

**Isolation and Sequencing of a Genomic Clone of ESI28: an
Early Salt-stress Induced Gene of the Salt-Tolerant Wild Wheatgrass
*Lophopyrum elongatum***

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ABSTRACT

Isolation and Sequencing of a Genomic Clone of ESI28: an Early Salt-stress Induced Gene of the Salt-Tolerant Wild Wheatgrass *Lophopyrum elongatum*

Amani Abu-Idrees

A genomic sequence corresponding to a putative actin-depolymerizing factor (ADF) was isolated from a wheat (*Lophopyrum elongatum*) genomic library. The genomic clone, designated ESI28-G-5, codes for the ESI28 gene (early salt-stress induced). The 5'-upstream sequence contains a putative TATA box and abscisic acid (ABA) response element (ABRE). The genomic clone contains a long open reading frame (ORF) which encodes a polypeptide of 143 amino acids with a predicted molecular mass of 15.95 kDa. The protein has two conserved domains identified as actin and phosphatidylinositol 4,5-bisphosphate (PIP₂) binding sites which are commonly found in members of the cofilin family. A search of DNA sequence databases indicated that the ESI28 sequence had the highest degree of sequence similarity to the *Triticum aestivum* low temperature regulated gene (Wcor719) which encodes an actin-binding protein (ABP). The induction of ESI28 by salt, the presence of ABRE in its promoter region, and the significant homology with ABP from different organisms suggest that this gene might be involved in reorganizing the cytoskeleton during water deficit stress. It may also serve as an essential factor in the salt stress signal transduction pathway that is regulated by ABA.

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ABBREVIATIONS

ABA: Absciscic acid.

abi: ABA-insensitive loci.

ABP: Actin-binding protein.

ABRE: Absciscic acid responsive element.

ADF: Actin depolymerizing factor.

BLAST: Basic Local Alignment Search Tool.

bp: Base pair.

CAM: Crassulacean acid metabolism.

cDNA: Complementary deoxyribonucleic acid.

CE: Coupling element.

cpm: Count (s) per minute.

dATP: 2' -Deoxyadenosine 5' -triphosphate.

dCTP: 2' -Deoxycytidine 5' -triphosphate.

dGTP: 2' -Deoxyguanosine 5' -triphosphate.

DNA: Deoxyribonucleic acid.

DRE: Dehydration-responsive element.

dTTP: 2' -Deoxythymidine 5' -triphosphate.

EmBp-1: Em-binding protein-1 (Wheat-leucine-zipper protein).

ESI: Early salt-stress-induced.

F-actin: Actin filaments.

G-actin: Globular actin.

Gpd1: Glyceraldehyde-P-dehydrogenase-1.

H⁺-ATPase: H⁺-adenosine triphosphatase.

H⁺-PPase: H⁺-pyrophosphatase.

Imt1: myo-inositol-O-methyl transferase-1.

lea, LEA: Late-embryogenesis-abundant.

Mdh1: Malate dehydrogenase-1.

Mod1: Malic enzyme-1.

mRNA: Messenger ribonucleic acid.

Na⁺-ATPase: Sodium pump ATPase

ORF: Open reading frame.

PCR: Polymerase chain reaction.

PEPCase: Phosphoenolpyruvate carboxylase.

pfu: Plaque forming unit.

P5CR: -Pyrroline-5-carboxylate reductase.

P5CS: -Pyrroline-5-carboxylate synthetase.

PI: Phosphatidyl inositol.

PIP: Phosphatidyl inositol-4-phosphate.

PIP₂: Phosphatidyl inositol-4,5-biphosphate.

PK: Protein kinase.

PMF: Proton-motive-force.

Rab: Responsive to ABA.

RNA: Ribonucleic acid.

Ta: Annealing temperature.

Tm: Melting temperature.

UV light: Ultraviolet light.

2-D gel electrophoresis: Two-dimensional gel electrophoresis.

PART I: INTRODUCTION

Plants are often exposed to diverse ecological conditions such as drought, temperature fluctuation, and saline soils which often have a detrimental influence on plant growth and survival. Of the world's 14 billion hectares of land, only 20%, or approximately 3.2 billion hectares, are considered arable or potentially arable land suitable for reasonable crop production (Nabors, 1983). Much of the remaining land is exposed to adverse conditions which make the establishment and maintenance of economically important crop species virtually impossible. These marginal lands experience mineral stress, drought, excess water, shallow soil or permanent freezing. Nearly 1.0 billion hectares of potentially arable land have salt toxicity mainly due to excess sodium. This is about 30% of the current or potential crop land. Sodium toxicity represents a serious agricultural problem especially in arid irrigated and estuarine areas. The build up of salt by irrigation systems limits the useful agricultural life of these regions. It is suggested that the demise of ancient Sumer in the southern Mesopotamian plain resulted in part in the gradual salinization of fields by irrigation water from the Tigris River (Nabors, 1983). Much effort has been directed toward understanding salt tolerance in plants, in order to improve agricultural production under saline conditions.

Enhancing salt tolerance in crops is an important objective for irrigated agriculture in areas subjected to or endangered by soil salinization. Genetic breeding and selection for stress tolerance is a major objective in crop improvement and it has been remarkably successful for many stresses but the selection for salt tolerance has met with limited success due mainly to the complex nature of the trait (Bohnert *et al.*, 1992 and Bray, 1997). Elucidation of the molecular and biochemical basis of salt tolerance can make a significant contribution to crop improvement by identifying both targets for genetic

engineering and for selection by classical breeding methods (Bohnert *et al.*, 1992 and Bray, 1997).

High salinity, drought and low temperature share a common feature in that they all cause reduced water potentials in plants and, therefore, are all osmotic stresses. Morphological, physiological and biochemical changes have been observed in plants when exposed to high salinity or osmotic stresses. These changes may contribute to adaptation that occur in both facultative halophytes which can grow in both saline and non-saline conditions, and in glycophytes which usually can not survive in high salinity. Therefore, it is important to identify genes encoding the physiological traits that can confer improved salt tolerance to the glycophytic plants.

Exposure of plant cells to a high concentration of salts leads to ionic imbalance, reduction of water potential, ion toxicity and metabolic disturbances (Kingsburg and Epstein, 1986). These cells would either cope with the stress or fail to tolerate the salt stress. To cope with the salt stress and become salt tolerant, halophytes exhibit several mechanisms. One of these mechanisms leads to the regulation of ion uptake and compartmentation (Binzel *et al.*, 1988). This involves selective ion accumulation in the cytoplasm (K^+) or in the vacuole (Na^+ and Cl^-) (Niu *et al.*, 1995). Thus, the cytoplasmic Na^+ concentration is reduced while the cellular ion homeostasis is maintained. Transport of Na^+ and K^+ are probably mediated by ion transporters and ion channels in plasma membranes and tonoplast membranes with selective activities for Na^+ and K^+ (Niu *et al.*, 1995). Plants may also exhibit differential salt stress response in different tissues as observed in the wheat x *Lophopyrum elongatum* amphiploid, in which young leaves maintain low Na^+ and high K^+ levels and accumulate glycine betaine, while old leaves have higher Na^+ levels (Colmer *et al.*, 1995).

