reverse 3' end vector primer

Table 3. Oligonucleotide primer sequences. Primer sequences used for PCR amplification and cloning of *ESI47* into pAS2 two-hybrid system vector, *OEE1* clones into pACT library vector, and primers used for sequencing cDNA in pACT library vector. The start codon, ATG and restriction sites are underlined.

CHAPTER 3. RESULTS

3.1. Northern analysis of *ESI*47 homologues in different wheat grasses

To investigate whether there is a correlation between the expression of *ESI*47 protein kinase homologues and the degree of salt tolerance among different genotypes (McGuire and Dvorak, 1981), eight accessions from five closely related wheat grass species (Table 1) were selected for northern analysis. The wheat grasses were grown hydroponically for 7 months and treated with 150 mM NaCl for 6, 12 and 24 hrs. RNA was extracted from the roots and total RNA was used for northern analysis. The expression of *ESI*47 homologues could not be detected. The level of *ESI*47 transcripts may have been insufficient for detection. However, to rule out the possibility of RNA degradation, the blots were re-probed with the *ESI*3 gene. The *ESI3* gene is known to be induced by salt treatment and to have expression levels 5 times greater than that observed with *ESI*47 in *L. elongatum* (Gulick and Dvorak, 1992). The *ESI*3 probe did detect an increase in gene expression in the wheat grasses (Figure 3-5), which indicates that the *ESI*47 signal was too low to be detected by northern analysis using total RNA samples.

Genes that are transcribed at levels too low to be detected by northern analysis with total RNA, can sometimes be detected by northern analysis with poly-$(A)^+$ RNA. Two species of wheat grasses, *Elytrigia intermedia* (PI281863) which has low levels of salt tolerance, and the highly salt tolerant *Elytrigia pontica* (PI276399) were chosen for poly-$(A)^+$ RNA isolation. Northern blots were prepared with 2 μg of poly-$(A)^+$ RNA from these two species and hybridized to the *ESI*47 probe (Figure 6). *ESI*47 expression was not detected in either of the wheat grass species tested.

3.2. The yeast two-hybrid screen

The yeast two-hybrid system was used to screen an *Arabidopsis* cDNA library made from mature leaf and root tissues to identify proteins that interact with the ESI47 protein kinase. When the *Arabidopsis* library was screened with the pAS2-ESI47 construct, in the yeast strain HF7C, no putative interacting proteins were detected. This screening was repeated with the pAS2-ESI47 construct in the yeast strain Y190. A total of 5.47×10^5 cDNA yeast transformants were screened in 4 separate transformation experiments. If a fusion protein with the *GAL4* DNA-activation domain interacts with the ESI47 protein fused to the *GAL4* DNA-binding domain, the transcription of *HIS3* and *lacZ* genes will be activated. Yeast transformants were selected by growth on media without histidine and by the X-Gal colony filter assay for β-galactosidase activity. Ninety four yeast transformants which activated the *GAL4* promoter and produced blue colonies were isolated. Putative positives where individually picked directly from the X-Gal filters and grown on fresh Sc/-leu/-trp/-his media. The X-Gal test was repeated and only 34 of the initial positive transformants gave blue colonies. From these 34 transformants, 13 failed to grow after two weeks of storage at 4°C and could not be regenerated.

The library plasmids from the remaining 21 yeast transformants were rescued and 14 of the library plasmids were subjected to a specificity test to eliminate any clones whose interactions were not specific to ESI47. The library plasmids were transformed into Y190 cells and then mated with the opposite mating type yeast strains, PCY3 and Y187, containing different expression constructs with unrelated "baits" (mousep21, lamin, actin, ribonucleotide reductase small subunit, and tRNA nucleotidyltransferase, Table 2). The X-Gal test was used to detect a positive interaction between unrelated

proteins and the putative positives that had been selected by screening with ESI47. None of the matings resulted in a positive X-Gal test, which indicates that the interactions between the positive clones and the ESI47 protein kinase were specific.

Of the 21 library plasmids rescued from yeast, 13 were successfully sequenced and the results were compared to sequences in the GenBank database. The construction of the cDNA *Arabidopsis* library was not unidirectional, which leads to the isolation of putative positives with cDNA inserts in the anti-sense orientation. Clones with anti-sense orientation, were considered false positives. The sequencing results (Table 4) showed that six positive clones, encoded an oxygen-evolving enhancer protein 1 precursor (OEE1) from photosystem II (Figure 6 and 7). These clones were in sense orientation and were in frame with the *GAL4* DNA-activation domain. These results suggest that the ESI47 protein kinase interacts with the OEE1 protein of photosystem II. Clone Y190-8, encoded a protein kinase C inhibitor in frame with the *GAL4* promoter, and may be another possible interacting protein (Table 4).

Four clones, Y190-E, Y190-F, Y190-G, and Y190-H, with approximately the same insert size were suspected also to encode the OEE1 protein, and were not sequenced. Four clones, Y190-6, Y190-13, Y190-14, and Y190-16, whose sequencing reactions failed were reassessed by X-Gal and were found to be negatives. Re-testing for positive interactions by X-Gal, confirmed a positive interaction for OEE1 but not for the protein kinase C inhibitor. To ensure that the yeast transformant containing the protein kinase C inhibitor library plasmid had not been contaminated, the library plasmid used for sequencing was re-transformed into the Y190 strain containing the pAS2-*ESI*47 construct. The library plasmid from the yeast transformant Y190-VII, the oxygen-

evolving enhancer protein 1 precursor was also re-transformed into the yeast strain Y190 containing the pAS2-*ESI*47 construct. The X-Gal assay showed that the Y190-VII transformant still produced blue colonies but the Y190-8 transformant which expressed the protein kinase C inhibitor did not.

The sequences of the positive clones that encoded the 33 kDa OEE1 protein, were compared to the full length sequence of the *Arabidopsis* 33 kDa oxygen-evolving protein, from the GenBank database. One of the *OEE1* clones, pACT-VII, was sequenced from both ends and the size of the *OEE1* clone was determined to be 645 base pairs. The pACT-VII, *OEE1* clone was missing the first 82 and last 35 amino acids, found in the full length *OEE1* precursor. The *OEE1* positive clones, pACT-B, pACT-C and pACT-D, sequenced from the 5' end were missing the first 82, 82, and 81 amino acids, respectively, at their amino termini. Restriction digestion of the clones indicated that the unsequenced ends of all three clones appear to be missing approximately 20 amino acids at their carboxy termini (Table 5, Figure 7). Two of the *OEE1* positive clones, pACT-IV, and pACT-VI, sequenced from the 3' end, were missing 11 and 12 amino acids, respectively at their carboxy termini. Restriction digestion indicated that both clones are missing approximately 80 amino acids at their amino termini (Table 5, Figure 7). All six *OEE1* positive clones therefore appeared to be missing the first 80 amino acids at the amino terminus (Figure 8). This region encodes the chloroplast targeting sequence (Görlach *et al.*, 1993). To determine whether the ESI47 protein kinase interacts only with the mature protein, or with the precursor protein, the full length *OEE1* clones from *Arabidopsis* and wheat were cloned into the pACT library vector. The interaction of ESI47 with the wheat OEE1 protein, was investigated since, the ESI47 protein kinase

was originally isolated from *L. elongatum*, a species closely related to wheat. In an X-Gal assay, neither the fusion construct with the full length *Arabidopsis* clone, nor that from wheat produced blue colonies, which indicates the ESI47 protein kinase does not interact with the precursor proteins.

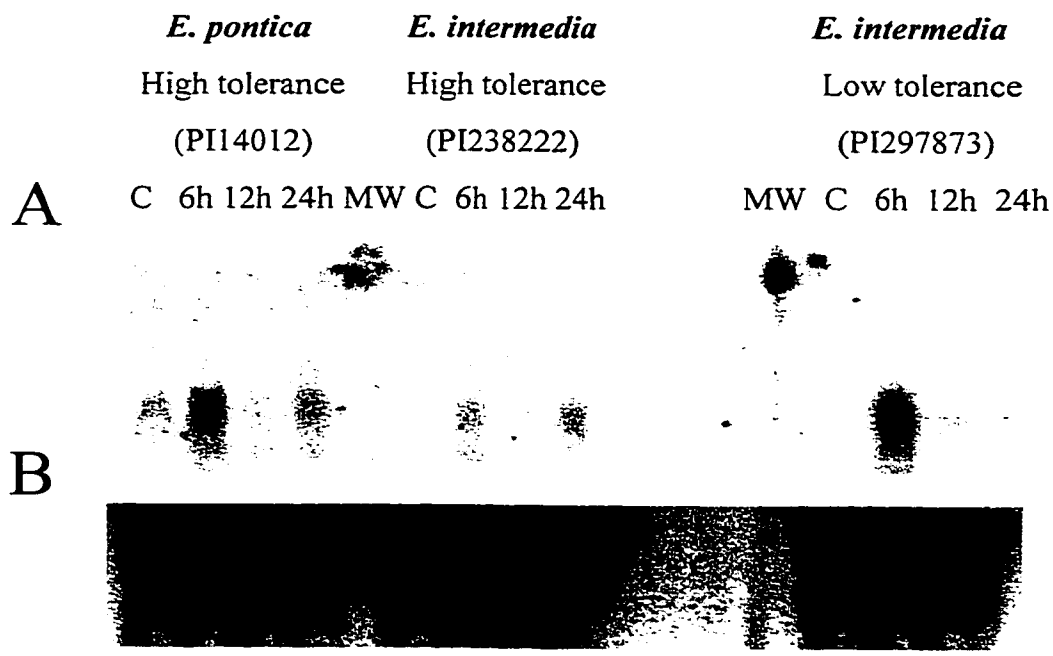


Figure 3. Northern blot analysis of the *ESI3* probe hybridyzed to total RNA from wheat grasses that differ in their degree of salt tolerance. (A) RNA from the highly salt tolerant *E. pontica* (PI14012), highly salt tolerant *E. intermedia* (PI238222), and salt sensitive *E. intermedia* (PI297873). RNA molecular weight markers were loaded in MW lanes. (B) The gel was stained with ethidium bromide and photographed as a negative image with *UV* illumination, to verify the amount of sample loaded in (A).

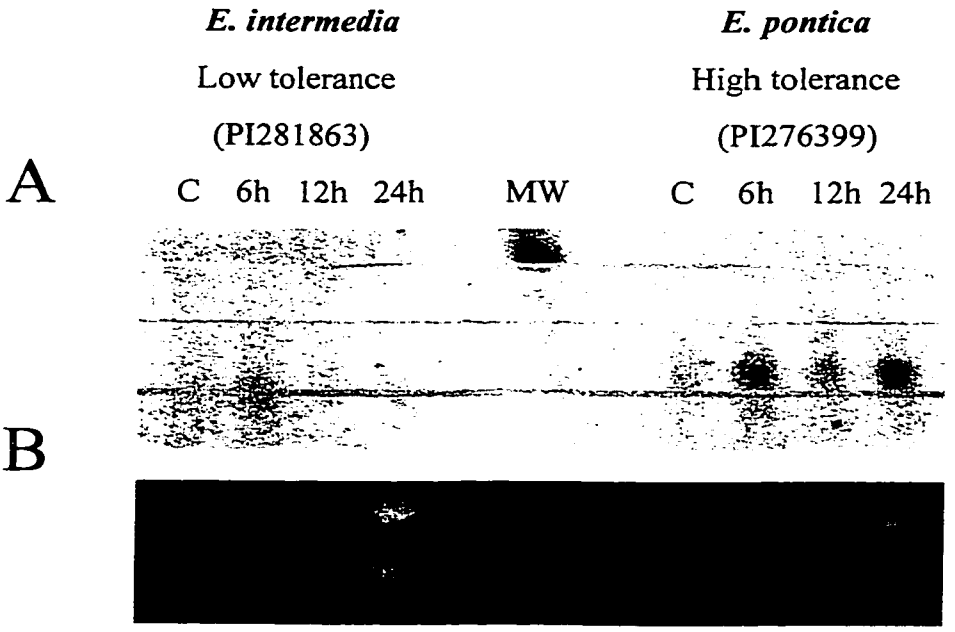


Figure 4. Northern blot analysis of the *ESI3* probe hybridyzed to total RNA from wheat grasses that differ in their degree of salt tolerance. (A) RNA from the salt sensitive *E. intermedia* (PI281863), and highly salt tolerant *E. pontica* (PI276399) accessions. RNA molecular weight markers were loaded in MW lane. (B) Gel stained with ethidium bromide and photographed with *UV* illumination to verify the amount of sample loaded in (A).