Another mechanism by which plants adapt to high salt stress is the

accumulation of organic osmolytes such as polyols, mannitol, sorbitol, proline and sucrose (Delauney and Verma 1993). It has also been found that plants respond to high salinity or osmotic stresses with an increase in the levels of the plant hormone, abscisic acid which causes reduced water loss through transpiration (Skriver and Mundy, 1990).

Molecular investigations will help understand the biochemistry and genetics of stress response and adaptation and will aid in the identification of genes which are responsible for stress-tolerance. The molecular genetic approach to understanding the genetics of salt tolerance has included three broad areas including the study of: (1)- genes that are implicated in physiological models of stress tolerance, such as ion transport and the synthesis of small organic molecules that are thought to act as osmolites or osmoprotectants, (2)- genes whose expression has been shown to be regulated by stress, though, at least initially, their metabolic function is not clear and (3)- genes that are implicated in signal transduction related to the response to salt and other environmental stresses. In the last ten years, the molecular genetic investigations have shown that many genes are regulated by high salinity and osmotic stresses. Gulick and Dvorak (1990) isolated a number of partial length cDNA clones of early-salt-stress induced (ESI) genes which will be mentioned in detail in other sections.

PHYSIOLOGICAL RESPONSES TO SALT STRESS

A. Synthesis of Organic Osmolytes

The induction of osmolyte biosynthesis is observed in plants exposed to drought and salt stress. These molecules function in two ways, in osmotic adjustment and osmoprotection. They act as osmolytes in maintaining osmotic balance between the cytoplasm and the vacuole and allow sodium sequestration to the vacuole or apoplast. They also are thought to function as osmoprotectants

for the cellular structures, which might be accomplished by their interactions with membranes or cytoplasmic proteins (Bohnert, 1995 and references therein).

The organic osmolytes such as amino acids (proline), quaternary ammonium compounds (glycine betaine), sugars (fructans) and sugar alcohols (mannitol, sorbitol and pinitol) have been related to adaptation to osmotic stress. During salt stress, synthesis of such osmolytes is elevated, resulting in the decrease of the osmotic potential and the maintenance of a favorable water potential to protect the cellular turgor (Delauney and Verma, 1993). Among the compatible organic solutes, proline is the most widely distributed osmolyte (Delauney and Verma, 1993). The production of proline is catalyzed by two enzymes P5C synthetase (P5CS) and P5C reductase (P5CR). In *Arabidopsis*, the expression of P5CS gene is induced by salt stress and dehydration (Bray, 1997). A number of studies showed that P5CS is a rate limiting enzyme in the biosynthesis of proline in plants, and transgenic plants which overexpressed P5CS overproduced proline and showed enhanced osmotolerance (Kavi-Kishor *et al.*, 1995).

B. Ion Transport

The physiological systems that are involved in salt tolerance in many other species include ion transport at both the plasmalemma and the tonoplast. The enhanced expression of a number of genes is required to adapt these physiological systems for function under salt stress. Transport of ions through channels in the cell membrane is dependent on membrane voltage and the concentration differences of the ions across the membrane. In higher plants anion channels play important roles in controlling cellular functions including turgor, maintenance of osmoregulation, anion transport, stomatal movement, and signal transduction. Ion transport across plant cell membranes occurs either passively or

actively depending on the electrical gradient or membrane potential and the chemical gradient (Niu *et al.*, 1995). When the transport of ions proceeds down the thermodynamic gradient it is passive, whereas transport against the gradient is active.

Ion transport is usually mediated by proteins responsible for ion flux which are categorized as pumps, carriers and channels (Niu *et al.*, 1995). Pumps utilize metabolic energy for vectorial transport, whereas carriers couple uphill transport of one solute to the downhill movement of another, either in the same (symporter) or opposite (antiporter) direction (Niu *et al.*, 1995). Channels, on the other hand, mediate passive transport (movement down a free energy gradient).

Under homeostatic conditions, the physiological concentrations in the cytosol are 100-200 mM for K^+ , 1-10 mM for Na^+ and Cl^- and 100-200 nM for Ca^{+2} (Binzel *et al.*, 1988). The K^+ homeostasis is controlled by K^+ channels and a high affinity K^+ - H^+ symporter, whereas Na^+ and Ca^{+2} uptake occurs passively across the plasma membrane and efflux is assumed to be controlled by the activities of Na^+ / H^+ antiporters and Ca^{+2} -ATPase, respectively. Cl^- uptake is assumed to be coupled to a H^+ symporter (Niu *et al.*, 1995). K^+ channels are also involved in regulating the closure of the stomata; this is mediated by K^+ and anion release from guard cells (Skerett and Tyerman, 1994). It has been found that in root cortical cells there are anion-influx channels and these channels are activated by membrane depolarization and by extracellular Cl^- . It is thought that these anion channels may be important for membrane potential regulation and Cl^- uptake in response to salt stress (Skerett and Tyerman, 1994).

In comparisons of salt tolerant and salt sensitive genotypes, derived from wheat and wild wheat grasses Omielan *et al.* (1991) demonstrated that the biomass and grain yield of plants exposed to salt stress are correlated positively with high K^+/Na^+ ratios. This correlation suggests that the accumulation of K^+

and the exclusion of Na^+ are related to the salt tolerance of the plant and are controlled dominantly by loci on the chromosomes of the wild wheat grass, *Lophopyrum elongatum* (Dvorak *et al.*, 1991).

It has been found that the single locus, Kna1, from *Triticum aestivum* controls Na^+ exclusion and K^+ accumulation when it placed in a *Triticum durum* genetic background. Under salt stress, this locus contributes to higher grain yields. Studies on this locus have shown that Na^+/K^+ is related to tolerance of salt stress (Dvorak *et al.*, 1994).

During the period of NaCl stress adaptation, the most important step is that Na^+ and Cl^- must be evacuated from the cytosol by efflux to the apoplast and to the vacuole, and this is achieved by active antiporters (Barkla and Pantoja, 1996). The translocation of Na^+ into the vacuole will result in the avoidance of the cytoplasmic Na^+ toxicity and maintenance of high cytoplasmic Na^+/K^+ ratio (Niu *et al.*, 1995). At the same time the vacuolar Na^+ will serve as an osmoticum necessary for cellular water homeostasis. In plants, several Na^+ and K^+ transport proteins have been identified and characterized physiologically and chemically and the genes encoding some of these proteins have been cloned (Niu *et al.*, 1995).

H^+ -ATPases

There are a number of proton pumps that have been characterized in plants, these include H^+ -adenosine triphosphatases (H^+ -ATPase) located in the plasma membrane and tonoplast, H^+ -pyrophosphatase (H^+ -PPase) located in the tonoplast, Ca^+ -ATPase located in the ER and Na^+ -ATPase located in the plasma membrane (Niu *et al.*, 1995). They all function by generating a proton-motive-force (PMF) by hydrolysing phospho-diester bonds of either ATP or pyrophosphate and thus transferring the driving energy for a wide range of

secondary active and passive transport processes. They also function in maintaining cellular pH (Barkla and Pantoja, 1996).

Ion Co-transporters

Ion co-transporters that have been identified include the Na^+/H^+ antiporter and K^+/H^+ symporter, which are both located in the plasma membrane (Niu *et al.*, 1995). These are likely to play an important role in salt tolerance in view of the observation that salt tolerant species and genotypes are able to maintain higher cytosolic K^+/Na^+ ratios than salt sensitive species, when they are subjected to salt stress (Gorham *et al.*, 1991 and references therein).

K^+ Channels

K^+ channels play a role in regulating both the influx and efflux of K^+ from cells, their activity is known to be changed by water relations and turgor. At least one K^+ channel has been identified which serves as an example of K^+ channels located in the plasma membrane (Niu *et al.*, 1995).

GENES ACTIVATED IN SALT STRESSED PLANTS

Late Embryogenesis Abundant (Lea) Proteins

The accumulation of a set of proteins and their corresponding mRNAs referred to as Late-Embryogenesis-Abundant (Lea) is a characteristic feature of seed desiccation (Galau *et al.*, 1986). The induction of expression occurs at the same time as the internal levels of ABA peak in the immature embryos (Dure, 1993). These genes appear to be regulated by mRNA levels during embryogenesis because the message levels increase at the same time as the increase of protein levels. They are also induced to high levels of expression in

other tissues, at other times by ABA, desiccation or osmotic stress (Galau *et al.*, 1986).

It has been hypothesized that these proteins may have a protective role in plant cells and may function as osmoprotectants for cellular structures during desiccation and that this protection is important for the survival of the plant under stress conditions. However a clear role for these genes in stress tolerance and adaptation remains unclear, although there is a correlation between their expression and the stress tolerance in a number of plants (Galau *et al.*, 1992). A recent report showed that transgenic rice expressing a group 3 Lea gene derived from barley showed tolerance to water deficit and salt stress (Xu *et al.*, 1996).

According to the primary structures of their gene products the Lea genes are divided into several groups:

Group 1 includes LeaD19, LeaD32L in cotton, and B19s in barley. Their products are hydrophilic. The expression of these genes can be induced by salt desiccation and mannitol treatment and contain a conserved 20- amino acid motif which is rich in glycine and charged amino acids (Galau *et al.*, 1992).

Group 2 includes Rab16A-D (responsive to ABA in rice), LeaD11 in cotton and dehydrins (dhns) in barley (Close *et al.*, 1993, Dure, 1993 and Yamaguchi-Shinozaki and Shinozaki, 1994). These proteins are hydrophilic, rich in threonine and glycine and characterized by the lysine-rich repeating amino acid sequence motif KIKEKLPG (Roberts *et al.*, 1993). Most of the genes of this group are widely induced by ABA and osmotic stresses such as NaCl treatment and dehydration.

Group 3 includes Lea-D7 and functions in resisting water deficiency. The amino acid sequence of its motif is T/A A/T Q/E A/T A/T K/R Q/E/D K/R A/T X E/D/Q (Dure, 1993). Other Lea genes products can be subdivided as hydrophobic or hydrophilic. The hydrophilic group includes LeaD113 (in

cotton), Lea25 (in tomato), and Hads11 (in sunflower) (Almoguera and Jordano, 1992) as well as group 1, 2 and 3. The hydrophobic group includes LeaD34 (in cotton), Rab28 (maize), and ECb31 (carrot) (Almoguera and Jordano, 1992).

INTERACTIONS OF CELLULAR SIGNALS WITH SALT STRESS RESPONSES

The cell is continuously altered by developmental and environmental events. In a stimulus-response coupling model, a stimulus is detected by the cell, a signal is generated and transmitted, and a biochemical change is instigated. The signal transduction usually requires the recognition of the stimulus by a receptor and the subsequent use of chemical second messengers and/or effector proteins to transmit a signal that will then trigger the appropriate response. Signal transduction can be characterized by its speed, sensitivity, specificity and its control by a complicated and specific network of pathways of positively and negatively acting elements. These properties are important for signal transduction propagation to ensure an appropriate, quantitatively and correctly timed response that is highly coordinated with other activities of the cell (Bowler and Chua, 1994).

Plant Stress and Absciscic Acid

ABA is a phytohormone that is involved in bud dormancy and leaf abscission. ABA levels increase during vegetative growth in tissues subjected to osmotic stress. Exogenous ABA can induce the expression of the genes which are inducible by environmental stresses (Skriver and Mundy, 1990), therefore, ABA is thought to play an important role in responses to environmental stresses such as osmotic and temperature extremes. Because elevated ABA levels occur very early in these responses, ABA may act as an internal signal to initiate the

stress response (Skriver and Mundy, 1990 and references therein). Though it is not clear how plant cells perceive high salinity or water deficiency, two hypotheses have been proposed to explain the role of salt and ABA in gene expression. The first assumes that Na^+ influx acts directly to alter cell structure through the osmotic or ionic changes induced by the stress. The second assumes that Na^+ influx acts indirectly via mediators like ABA through signal transduction (Thomas *et al.*, 1992). There is evidence that ABA-receptors may exist since binding of ABA shows saturable kinetics (Hornberg and Weiler, 1984). Secondary messengers seem to participate in the ABA action as well; in guard cells, stress- or ABA-evoked stomatal closure mediated by the net efflux of K^+ . This requires both the elevation of cellular Ca^{2+} concentration for the inhibition of the inward rectifying K^+ channels and the Ca^{2+} -independent increase of cytoplasmic pH for the activation of the outward rectifying K^+ channels (Giraudat *et al.*, 1994). The characterization of biosynthetic mutants has demonstrated that endogenous ABA is required for the regulation of numerous genes by salt, drought and cold (Shinozaki and Yamaguchi-Shinozaki, 1996). Biochemical analysis of mutants that are deficient in ABA has facilitated the elucidation of the biosynthetic pathway of ABA in higher plants. Most of these mutants have been isolated on the basis of their wilted phenotype or seed dormancy characteristics (Koornneeff, 1986 and references therein).

Genetic analysis of *A. thaliana* and maize (*Zea mays*) helped in understanding the ABA biosynthesis, physiology and signal transduction. Embryos of the ABA-biosynthetic vp mutants of maize vp2, vp5, vp7, and vp9, are blocked in early steps of carotenoid biosynthesis. (Marin *et al.*, 1996 and McCarty, 1995). aba mutants from *Arabidopsis* (aba1 and aba2, aba3), have been identified among the revertants of non-germinating gibberellin-deficient mutants (Leon-Kloosterziel *et al.*, 1996). Leon-Kloosterziel *et al.*, (1996) found that both

aba2 and aba3 mutants had reduced seed dormancy, lower endogenous ABA levels than the wild-type, and suffer excessive water loss.