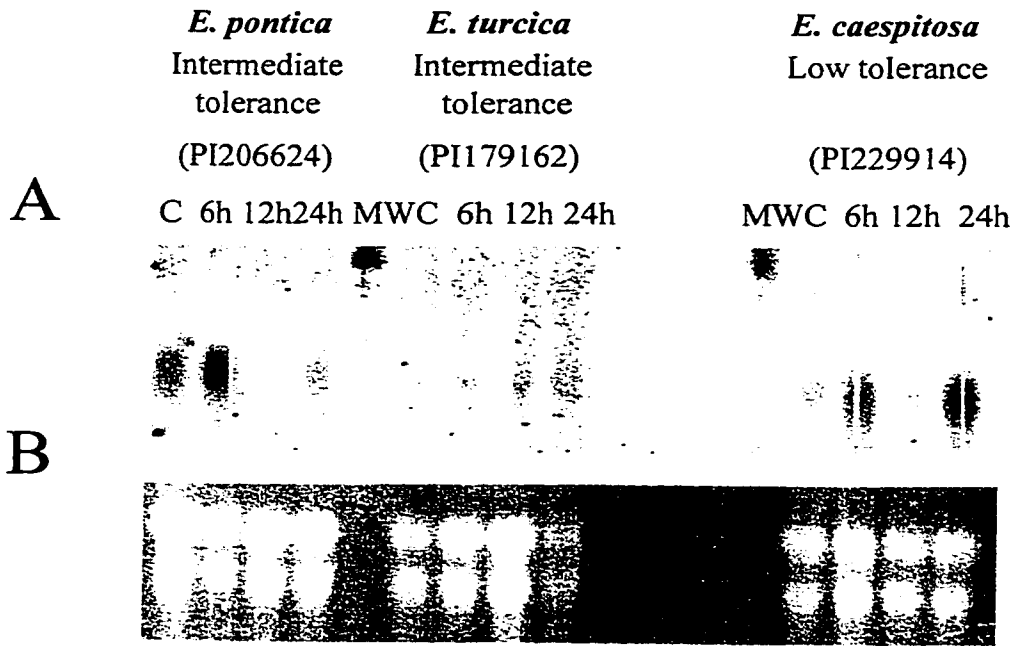


Figure 5. Northern blot analysis of the *ESI3* probe hybridyzed to total RNA from wheat grasses that differ in their degree of salt tolerance. (A) RNA from the moderately salt tolerant *E. pontica* (PI206624), moderately salt tolerant *E. turcica* (PI179162), and salt sensitive *E. caespitosa* (PI229914). RNA molecular weight markers were loaded in MW lanes. (B) Gel stained with ethidium bromide and photographed with *UV* illumination to verify the amount of sample loaded in (A).

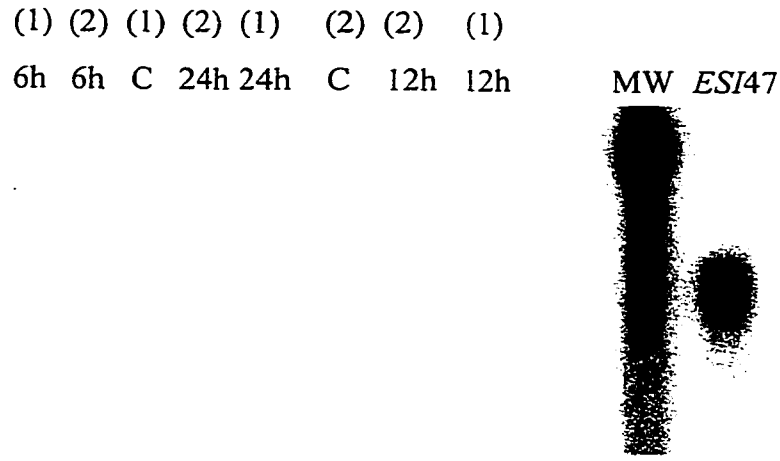


Figure 6. Northern analysis of mRNA from wheat grasses. Lanes labeled with (**1**) are from the *E. intermedia* (PI281863) with low salt tolerance and those labeled (**2**) from highly tolerant *E. pontica* (PI276399). RNA blots were hybridized to *ESI*47 probe. The last lane contained an 8 pg sample of *ESI*47 DNA as control. MW lane contains RNA molecular weight markers.

Yeast Transformants	X-Gal Assay 1	X-Gal Assay 2	Sense orientation	Sequencing results
Y190-1	Dark blue	Blue	No	v-ATPase subunit D
Y190-4	Dark blue	Blue	No	18S ribosomal protein
Y190-6	Pale blue	White		
Y190-8	Pale blue	White	Yes	Protein kinase C inhibitor
Y190-9	Pale blue	White	No	Hypothetical protein
Y190-11	Pale blue	Blue		No insert
Y190-13	Pale blue	White		
Y190-14	Pale blue	White		
Y190-16	Pale blue	White		
Y190-32*	Dark blue	White		No insert
Y190-IV	Dark blue	Blue	Yes	Oxygen-evolving enhancer protein 1 precursor (OEE1)
Y190-V	Pale blue	Blue		No insert
Y190-VI	Pale blue	Blue	Yes	Oxygen-evolving enhancer protein 1 precursor (OEE1)
Y190-VII	Dark blue	Blue	Yes	Oxygen-evolving enhancer protein 1 precursor (OEE1)
Y190-B	Dark blue	Blue	Yes	Oxygen-evolving enhancer protein 1 precursor (OEE1)
Y190-C	Dark blue	Blue	Yes	Oxygen-evolving enhancer protein 1 precursor (OEE1)
Y190-D	Dark blue	Blue	Yes	Oxygen-evolving enhancer protein 1 precursor (OEE1)
Y190-E	Dark blue	Blue		
Y190-F	Dark blue	Blue		
Y190-G	Dark blue	Blue		
Y190-H	Dark blue	Blue		

Table 4. Putative positive clones from yeast two-hybrid screen. The X-Gal test and sequencing results for selected positive plasmids isolated with the yeast two-hybrid system. X-Gal assay 1 was used to detect a positive interaction between positive clones and ESI47. X-Gal assay 2 was performed to verify the interaction with ESI47. The nucleotide sequence for 13 of the clones was determined and compared to sequences in the GenBank database. Putative positives were scored for sense or anti-sense orientation, relative to the gene promoter and *GAL4* DNA-activating domain.