Mutations that affect ABA signaling by either decreasing or increasing the ABA sensitivity have also been characterized in *Arabidopsis*. The ABA-insensitive mutants helped to identify genes participating in ABA signaling through cloning of the four ABA-insensitive loci (abi1, abi2, abi3, and abi4) (Merlot and Giraudat, 1997). ABI1 and ABI2 from *A. thaliana* are the genes for 2C-type Ser/Thr protein phosphatases containing an EF-hand Ca²⁺-binding site (Leung *et al.*, 1994; Leung *et al.*, 1997). ABI1 and ABI2 have very high sequence similarity and may share common functions in the signaling pathway of ABA or alternatively, each of these proteins may have a specific function through the ABA signaling pathway by interacting with specific elements and/or substrates through their N-termini (Merlot and Giraudat, 1997). The ABI3 gene is the ortholog of the maize VP1 (Giraudat *et al.*, 1992); and by homology, ABI3 is thought to be a transcriptional activator (McCarty *et al.*, 1991).

ABA Responsive Genes

Multiple ABA-responsive genes have been isolated and their analysis has provided insights into the biological function of the encoded proteins as well as the nature of the cis and trans-acting factors involved in ABA-responsiveness (Ingram and Bartels, 1996). Stress induced genes are divided into three groups: 1) primary ABA-induced genes, the expression of which is independent of protein synthesis (they do not require the expression of other genes); 2) ABA-induced genes whose expression is dependent on protein synthesis; and 3) stress-induced genes whose induction is independent of increases in ABA levels (Quatrano *et al.*, 1997).

A number of stress- and ABA-responsive elements in the promoter regions of ABA- or stress-regulated genes

have been identified. These elements are part of the terminal components of the signal transduction pathways. The rice Rab16A gene promoter and the wheat Em promoter contain G-box-like elements, which have a core sequence of C/TACGTGG and confer ABA-responsiveness (ABREs) (Skriver *et al.*, 1991; Marcotte *et al.*, 1989). The ABRE is required for the ABA-responsive expression of certain genes that have been identified in wheat (Em) (Marcotte *et al.*, 1989), rice (rab16) (Mundy *et al.*, 1990), maize (rab17 and rab28) (Pla *et al.*, 1991), and barley (HVA22) (Shen and Ho, 1995). It is now clear that in some genes, for example, barley HVA22 (which encodes a potential regulatory protein), the G-box like element is necessary but not sufficient, and a coupling element is required to form a responsive complex for ABA-induction (Shen and Ho, 1995).

In *A. thaliana*, the rd29A gene has two cis-acting elements; the first element which is necessary for the dehydration response has been shown to be independent of ABA. This element is referred to as a dehydration response element (DRE) and is composed of a 9-bp sequence (TACCGACAT) (Yamaguchi-Shinozaki and Shinozaki, 1994). The second element is responsible for or participates in the slower ABA response which is mediated by another fragment that contains an ABRE-like sequence in its promoter (Yamaguchi-Shinozaki and Shinozaki, 1994; Ingram and Bartels, 1996). In HVA22, rd29A and probably many other genes, multiple cis-acting complexes are present in the promoter regions with differential responsiveness to different environmental conditions. For example the coupling element (CE1) acts together with a G-box-type ABRE in showing high ABA induction, whereas the ABRE alone is not sufficient for transcriptional activation (Ingram and Bartels, 1996).

Binding of nuclear proteins to the cis-elements or to the promoter regions has been reported for many ABA or stress regulated genes. In the Em gene, the wheat-leucine-zipper protein (EmBp-1) binds specifically to the G-box-like

ABRE although it is not known if it confers ABA-induction *in vivo* (Guiltinan *et al.*, 1990). EmBp-1 belongs to the family of bzip transcription factors which contain a basic DNA-binding domain and a leucine zipper domain for dimerization. It is not clear how the expression of the gene encoding Embp-1 is regulated. On the other hand, the *A. thaliana* Atmyb2 gene encodes a MYB-related protein and is induced by dehydration, salt stress and by ABA (Urao *et al.*, 1993). Another *A. thaliana* gene rd22 has a promoter with no ABRE, but with two recognition sites for the transcription factors MYC and MYB (Ingram and Bartels, 1996). These transcription factors are likely to be components of the signal transduction pathways leading to the expression of the ABA or stress responsive genes.

Protein Kinases

The protein kinases (PK) are especially important components in signal transduction. Gene expression in cells can be regulated by phosphorylation of transcription factors that is catalyzed by protein kinases, which are in turn activated by phosphorylation or dephosphorylation stimulated by a signal cascade beginning from a receptor protein kinase. Based on their ability to phosphorylate specific amino acid residues, protein kinases are divided into two major groups: 1- protein serine/threonine kinases, which can phosphorylate both serine and threonine residues. 2- protein tyrosine kinases which phosphorylate tyrosine residues (Hanks and Quinn, 1991).

Four protein kinase genes have been reported to be up-regulated by ABA, dehydration, NaCl and low temperature. These include the wheat gene PKABA1 (Holappa and Walker-Simmons, 1995), the *Arabidopsis thaliana* PKS, ATCDPK1 and ATCDPK2 genes (Urao *et al.*, 1994), which requires Ca^{+2} for activation of

kinase activity, and a gene from *A. thaliana*, ARSK1 which shows root-specific expression (Hwang and Goodman, 1995).

ACTIN and ACTIN-DEPOLYMERIZING FACTOR

Actin filaments perform essential functions in eukaryotic cells, they are responsible for cytoskeletal elements, for the structural integrity of the cell, and for many of the cellular movements including cytokinesis, cytoplasmic streaming and protein organelle interactions (Reisler, 1993). Actin is found in the cytoplasmic compartment of cells. The actin monomers, globular actin (G-actin), assemble in cells to form actin polymers; F-actin (actin filaments) assembles into microfilaments (Hennessey *et al.*, 1993). In plants, the F-actin filaments are arranged in specific arrays under the plasma membrane with functions that are unique to the plant cytoskeleton (Ruhlandt *et al.*, 1994). The assembly of actin filaments is regulated by numerous types of actin-binding proteins (ABPs) such as capping proteins, monomer-binding proteins, and side-binding proteins (Staiger *et al.*, 1997). The turnover of actin filaments and the properties of ABP that determine the actin cytoskeleton organization are modulated by Ca^{2+} , pH, and phosphorylation (membrane polyphosphoinositols) (Staiger *et al.*, 1997).

In plant cells, actin filaments have been implicated in several cellular processes including division, light-induced plastid migration, differentiation, wound repair, and response to pathogen attack, and more recently they have been reported to be involved in the regulation of K^+ ion channels (Hwang *et al.*, 1997). The plant cell contains a dynamic cytoskeleton that re-modulates cell architecture and motility in response to external and internal stimuli (Staiger *et al.*, 1997). For example, when *Vallisneria gigantea* cells are exposed to low light intensity, they change their chloroplast position in order to maximize their exposure to light (Staiger *et al.*, 1997 and references therein).

Reorganization of actin is observed in response to wounding and pathogen attack in the leaf epidermal cells of *Tradescantia albobittata*. When these cells are wounded, the actin filaments in neighbouring cells re-orient and take the form of cortical actin bands parallel to the wound within 30 min. This event modulates the tissue to prepare for another cell division to exclude the wounded region (Staiger *et al.*, 1997).