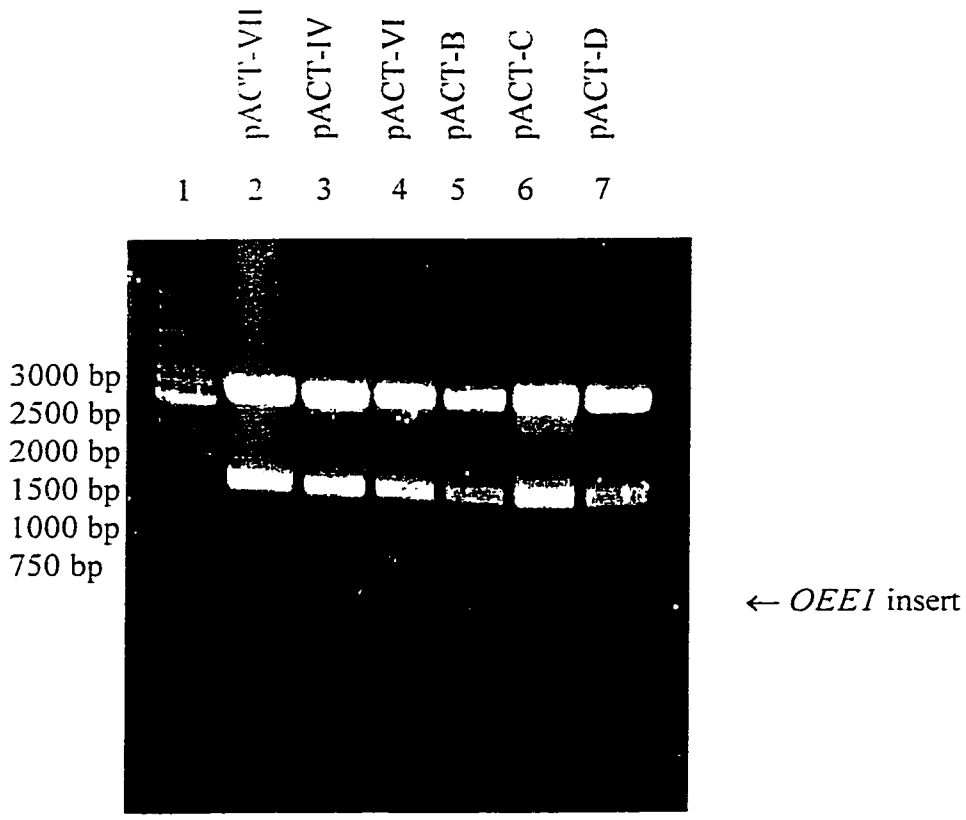


Figure 7. Restriction analysis of *OEE1* positive clones. Positive two-hybrid interacting clones of *OEE1* were digested with *Eco*RI and *Bgl*II and electrophoresed on an agarose gel. Lane 1 shows a 1 kb DNA molecular weight marker and lanes 2-7 show the digested pACT-VII, IV, VI, B, C, D, respectively.

Positive Clone	Approximate Clone Size (bp)	Approximate Clone Size (aa)	Portion of *OEE1* missing from amino-termus	Portion of *OEE1* missing from carboxy-terminus
Sequenced from 3' and 5'end				
PACT-VII	645 bp	215 aa's	82 aa's	35 aa's
Sequenced from 5'end				
PACT-B	700 bp	233 aa's	82 aa's	~ 20 aa's
PACT-C	700 bp	233 aa's	82 aa's	~ 20 aa
PACT-D	700 bp	233 aa's	81 aa's	~ 20 aa
Sequenced from 3'end				
PACT-IV	750 bp	250 aa's	~ 80 aa's	11 aa's
PACT-VI	750 bp	250 aa's	~ 80 aa's	12 aa's

Table 5. *OEE1* fragments in pACT cDNA library plasmids. The plasmids were digested with *Eco*RI and *Bgl*II restriction enzymes. The OEE1 insert size was approximated from the gel (Figure 7). The pACT-VII clone was sequenced from both ends and the exact size of the clone is known. For clones sequenced from only one end, the number of missing amino acids at the unsequenced end was estimated from the insert sizes.

33kDa oxygen-evolving protein *A. thaliana*:

MAASLQSTATFLQSAKIATAPSRGSSHLRSTQAVGKSFGLETSSARLTCSFQSDFK

D (B,C,VII)

DFTGKCSDAVKIAGFALATSALVVSIGIASA▼EGAPKRLTYDEIQSKTYMEVKGT

GTANHSPTIDGGSETFSFKPGKYAGKKFCFEPTSFTVKADSVSKNAPPEFQNTKL

MTRLTYTLDEIEGPFEVASDGSVNFKEEDGIDYAAVTVQLPGGERVPFLFTVKQL

DSGKPDSFTGKFLVPSYRGSSFLDPKGRGGSTGYDNAVALPAGGRGDEEELVKE

VII **VI IV**

NVKNTAASVGEITLKVTKSKPETG|EVIGVFESLQPSDTDLGAKVPKD|V|KIQGV

WYGQLE

Figure 8. Oxygen-evolving protein sequence of *Arabidopsis* compared to *OEE1* positive clones. ([) Start of *OEE1* sequence in positive clones sequenced from 5' end. These clones were estimated by restriction digestion to end in the underlined region. (]) End of *OEE1* sequence in clones sequenced from 3'end, and the 5' end estimated by restriction digest is shaded.
(▼) Peptide cleavage site, which produces the mature OEE1 protein located in the chloroplast.

```
OEE1(GenBank) FQSDFKDFTGKCSDAVKIAGFALATSALVVSGASAEGAPKRLTYDEIQSKTYMEVKGTG 109
Y190-VII (5')  ------------------------------ASAEGAPKRLTYDEIQSKTYMEVKGTG
Y190-VII (3')  ------------------------------------------------------------
Y190-B (5')    ------------------------------ASAEGAPKRLTYDEIQSKTYMEVKGTG
Y190-C (5')    ------------------------------ASAEGAPKRLTYDEIQSKTYXGXSERN
Y190-D (5')    ------------------------------GASAEGAPKRLTYDEIQSKTYMEVKGTG
Y190-IV (3')   ------------------------------------------------------------
Y190-VI (3')   ------------------------------------------------------------

OEE1(GenBank) TANHSPTIDGGSETFSFKPGKYAGKKFCFEPTSFTVKADSVSKNAPPEFQNTKLMTRLT 168
Y190-VII (5')  TANQCPTIDGGSETFSFKPGKYAGKKFCFEPTSFTVKADSVSKNAPPEFQNTKLMTRLT
Y190-VII (3')  ------------------------------------------------------------
Y190-B (5')    TANQCPTIDGGSETFSFKPGKYAGKKFCFEPTSFTVKADSVSKNAPPEFQNTKLMTRLT
Y190-C (5')    WNG-------------------------------------------------------
Y190-D (5')    TANQCPTIDGGSETFSFKPGKYAGKKFCFEPTSFTVKADSVSKNAPPEFQNTKLMTRLT
Y190-IV (3')   ------------------------------------------------------MTXLT
Y190-VI (3')   ------------------------------------------------------------

OEE1(GenBank) YTLDEIEGPFEVASDGSVNFKEEDGIDYAAVTVQLPGGERVPFLFTVKQLDASGKPDSF 227
Y190-VII (5')  YTLDEIEXPFXVASDGSVNFKEEDGIDYA----------------------------
Y190-VII (3')  ------------------------------------------TVKQLDASGKPDSF
Y190-B (5')    YTLDEIEGPFEVASDGSVNFKEEDGIDYAAVTVQLPGGERVPXLFTVKQLDASGKPDSX
Y190-C (5')    ------------------------------------------------------------
Y190-D (5')    YTLDEIEGPFEVASDGSVNFKEEDGIDYAAVTVQLPGGERVPFLFTVKQXDASGKPDSF
Y190-IV (3')   YTXDEIEGPXEVASDGSVDFKEEDGIDYAAVTVQLPGGERVPFLFTVKQLDASGKPDSF
Y190-VI (3')   ----------------------------------------------------SGKPDSF

OEE1(GenBank) TGKFLVPSYRGSSFLDPKGRGGSTGYDNAVALPAGGRGDEEELVKENVKNTAASVGEIT 286
Y190-VII (5')  ------------------------------------------------------------
Y190-VII (3')  TGKFLVPSYRGSSFLDPKGRGGSTGYDNAVALPAGGRGDEEELVKENVKNTAASVGEIT
Y190-B (5')    ------------------------------------------------------------
Y190-C (5')    ------------------------------------------------------------
Y190-D (5')    ------------------------------------------------------------
Y190-IV (3')   TGKFLVPSYRGSSFLDPKGRGGSTGYDNAVALPAGGRGDEEELVKENVKNTAASVGEIT
Y190-VI (3')   TGKFLVPSYRGSSFLDPKGRGGSTGYDNAVALPAGGRGDEEELVKENVKNTAASVGEIT

OEE1(GenBank) LKVTKSKPETGEVIGVFESLQPSDTDLGAKVPKDVKIQGVWYGQLE 332
Y190-VII (5')  ------------------------------------------------
Y190-VII (3')  LKVTKSKPET--------------------------------------
Y190-B (5')    ------------------------------------------------
Y190-C (5')    ------------------------------------------------
Y190-D (5')    ------------------------------------------------
Y190-IV (3')   LKVTKSKPETGEVIGVFESLQPSDTDLGAKVPKDV--------------
Y190-VI (3')   LKVTKSKPETGEVIGVFESLQPSDTDLGAKVPKD---------------
```

Figure 9. The amino acid sequence from amino acid 51 to 332 of the *OEE1* gene from *Arabidopsis*, aligned with the complete sequence of Y190-VII and the partial sequences of the other *OEE1* clones. The 5' and 3' end sequences show the coding region missing at the respective ends. The 5' end sequences indicate the exact beginning of 4 clones and the 3' end sequences indicate the exact end of 2 clones and Y190-VII.

CHAPTER 4. DISCUSSION

Plant protein kinases are believed to play key roles in metabolic functions, stress signaling pathways, growth and development (Braun and Walker, 1996). The first complete genomic sequence of a plant, that of *Arabidopsis* has revealed the presence of 860 genes encoding serine/threonine protein kinases (The Arabidopsis Genome Initiative, 2000). The vast majority of these are yet to be characterized. The study of protein kinase interactions with other kinases, ion channels, and transcription factors will provide a deeper understanding of the mechanisms involved in salt-stress signaling pathways. In this study, the role of the salt stress induced kinase, *ESI*47 from *L. elongatum*, in signaling and salt tolerance was investigated.

Northern blot analysis was used to compare the expression levels of *ESI*47 homologues, in species of wheat grasses that differ in their degree of salt tolerance (Table 1) to determine whether there is a correlation between the expression of *ESI*47 and salt tolerance. Though we have kept the "*Elytrigia*" designation for these species that was assigned by the USDA National Small Grains Collection in this thesis, *Elytrigia* is synonymous with the "*Lophopyrum*" name we use for our subject species. The results obtained from northern blots of total RNA samples extracted from the roots of 8 accessions from 5 species of wheat grasses and probed with *ESI*47 did not allow the detection of *ESI*47 expression. The expression of *ESI*3 as observed in Figures 3, 4 and 5 confirmed the quality of the RNA used in the blots and suggested that transcript levels of *ESI47* were too low to allow the detection on these blots.

Previous northern analyses of *ESI47* in *L. elongatum*, wheat, and their amphyploid product, were performed with poly-$(A)^+$ RNA (Gulick and Dvörák, 1990;

Galvez *et al.*, 1993), suggesting that *ESI47* transcripts levels are too low to allow detection from total RNA. Poly-$(A)^+$ RNA was isolated from one of the species with high salt tolerance, *E. pontica* (PI276399) and one species with low tolerance, *E. intermedia* (PI281863). Northern analysis was carried out with the poly-$(A)^+$ RNA and an *ESI47* probe. Expression of *ESI47* was not observed (Figure 4), indicating the *ESI47* transcript levels are very low in the wheat grass species being studied. These results suggest that the detection of *ESI47* expression may require greater than the 2 μg of poly-$(A)^+$ RNA used in this study. *ESI47* transcripts, may be detectable by more sensitive means, such as quantitative RT-PCR, however, this method requires knowledge of DNA sequences for the target gene. This would require the sequence of homologues from the wheat grass species, or perhaps preliminary testing of candidate primers based on the *L. elongatum* sequence. Since the *ESI47* sequence from *L. elongatum* is 97% similar to a homologue in wheat, *Triticum aestivum*, it seems likely that primers designed from the *L. elongatum* gene would function in these closely related species.

The yeast two-hybrid system was used to screen an *Arabidopsis* cDNA library from leaf and root tissues to identify proteins that interact with ESI47. The yeast two-hybrid system has been used in tomato for the successful identification of several *Pti* genes encoding proteins that interact with the Pto serine/threonine protein kinase involved in pathogen disease resistance (Zhou *et al.*, 1995; Zhou *et al.*, 1997). These findings, as well as numerous other examples of protein kinases used in the two-hybrid system (Patharkar and Cushman, 2000; Ichimura *et al.*, 1998; Clark *et al.*, 1998), indicate that the two-hybrid system can identify proteins interacting with protein kinases. In this study, a total of 5.47×10^5 cDNA yeast transformants were screened and 34 yeast

transformants were identified as putative positives after two rounds of X-Gal testing, 21 of which were used for further analysis. Even though the X-Gal test can be used to efficiently eliminate most false positives, the results obtained can also be misleading. Yeast transformants that are subjected to the X-Gal test are incubated at 30°C for up to 24 hours to allow color development, which is dependent on the strength of protein interactions and the growth rate of the transformants. After a certain time, yeast colonies accumulate the blue color even though there is no positive interaction. The blue color development observed in false positives may result from the non-enzymatic degradation of X-Gal. In this study, yeast transformants that produced pale blue colonies when assayed with X-Gal, were selected as putative positives based on the assumption that the ESI47 protein kinase may display a transient, weak interaction with its substrate, inadvertently leading to the selection of false positives.

To verify the specificity of the interaction between the positive clones and the ESI47 protein kinase, plasmids were recovered for 14 of the positives and re-transformed into the Y190 strain, which was mated with PCY3 and Y187 yeast strains containing "bait" constructs for five unrelated proteins. None of the yeast transformants mated activated the *GAL4* promoter in the X-Gal test, thus, the interaction with ESI47 was specific. DNA sequence analysis indicated that six of the clones encoded an oxygen-evolving protein enhancer 1 precursor (OEE1) from the photosystem II oxygen-evolving complex and one clone encoded a protein kinase C inhibitor. These clones were in the proper reading frame to be expressed as fusion proteins with the *GAL4* DNA-activating domain. Seven clones were reclassified as false positives because they contained fragments cloned in the anti-sense orientation or were plasmids without cDNA inserts

(Table 4). The positive clones, Y190-8 encoding the protein kinase C inhibitor and Y190-VII encoding the OEE1 were re-transformed into the Y190 strain containing the pAS2-*ESI*47 construct to further verify the interaction. The X-gal test resulted in a positive interaction between the ESI47 protein kinase and the OEE1 protein, but not with the protein kinase C inhibitor, which suggests that the protein kinase C inhibitor was likely a false positive.