Cofilin is a member of the cofilin-actin depolymerizing factor (actin-ADFs) family of small ABP (15-20 kDa). It is widely distributed and identified as an actin monomer-binding protein. These proteins bind to G-actin and F-actin. Cofilin depolymerizes the F-actin in a pH-dependent manner and is found in most eukaryotic cells (Yonezawa *et al.*, 1991, Hartwig, 1994). The interaction of cofilin with both G-actin and F-actin can be inhibited by the phosphatidylinositides such as phosphatidyl inositol 4,5-bisphosphate (PIP₂), Phosphatidylinositol 4-phosphate (PIP) and phosphatidyl inositol (PI). These phosphatidylinositides are protected from hydrolysis by phospholipase C by binding to cofilin (Yonezawa *et al.*, 1991). The interaction of cofilin with G- and F-actin may be affected by signal transduction pathway in a cofilin-dependent manner which is conversely modulated by cofilin (Aizawa *et al.*, 1995).

The cofilin and related proteins such as destrin, human ADF, chicken cofilin and other ADFs, lily ADF, and yeast cofilin are widely distributed among eukaryotes (Aizawa *et al.*, 1995). The expression of mammalian cofilin or destrin in mutant yeast rescues these cells from lethality which indicates that cofilin function is conserved among eukaryotes (Aizawa *et al.*, 1995).

A number of plant genes were found to encode actin-binding proteins including the BMP1 from *Brassica napus* which is expressed specifically in mature anthers and codes for Actin Depolymerization Factor (ADF). This putative ADF may play an important function in anther (Kim *et al.*, 1993). LMP131A, a

gene from *Lilium longiflorum*, encodes ADF and is expressed in mature pollen (An, 1993). AtADF1 and AtADF2 from *A. thaliana* encode ABP (Newman *et al.*, 1994). ZmABP1 and ZmABP2 from *Zea mays* are involved in germinating pollen and encode ADFs which may be involved in pollen actin reorganization (Rozycka *et al.*, 1995; Lopez *et al.*, 1996). Recently, a gene from *T. aestivum* which is cold inducible (Wcor719) has been identified and reported to encode for an actin-binding protein. The high amino acid similarity of this gene to different ABP from different organisms suggests that the product of this gene may be involved in the reorganization of the cytoskeleton under cold stress (Danyluk *et al.*, 1996).

THE GENETIC RESPONSE TO SALT STRESS OF *Lophopyrum elongatum*

Some species of wild wheatgrass, which are phylogenetically related to cultivated wheats, are highly salt tolerant. Among them is the diploid species *L. elongatum* (host) A.love (syn. *Elytrigia elongata* [Host] Nevski, *Agropyrum elongatum* Host, (2n=2x=14)), which is commonly known as tall wheat grass (McGuire and Dvorak, 1981). *L. elongatum* naturally occurs in salt marshes in the Mediterranean region. It has been hybridized with a salt-sensitive bread wheat *T. aestivum* L. (2n=6x=42) cultivar Chinese Spring to generate a stable octoploid amphiploid (2n=8x=56) (Omielan *et al.*, 1991). The latter is more salt tolerant than *T. aestivum* when tested in hydroponic culture and in saline field conditions indicating the inheritance of the genetic character of salt tolerance in *L. elongatum* and that the character is dominant over sensitivity (Dubcovsky *et al.*, 1994). The *L. elongatum* genome was also shown to contribute Na⁺, K⁺, Cl⁻, Mg⁺² and SO₄⁻² tolerance to the *L. elongatum* x Chinese Spring amphiploid (Dvorak and Ross, 1986). This makes *L. elongatum* an excellent material to

study how plants tolerate saline environments, and such studies could be significant for the improvement of cultivated cereals. Exposure of the plants to mild NaCl stress enhances their tolerance to high levels of NaCl. This acclimation suggests the possible requirement of induction of gene expression for growth to occur under salt stress. Analysis by two-dimensional gel electrophoresis of *in vitro* translation products of poly-A RNA from amphipoid (salt tolerant) and Chinese Spring (sensitive) plants showed the root-specific accumulation of differentially expressed messages from the salt-tolerant genotype when it was stressed for seven days with 250mM NaCl (Gulick and Dvorak, 1987). No changes were observed in the translatable mRNA population from leaves. Since *L. elongatum* is a salt excluder, the subsequent studies on its genetic response to salt stress were mainly focused on the roots. A cDNA subtractive-enrichment method was developed to selectively isolate cDNA clones of the differentially expressed genes from *L. elongatum* roots after exposure to salt stress for six hours (Gulick and Dvorak, 1990).

Eleven partial cDNA clones representing eleven different genes or gene families were isolated and showed increased steady-state mRNA levels in response to salt stress (Gulick and Dvorak, 1992). These clones were used as probes to characterize their temporal gene expression in roots after initiation of salt stress (250mM NaCl for 2, 6, 12, 24 h, and 3 and 7 days). All eleven genes were induced after the first two hours of stress and reached peak expression after about six hours. All the genes are induced only in roots except ESI15 which is also induced in leaves. Two groups of genes were distinguished by their mRNA decline one group reached the basal level by 24 h and the other group required between 3 and 7 days to return to the level of the controls. These genes were referred to as early salt-stress-induced (ESI) genes. The expression of the eleven clones in the three genotypes (*L. elongatum*, salt-sensitive wheat, and the

amphiploid which was derived from a cross between these two species) was compared in plants exposed to salt stress. This study showed that there is a positive correlation between the expression of these genes and salt tolerance.

The response of these genes could be duplicated to the approximate level of NaCl induction, when ABA was applied exogenously to the three genotypes. Moreover, both KCl and mannitol were as effective as NaCl to induce the ESI genes (Galvez *et al.*, 1993). This indicates that the induction of these genes may represent a general response to osmotic stress and such response may be mediated by ABA.

The full-length cDNA clones for the genes ESI3 (Gulick *et al.*, 1994), ESI35 (Gulick and An, 1993), ESI47 and four members in the ESI18 multigene family (Shen and Gulick, pers. com.) have been isolated. ESI35 encodes a hydrophilic protein of 253 aa. The amino acid composition includes 17% glutamic acid, 14% lysine, 8% alanine and contains no cystine or tryptophan residues. Amino acid sequence shows high localized similarity to conserved lysine-rich and serine-repeat regions of dehydrin genes (Gulick and An, 1993). ESI3 encodes a putative hydrophobic protein of 54 aa with two potential transmembrane domains (Gulick *et al.*, 1994). It has identical a.a sequence and 92% nucleotide sequence identity to the barley blt101 gene product, a low-temperature-inducible gene (Goddard *et al.*, 1993 and Gulick *et al.*, 1994). The aa composition includes 19% leucine, 17% valine and 15% isoleucine and contains no histidine, glutamine, asparagine or aspartic acid residues. This protein is thought to be a membrane-integrated protein that may play a role in membrane function or in the maintenance of membrane integrity in water-stressed tissues (Gulick *et al.*, 1994). ESI18 was found to be homologous to dehydrin genes (Gulick and Dvorak, 1992). ESI18 multigene family includes ESI18-1, ESI18-2, ESI18-15, ESI18-17, and ESI18-10 (Shen, 1994). A positive correlation

between the expression of the dehydrin-like genes and salt-tolerance among different *Lophopyrum* species has been observed (Shen, 1994). The ESI47 gene encodes a ser/thr protein kinase. Partial sequences of cDNAs for other ESI genes revealed that ESI14 encodes a cysteine protease; ESI48 encodes an aspartate amino transferase; and ESI2 encodes a proline-rich protein. ESI2 is of particular interest in that this is the only ESI gene showing differential regulation by NaCl and KCl (Galvez *et al.*, 1993).