One clone, pACT-VII, encoding the OEE1 protein was sequenced entirely, the other clones were sequenced from only one end and restriction digestion was used to estimate the size of the *OEE1* fragments contained in the partially sequenced clones. The sequence analysis revealed that four of the *OEE1* clones isolated were missing 81 or 82 amino acids at the amino-terminus, present in the *OEE1* full-length sequence (Table 5, Figure 8). Restriction digestion indicated that the other two clones also lacked approximately 80 amino acids of the coding region for the OEE1 precursor protein. The OEE1 precursor protein, which is synthesized in the cytosol, requires the proteolytic cleavage of a transit peptide to be imported into the chloroplast (James *et al.*, 1989). The first 82 amino acids at the amino-terminus of the OEE1 precursor protein constitute the chloroplast targeting peptide (Görlach *et al.*, 1993), this corresponds very closely to the amino-terminal region absent from the *OEE1* clones isolated in the two-hybrid screen. The *Arabidopsis* library that was screened was constructed with random primers and therefore should contain a random assortment of sizes and 5' end starting points for the cDNA clones. All six *OEE1* clones isolated in the two-hybrid screen had similar 5' ends indicating a non-random selection. This indicates that the ESI47 kinase likely interacts with the mature OEE1 and not with the precursor protein containing the 80 amino acid

signal peptide. Studies in *Arabidopsis* and tomato have shown that the OEE1 protein is expressed in photosynthetically active tissues such as leaves, stems, cotyledons but not in roots (Jain *et al.*, 1998). In contrast, the *ESI47* kinase expression was previously observed using northern analysis, in the roots of *L. elongatum* but not in the photosynthetic tissues (Galvez *et al.*, 1993). This raises the question of whether the interaction with the OEE1 protein observed in yeast, actually occurs *in vivo*. Three *Arabidopsis* protein kinases, F8A24.12, F12E4.50, and T7F6.28, show high amino acid sequence similarity to ESI47 (Shen *et al.*, 2001). One of these kinases, F8A24.12, is induced by salt stress in both root and leaf tissues, thus it seems possible that the ESI47 protein homologue in *Arabidopsis* may interact with the OEE1 protein in the leaves.

The possible role of ESI47 in photosynthesis had not been considered prior to this study. There is evidence that salt stress causes a decrease in photosynthesis by reducing the electron transport and oxygen-evolving activities of photosystem I and photosystem II (Allakhverdiev *et al.*, 2000). Salt stress primarily reduces the activity of the oxygen-evolving complex of photosystem II. It has been reported that the transcript levels of the OEE1 (33 kDa), OEE2 (23 kDa) and OEE3 (16 kDa) proteins, which form part of the oxygen-evolving complex responsible for the oxygen-evolving activity of photosystem II, are induced by salt stress (Sugihara *et al.*, 2000). Given that ESI47 is also induced by salt stress, it can be hypothesized that ESI47 participates in the reactivation of photosynthesis through its interaction with OEE1. In the chloroplast, removal of the three OEE's from the oxygen-evolving complex results in decreased oxygen evolution and instability of the manganese cluster. During salt stress the D1 and D2 proteins in the reaction center of the photosystem II complex are damaged. The turnover rate of these proteins appears to be

regulated by a phoshorylation/dephosphorylation cycle (Rokka *et al.*, 2000), and the OEE1 protein is thought to play an important role in stabilizing the reaction center of the photosystem II complex. Until now, only DI and D2 have been shown to be phosphorylated as they dissociate from the photosystem II complex. The OEE1 protein has been found to dissociate from the photosystem II complex along with the D1 protein during photoinhibition however, phosphorylation of the mature OEE1 protein when it is released from the photosystem II complex has not been investigated. It has been suggested that the OEE1 protein may be involved in the regulation of D1 protein turnover, helping to maintain the structural integrity of photosystem II during photoinhibition (Yamamoto *et al.*, 1998). Proteins required for photosynthetic activity may be subject to regulation by genes induced by salt stress. The salt-induced ESI47 protein kinase could play a role in regulating the re-association of the OEE1 protein to the oxygen-evolving complex following its dissociation after photoinhibition resulting from salt stress (Allakhverdiev *et al.*, 2000).

Alternatively, ESI47 could regulate the import of the OEE1 precursor protein into the chloroplast or the assembly of the OEE1 mature protein into the photosystem II complex. In pea mesophyll cells, studies have shown that the preOE23 (OEE2) and preOE33 (OEE1), thylakoid lumen-localized precursor proteins of the oxygen-evolving complex, are phosphorylated in the cytosol, on serine or threonine residues of the transit peptide, before being imported into the chloroplast (Waegemann and Soll, 1996). The proteins targeted to the thylakoid lumen possess bipartite transit sequences consisting of the stroma-targeting domain and the thylakoid-targeting domain. Waegamann and Soll (1996) have shown that the phosphorylation of these precursor proteins occurs within the

stroma-targeting domain of the transit peptide. The cytosolic kinases that phosphorylate the chloroplast precursor proteins have yet to be characterized. The interaction of the ESI47 protein kinase with the OEE1 precursor protein might involve the modification of the OEE1 protein structure by phosphorylation. However, the yeast two-hybrid assays reported here did not show that the ESI47 protein kinase interacts with the OEE1 precursor proteins from either *Arabidopsis* or from wheat when the chloroplast targeting peptide was present. In pea mesophylls, the preOE23 is cleaved by a peptidase in the stroma following phosphorylation, to yield an intermediate OE23 precursor which is later processed in the thylakoid membrane (Waegemann and Soll, 1996). The ESI47 protein kinase could interact with an intermediate OEE1 form, to play a role in the targeting of the OEE1 protein to the thylakoid membrane. However, the fact that none of the positive clones selected in the two-hybrid screen include the coding region for the transit peptides, does not support this hypothesis. Since the ESI47 kinase does not contain a chloroplast targeting sequence, it is difficult to assess its involvement in chloroplast-localized photosystem reactivation following salt stress.

It is possible that phosphorylation of the OEE1 precursor protein by the ESI47 kinase, involves a transient interaction, where ESI47 binds to an internal domain in the OEE1 precursor. This interaction could result in a conformational change, followed by subsequent phosphorylation and cleavage of the transit peptide. The yeast two-hybrid system may not be sufficiently sensitive to allow the detection of the transient interaction. Perhaps, the absence of the targeting peptide stabilizes the interaction between the ESI47 kinase and the mature OEE1 protein resulting in a positive X-Gal test. Further characterization of the interaction between ESI47 and OEE1 using phosphorylation

assays could reveal the role of ESI47 in regulating OEE1 import into the chloroplast. *In vitro* phosphorylation assays, as well as *in vivo* labeling using yeast expression vectors, with both the precursor and mature OEE1 proteins could be used to further investigate the function of ESI47 in its interaction with OEE1.

The study of salt-induced genes such as *ESI*47 from salt tolerant wild wheat grass species will help to elucidate the multigenic mechanisms implicated in the salt tolerance pathways. Further two-hybrid screening of *Arabidopsis* cDNA and wheat cDNA libraries will likely lead to the discovery of other ESI47 substrates that could in turn, reveal information concerning the regulation and function of the ESI47 protein kinase. The characterization of the ESI47 protein kinase and its substrates will provide a better understanding of the mechanisms involved in salt stress pathways. These studies could also lead to the identification of genes that are responsible for the high level of salt tolerance observed in *L. elongatum*. Gene transfer made possible by genetic engineering could lead to the improvement of salt tolerance in agricultural crops such as wheat.

LITERATURE CITED

Ädelroth, P., Lindberg, K., and Andréasson, L.-E. (1995) Studies of Ca^{2+} Binding in spinach photosystem II using $^{45}Ca^{2+}$. *Biochemistry.* **34**, 9021-9027

Allakhverdiev, S. I., Sakamoto, A., Nishiyama, Y., Inaba, M., and Murata, N. (2000) Ionic and osmotic effects of NaCl-induced inactivation of photosystems I and II in *Synechococcus sp. Plant Physiol.* **123**, 1047-1056

Apse, M. P., Aharon, G. S., Snedden, W. A., and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in *Arabidopsis. Science.* **285**, 1256-1258

Barkla, B. J., Vera-Estrella, R., and Pantoja, O. (1999) Towards the production of salt-tolerant crops. *Adv Exp Med Biol.* **464**, 77-89

Bai, C. and Elledge, S. J. (1997). Gene identification using the yeast two-hybrid system. *Methods Enzymol.* **283**, 141-56

Bañuelos, M. A. and Rodríguez-Navarro, A. (1998) P-type ATPases mediate sodium and potassium effluxes in *Schwanniomyces occidentalis. J. Biol. Chem.* **273**, 1640-1646

Blumwald, E. (2000) Sodium transport and salt tolerance in plants. *Curr. Opin. Cell Biol.***12**, 431-434

Braun, D. M. and Walker, J. C. (1996) Plant transmembrane receptors: new pieces in the signaling puzzle. *Trends Biochem Sci.* **21**, 70-73

Busk, K. and Pagès, M. (1998) Regulation of abscisic acid-induced transcription. *Plant Mol Biol.* **37**, 425-35

Chrispeels, M. J., Crawford, N. M., and Schroeder, J. I. (1999) Proteins for transport of water and mineral nutrients across the membranes of plant cells. *Plant Cell.* **11**, 661-675

Clark, K. L., Larsen, P. B., Wang, X., and Chang, C. (1998) Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. *Proc. Natl. Acad. Sci. USA.* **95**, 5401-5406

de Nadal, E., Calero, F., Ramos, J., and Anno, J. (1999) Biochemical and genetic analyses of the role of yeast casein kinase 2 in salt tolerance. *J. Bacteriol.* **181**, 6456-6462

Elledge, S. Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030, November 18, 1993

Epstein, E., Norlyn, J. D., Rush, D. A., Kingsbury, R. W., Kelley, D. B., Cunningham, G. A., and Wrona, A. F. (1980) *Science*. **210**, 399-404

Galvez, A. F., Gulick, P. J., and Dvo9ák, J. (1993) Characterization of early stages of genetic salt-stress responses in salt-tolerant *Lophopyrum elongatum*, salt-sensitive wheat, and their amphiploid. *Plant Physiol.* **103**, 257-265

Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L., and Fink, G. R. (1999) The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proc Natl Acad Sci U S A.* **96**, 1480-1485

Gietz, R. D. and Woods, R. A. (1994) High Efficiency transformation in Yeast. (Invited Book Chapter) In; **Molecular Genetics of Yeast: Practical Approaches**, ed. J. A. Johnston, Oxford University Press pp. 121-134

Görlach, J., Schmid, J., and Amrhein, N. (1993) The 33 kDa protein of the oxygen-evolving complex: a multi-gene family in tomato. *Plant Cell Physiol.* **34**, 497-501

Greenway, H. and Munns, R. (1980) Mechanisms of salt tolerance in non-halophytes. *Annu. Rev. Plant. Phy*siol. **31**, 149-655

Gulick, P. J. and Dvo9ák, J. (1990) Selective enrichment of cDNAs from salt-stress-induced genes in the wheatgrass, *Lophopyrum elongatum*, by the formamide-phenol emulsion reassociation technique. *Gene*. **95**, 173-177

Gulick, P. J. and Dvo9ák, J. (1992) Coordinate Gene response to salt stress in *Lophopyrum elongatum. Plant Physiol.* **100**, 1384-1388

Hardie, D. G. (1999) Roles of the AMP-activated/SNF1 protein kinase family in the response to cellular stress. *Biochem. Soc. Symp.* **64**, 13-27

Holmström, K-O., Somersalo, S., Mandal, A. Palva, T., and Welin, B. (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. *J. Exp. Bot.* **343**, 177-185

Hong, S. W., Jon, J.H., Kwak, J. M., Nam, H. G. (1997) Identification of a receptor-like protein kinase gene rapidly induced by abscisic acid, dehydration, high salt, and cold treatments in *Arabidopsis thaliana. Plant Physiol.* **113**, 1203-12

Hwang, I. and Goodman, H. (1995) An *Arabidopsis thaliana* root-specific kinase homolog is induced by dehydration, ABA, and NaCl. *Plant J.* **8**, 37-43

Ichimura, K., Mizoguchi, T., Irie, K., Marris, P., Giraudat, J., Matsumoto, K., and Shinozaki, K. (1998) Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in *Arabidopsis. Biochem. Biophys. Res. Commun.* **253**, 532-543

Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J-K. (1997) Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell.* **9**, 1935-1949

Iwasaki, T., Yamaguchi-Shinozaki, K., Shinozaki, K. (1995) Identification of a cis-regulatory region of a gene in *Arabidopsis thaliana* whose induction by dehydration is mediated by abscisic acid and requires protein synthesis. *Molec. Gene Genet.* **247**, 391-398

Jain, P. K., Kochhar, A., Khurana, J. P., and Tyagi, A. K. (1998) The psbO gene for 33-kDa precursor polypeptide of the oxygen-evolving complex in *Arabidopsis thaliana* nucleotide sequence and control of its expression. *DNA Res.* **5**, 221-228