The genetic organization of *L. elongatum* and related wheat species are very similar, and the ESI genes have been mapped in wheat. Nine genes complementary to clones ESI4, ESI14, ESI15, ESI28 and ESI32 were found in homologous chromosomal group 5. Chromosome group 6 contains those genes complementary to ESI18 and ESI35. Chromosome group 1 contains the ESI47 gene, chromosome group 3 contains the ESI48 gene, chromosome group 4 contains the ESI3 gene and chromosome group 7 contains the ESI2 gene. These genes exist in a single-copy in *L. elongatum* except ESI2 and ESI18 which are present in more than one copy (Dubcovsky *et al.*, 1994). These results indicate that some changes must have taken place in *L. elongatum* ESI genes relative to wheat genes which cause higher expression under salt stress (Dubcovsky *et al.*, 1994).

ESI28 GENE:

The ESI28 gene, the subject of the work presented here, is a single copy salt-induced gene in *L. elongatum*. The induction of this gene in the roots of the salt-tolerant *L. elongatum* by 250mM NaCl is 2.69 fold; whereas in the salt-sensitive wheat it is only 1.31 fold (Galvez *et al.*, 1993). The originally cloned cDNA is 179bp in length, apparently a partial cDNA clone (Gulick and Dvorak, 1992).

The objectives of the work presented here were to obtain the genomic clone and sequence of ESI28. This will provide an opportunity to predict the amino acid sequence and the identity of its gene product, and to identify possible regulatory elements from the gene promoter. Work presented here shows that ESI28 encodes an actin depolymerizing protein.

PART II. MATERIAL AND METHODS

Screening of Genomic Library

A *L. elongatum* genomic library was previously constructed in our laboratory by Wei Shen. Leaf genomic DNA was partially digested by *Sau3A* enzyme and cloned into *Bam*HI-digested arms of lambda phage FixII (Stratagene). 1×10^6 plaques of the unamplified library were screened with a mixture of four labeled probes representing ESI3, ESI14, ESI28 and ESI48 cDNAs and nineteen primary positive clones were isolated. In the work presented here, the primary positive clones were rescreened using single cDNA probes to isolate and purify the individual genomic clone.

Restriction Digestion of DNA

The restriction endonucleases were obtained from BIO/CAN, Promega and Pharmacia. The conditions for the enzymatic reaction were as recommended by the supplier. In general, DNA digestion was done using 2-10 units (u) of enzyme per microgram (μ g) of DNA.

Agarose Gel Electrophoresis and Purification of DNA Fragments

Agarose gel electrophoresis was carried out in 1x TAE buffer [0.04 M Tris-acetate (pH 8.0), 1 mM EDTA]. The concentration of agarose ranged from 0.7% to 1.5% (w/v) according to the size of the fragments being analyzed. The electrophoresed DNA fragments were observed after ethidium bromide staining by UV light. If necessary, agarose regions containing the fragment of interest were cut out and DNA was extracted from the gel using QIAEX Extraction Kit (QIAGEN) following the manufacturer's instructions. The purified DNA was dissolved in TE buffer.

Preparation of Radioactive Probes

The Amersham Multiprime labelling system was used for labeling DNA. Briefly, 25 nanograms (ng) of gel purified DNA were heat-denatured for 10 min, rapidly chilled on ice and the DNA labelling reaction was done according to manufacturer's instructions. The unincorporated radioactive nucleotides were removed from the DNA probe by passing the mixture through a Sephadex G-50 (Pharmacia) column packed in a Pasteur pipette. The probe was collected in the exclusion volume. The total activity of the probe per 25 ng was measured; probes usually contained between 1×10^7 to 4×10^7 counts per minute (cpm)/ μg DNA.

Hybridization

Hybridization was performed according to the instructions of the supplier of the Hybond-N nylon filter (Amersham). The filters with the immobilized DNA either from plaque lifts or Southern blots were prehybridized at 55°C for 3h in the prehybridization solution containing 5x SSPE (5x SSPE: 0.9M NaCl, 0.05M sodium phosphate buffer, pH 7.7, 5mM EDTA), 5x Denhardt's solution (5x : 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% SDS, and 100 $\mu\text{g}/\text{ml}$ sonicated denatured salmon sperm DNA. The radioactive probe was heat denatured and added to a hybridization solution of the same composition as the prehybridizing solution and filters were incubated at 55°C for 24h, then the filters were washed two to three times in 1x SSPE/ 0.1% SDS solution at 42°C for 5 to 10 min, each. Fuji x-ray films were used for autoradiography and exposure was at -80°C . Intensifying screens were used to enhance signals.

Lambda Phage Clone Purification

E.coli XL-1 Blue host cells were grown in LB medium to OD_{600} between 0.4 and 0.8. Cells were collected by centrifugation at 2000 rpm for 2 min,

resuspended in 10 mM MgSO_4 and diluted to $\text{OD}_{600} = 0.5$ in the same solution. The phage suspension was used for infection by diluting 1 μl of the phage stock into 1 ml of SM buffer (100 mM NaCl, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mM Tris-Cl pH 7.4 and 0.01% gelatin) and 3 μl of this was added to 200 μl of bacterial suspension. The mixture was incubated for 15 min at 37°C, then rapidly mixed with 3 ml of melted soft top-agar (0.7% agarose), poured onto 100-mm plates containing LB-solid medium (1.5% agar, 0.5% NaCl, 0.5% yeast extract, 1% tryptone, pH 7.5) and incubated overnight at 37°C. Plates with 100-1000 plaques were selected for screening. Plaques were lifted onto Hybond-N nylon filters (Amersham), denatured in 1.5M NaOH, 0.5 NaCl, neutralized twice in 1.5M NaCl, 0.5M Tris-HCl (pH 7.2 buffer), briefly washed into 2x SSC (1x SSC: 0.15M NaCl, 15mM sodium citrate, pH 7.0), dried in air and baked for 2 hr at 80°C according to Amersham instructions. Filters were hybridized with a radioactively labeled probe made from the ESI28 partial cDNA clone as described above. Plaques are usually isolated from the agar plates as a single plaque using a cut sterile micropipette tip and resuspended into SM solution.

DNA Isolation from Lambda phage.