James, H. E., Bartling, D., Musgrove, J. E., Kirwin, P. M., Herrmann, R. G., and Robinson, C. (1989) Transport of proteins into chloroplasts. Import and maturation of precursors to the 33-, 23-, and 16-kDa proteins of the photosynthetic oxygen-evolving complex. *J. Biol. Chem.* **264**, 19573-1976

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P. J., Huskisson, N. S., and Hirt, H. (1996) Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci. USA.* **93**, 11274-11279

Knetsch, M. L. W., Wang, M., Snaar-Jagalska, B. E., and Heimovaara-Dijkstra, S. (1996) Abscisic acid induces mitogen-activated protein kinase activation in barley aleurone protoplasts. *Plant Cell.* **8**. 1061-1067

Liu, J. and Zhu, J.-K. (1997) An *arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. *Proc. Natl. Acad. Sci. USA.* **94**, 14960-14964

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell.* **10**, 1391-1406

McGuire, P.E. and Dvorak, J. (1981) High salt-tolerance potential in wheat grasses. *Crop Science.* **21**, 702-705

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., and Kamada, K. S. (1993) ATMPKs: a gene family of plant MAP kinases in *Arabidopsis thaliana. FEBS Lett.* **336**, 440-444

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., and Shinozaki, K. (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated

protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*. **93**, 765-769

Moons, A., Prinsen, E., Bauw, G., and Montagu, M. V. (1997) Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. *Plant Cell*. **9**, 2243-2259

Moran, T. V., and Walker, J. C. (1993) Molecular cloning of two novel protein kinase genes from *Arabidopsis thaliana*. *Biochim Biophys Acta*. **1216,** 9-14

Nakashima, K., Shinwari, Z. K., Sakuma, Y., Seki, M., Miura, S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Organization and expression of two *Arabidopsis* DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. *Plant Molec Biol.* **42**, 657-665

Nanjo, T., Kobayasi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. *Plant J.* **18**, 185-193

Nield, J., Kruse, O., Ruprecht, J., da Fonseca, P., Buchel, C., and Barber, J. (2000) Three-dimensional structure of *Chlamydomonas reinhardtii* and *Synechococcus elongatus* photosystem II complexes allows for comparison of their oxygen-evolving complex organization. *J. Biol. Chem.* **275**, 27940-27946

Ono, T.-A. and Inoue, Y. (1983) Mn-preserving extraction of 33-, 24- and 16-kDa proteins from O_2-evolving PSII particles by divalent salt-washing. *FEBS Lett.*. **164**, 255-260

Patharkar, O. R. and Cushman, J. C. (2000) A stress-induced calcium-dependent protein kinase from *Mesembryanthemum crystallinum* phosphorylates a two-component pseudo-response regulator. *Plant J.* **24**, 679-91

Piao, H. L., Pih, K. T., Lim, J. H., Kang, S. G., Jin, J. B., Kim, S. H., and Hwang, I. (1999) An *Arabidopsis* GSK3/shaggy-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and abscisic acid. *Plant Physiol.* **119**, 1527-1534

Quertermous, T., (1996) Plating libraries and transfer to filter membranes. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., supplement 34

Rokka, A., Aro, E. M., Herrmann, R. G., Andersson, B., and Vener, A. V. (2000) Dephosphorylation of photosystem II reaction center proteins in plant photosynthetic membranes as an immediate response to abrupt elevation of temperature. *Plant Physiol.* **123**, 1525-36

Rubio, F., Schwarz, M., Gassmann, W., and Schroeder, J. I. (1999) Genetic selection of mutations in the high affinity K+ transporter HKT1 that define functions of a loop site for reduced Na+ permeability and increased Na+ tolerance. *J. Biol. Chem.* **274**, 6839-6847

Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning. A Laboratory Manual. Second edition, Cold Spring Harbor Laboratory Press, New York, 1989.

Sanders, D. (2000) The salty tale of *Arabidopsis*. *Curr Biol.* **10**, R486-8

Schroeder, J. I., and Hedrich, R. (1989) Involvement of ion channels and active transport in osmoregulation and signaling of higher plant cells. *Trends Biochem Sci.* **14**, 187-92

Shen, B., Hohmann, S., Jensen, R. G., and Bohnert, H. J. (1999) Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. *Plant Physiol.* **121**, 45-52

Shen, W., Gómez-Cadenas, A., Routly, E. L., Ho, T.-H. D., Simmonds, J. A., and Gulick, P. J. (2001) The salt-stress-inducible protein kinase gene, Esi47, from the salt-tolerant wheatgrass *Lophopyrum elongatum* is involved in plant hormone signaling. *Plant Physiol.* **125**, 1429-1441

Shi, H., Ishitani, M., Kim, C., and Zhu, J. K. (2000) The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. *Proc Natl Acad Sci U S A.* **97**, 6896-6901

Sugihara, K., Hanagata, N., Dubinsky, Z., Baba, S., and Karube, I. (2000) Molecular characterization of cDNA encoding oxygen evolving enhancer protein 1 increased by salt treatment in the mangrove *Bruguiera gymnorrhiza*. *Plant Cell Physiol.* **41**, 1279-1285

Taiz. L. (1992) The plant vacuole. *J. Exp. Biol.* **172**, 113-122

Waegemann, K. and Soll, J. (1996) Phosphorylation of the transit sequence of chloroplast precursor proteins. *J. Biol. Chem.* **271**, 6545-6554

Walker, J. C. (1994) Structure and function of the receptor-like protein kinases of higher plants. *Plant Molec. Biol.* **26**, 1599-1609

Wilson, I., Vogel, J., and Somerville, S. (1997) Signalling pathways: a common theme in plants and animals? *Curr. Biol.* 7, R175-R178

Xiong, L., Ishitani, M., Zhu, J-K. (1999) HOS5-a negative regulator of osmotic stress-induced gene expression in *Arabidopsis thaliana*. *Plant Physiol.* **119**, 205-211

Yamamoto, Y., Ishikawa, Y., Nakatani, E., Yamada, M., Zhang, H., and Wydrzynski, T. (1998) Role of an extrinsic 33 kilodalton protein of photosystem II in the turnover of the reaction center-binding protein D1 during photoinhibition. *Biochemistry.* **37**, 1565-74

Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1997) Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.* **38**, 1095-1102

Zhou, J., Loh, Y., Bressan, R. A., and Martin, G. B. (1995) The tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell.* **83**, 925-935

Zhou, J., Tang, X., and Martin, G. B. (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *Embo J.* **16**, 3207-3218

Zhu, J. K., Liu, J., Xiong, L. (1998) Genetic analysis of salt tolerance in *arabidopsis*. Evidence for a critical role of potassium nutrition. *Plant Cell.* **10**, 1181-91