To obtain high titre phage suspension, positive phage isolates were used for replating at densities of $10\text{-}50 \times 10^3$ plaques per plate. Four ml of SM solution was added to the petri plate which was gently shaken at 4°C overnight. The phage suspension was transferred to a 50-ml conical tube and 125 μl of chloroform was added and centrifuged for 10 min at 5000 rpm. The supernatant was removed and saved. Twenty microliter of chloroform was added to the phage stock and was stored at 4°C. The recombinant phage DNA preparation was prepared by transferring 0.5 ml of overnight bacterial culture into a 50 ml of LB-medium containing 0.5 ml of 1M MgSO_4 and 0.5 ml of 20% maltose and shaken

at 37°C for 2hrs to reach OD₆₀₀= 0.5. The bacteria were infected with 200µl of the phage stock by incubating the mixture at room temperature for 10 min and incubated for 5h to overnight at 37°C with shaking at 350 rpm. Culture lysate was treated with 1ml chloroform and 3.0 g of NaCl and shaken at room temperature for 20 min. The cellular debris were removed by centrifugation at 10,000 rpm for 30 min. The supernatant was treated with 100 µg each of DNase I and RNase-A and incubated at 37°C for 30 min. Polyethylene glycol (PEG; molecular weight of 8000) at a rate of 3.75g/50ml culture was added to the solution and was shaken for 1hr at RT, then the sample was cooled on ice for 30 min and finally centrifuged as above. The pellet was resuspended in 500 µl SM solution; 5 µl of 10% SDS and 5 µl of 0.5 M EDTA were added and incubated at 65°C for 10 min. The lysate was phenol-chloroform extracted twice, followed by one chloroform extraction. One tenth volume of 3M sodium acetate pH 7.0 was added followed by two volumes of 95% ethanol at RT. The DNA was spooled out with a glass pipette, washed with 70% ethanol, air dried and dissolved in 100 µl H₂O.

Southern Blot Analysis of Lambda Clones

DNA was digested with restriction endonucleases and electrophoresed in an agarose gel. The gel was then denatured by soaking in a solution of 0.5 M NaOH and 1.5 M NaCl for 30 min then placed in dH₂O for 5 min and neutralized by two treatments of 15 min with a solution of 1.5 M NaCl/0.5 M Tris-HCl (pH 7.2)/ 1mM EDTA. The DNA fragments were transferred to a Hybond-N⁺ nylon membrane (Amersham) by the capillary transfer method with 20x standard saline citrate (SSC) (20x SSC: 3M NaCl, 300mM sodium citrate, pH 7.0). The DNA was fixed to the surface of the membrane by UV irradiation at 254 nm for 2 min. Prehybridization and hybridization of Southern blots were carried out in a

rotisserie hybridization oven (HYBAID, HB-OV-BL, BIO/CAN) as previously described in the hybridization section.

DNA Ligation

DNA ligation was performed by using 1:3 molar ratio of the digested plasmid (pBluescript): insert DNA. The reaction was done in a total volume of 10 μ l including the plasmid DNA and the insert DNA. The mixture solution was warmed at 42°C for 5 min and chilled on ice. Ligase buffer and 1 μ l of T₄ DNA ligase (2u/ μ l) (GIBCO/BRL) were added to the mixture, and the solution was spun by pulse centrifugation and incubated overnight at 16°C.

Transformation of *E. coli*

5 μ l of DNA solution containing 10-100ng of ligation products was added to 100 μ l of competent cells and incubated on ice for 30 minutes. Cells were then heat shocked at 37°C for 5 min. Then 1.85 ml of LB medium were added and the cells shaken for 1h at 37°C. One hundred to 200 μ l of the transformed cells were transferred to an LB-ampicillin plate and incubated overnight at 37°C.

Preparation of Plasmid DNA from Transformed Bacteria

Half milliliter of LB medium with 50 μ g/ml ampicillin was inoculated with a clone of interest and shaken at 37°C for 6-8h. The culture was transferred to 50 ml of LB with 50 μ g/ml ampicillin and left to shake overnight at 37°C at 250 rpm. Plasmid DNA was purified by the Qiagen Plasmid Purification System (Qiagen Inc.) according to the manufacturer's instructions. DNA yield was quantitated by absorbance OD₂₆₀ and quality was assessed by a scan from 230-320 nm.

Polymerase Chain Reaction (PCR)

Polymerase chain reactions were carried out using the methods described in the manual by Innis *et al.* (1990). The annealing temperature (T_a) was selected as 5°C below the lower melting temperature (T_m) of the two primers, while the (T_m) for a primer with a length of 17-28 nucleotides was calculated according to the formula T_m (in °C) = 4(G + C) + 2(A + T). Typical annealing temperatures were between 55°C and 66°C. In a typical reaction, there were 10^4 to 10^8 copies of target DNA, 0.2 mM of each primer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.1 mg/ml bovine serum albumin, 25 u/ml Taq DNA polymerase (BIO/CAN), 50 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, 0.1% Triton X-100. The reaction final volume was brought up to 50 µl with dH₂O and overlaid by an equal volume of mineral oil. The thermal cycles were carried out in an automated thermal cycler (HYBAID). In the initial cycle, the reaction was at 95°C for 5 min of denaturation, then at the annealing temperature for 30 sec, and at 72°C for 30 to 60 sec for extension. This was followed by 29-34 cycles of 95°C; 30 sec; annealing at a primer specific temperature; 30 sec; 72°C/ 30-60 sec. Finally, the reaction mixture was incubated at 72°C for a further 10 min to complete the synthesis of DNA molecules.

DNA Sequencing

DNA sequencing was done using both vector-specific and clone-specific oligonucleotide primers (see Table 1 for list of primers). Three micrograms of plasmid DNA in 36 µl was denatured using 4 µl of a solution containing 2M NaOH for 30 min at 37°C. Four µl of 3M sodium acetate (pH 5.0) was added to the mixture and precipitated with 100 µl of 95% ethanol at -20°C for 15 min. The precipitated DNA was microcentrifuged for 15 min and the pellet was washed with 70% ethanol, dried and redissolved in dH₂O for sequencing reaction using

the sequenase dideoxy DNA sequencing kit (Amersham) according to the manufacturer's instructions. The samples were heated at 75°C for 2 min before loading onto a 6% (w/v) polyacrylamide sequencing gel. After electrophoresis, the gel was fixed in 10% (v/v) acetic acid, 10% (v/v) methanol 3 times for 2 min each and dried. Fuji X-ray films were exposed to the dried gels at room temperature for autoradiography. Some of the sequencing was done at the Sheldon center for Biotechnology, McGill University.

Computer-Assisted Data Analysis

Analysis of nucleotide sequences of genes and amino acid sequences of proteins was mainly done with PC/Gene computer programs (Release 6.70, by A. Bairoch, University of Geneva, Switzerland). The software was used for data entry, manipulation, comparison and identity search, as well as for other applications. The access to DNA databases was through the NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) E-mail server system (Altschul *et al.*, 1990). Sequences were sent for similarity comparison. Also, sequences and related information were obtained from databases through the NCBI Retrieve E-mail Server.

Table 1: List of Primers

Gene-specific primers used in sequencing and PCR amplification of ESI28 clone.

Primer Name	Sequence from 5' to 3'
ESI 28 R3KB	TTGCTCTGTTTCGAGCTG
ESI 28 R25KBA	TACGCGGTGTACGACCT
ESI28 R25KBB	CTCAAGGACTACACCAC
ESI F 1050	GTGCAGAATCTTCTACTG
ESI 28 F25KB	GCGACCAGTGGATGAAG
ESI 28	ACACATCGCACATCCAACAG

RESULTS:

1- SCREENING AND PURIFICATION OF ESI28 GENOMIC CLONES

After four rounds of purification, a single genomic clone ESI28-G-5 was isolated. The agarose gel analysis of a *Sal I*-digestion of ESI28-G-5 had DNA fragments of 5.5, 4.0, 3.5 and 2.0 kb, which were derived from the cloned insert,, indicating a total clone size of ~ 15 kb. The 4.0 kb fragment showed a hybridization signal in Southern analysis of this digestion (Figure 1). The 4.0 kb fragment was gel purified and the DNA was ligated into *Sal I*-digested pBluescript KS⁻ plasmid vector to generate the ESI28 genomic subclones. Three genomic subclones were purified ESI28-S-5-21, ESI28-S-5-20 and ESI28-S-5-17.

2- SUBCLONING OF ESI28-G-5 GENOMIC CLONES

Three subclones ESI28-S-5-21, ESI28-S-5-20 and ESI28-S-5-17 of the 4.0 kb fragment all hybridized to the ESI28 cDNA probe. A gene specific primer (ESI28) gave a positive product in a PCR reaction when used in conjunction with a vector specific primer (M13) (Table 1). The amplification also showed that two subclones (ESI28-S-5-17) and (ESI28-S-5-21) were cloned in the same orientation and that ESI28-S-5-20 was cloned in the opposite orientation in the pBluescript vector.

The restriction map of the three subclones is illustrated in (Figure 2). The entire coding region of ESI28 is encompassed in the 4.0 kb *Sal I*-digestion fragment and it is located between the *Hind III* site (at 2.8 kb) and the *Sal I* site (at 4.0 kb) of clones ESI28-S-5-17 and ESI28-S-5-21. The remainder of the sequence downstream from the 3' end of ESI28 gene (about 2.8 kb) has several unique restriction sites. No restriction sites were detected for *Kpn I*, *Apa I*, *Xba I* or *EcoR I*.

Figure 1:

Southern Blot of ESI28-G-5

Southern blot was prepared from the genomic clone ESI28-G-5 which is contained in the lambda cloning vector Lambda Fix II. The blots were probed with a ^{32}P -labeled probe prepared from the partial cDNA clone ESI28.

(a) DNA was digested with *Sall* and *SacI*, lanes 2 and 3 respectively. The positive signal in the *Sall* lane represents a 4.0 kb fragment. The 2.0 kb fragment in the marker's lane represents a non-specific hybridization between the probe and the marker's DNA.

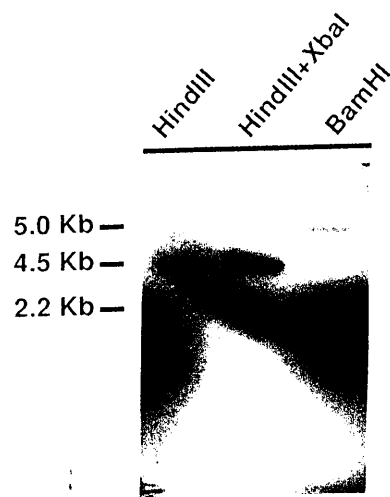
(b) DNA was digested with: lane 1, *HindIII*; lane 2 *HindIII* and *XbaI*; lane 3 *BamHI*.

The probe hybridized with a fragment of 4.5 kb in lanes 1 and 2, and with two fragments of 5 and 2.2 kb respectively in lane 3.

(a)



(b)



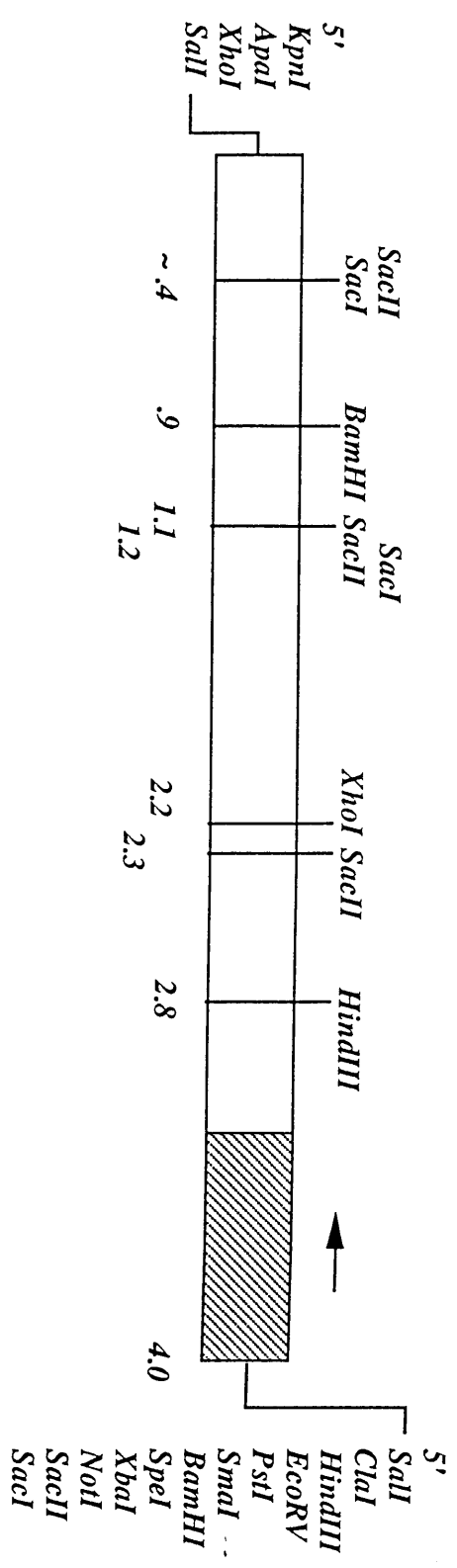
3- ESI28 ENCODES A PUTATIVE ACTIN DEPOLYMERIZING FACTOR

A 1349 bp portion of the 4.0 kb *SalI* subclone was sequenced (Figure 3). The sequence in this region was determined in entirety on both strands by using a series of vector-specific and gene specific oligonucleotide primers (Table 1). The strategy used is shown in Figure 4. The ORF of the gene starts at nucleotide 219 and ends with a stop codon at nucleotide 769. Immediately after the start codon, an intron of 117 bp is present. The sequence of the original ESI28 partial cDNA sequence was found in the untranslated region of the gene sequence with 100% sequence similarity that starts at nucleotide 768 and ends at nucleotide 935. A Genbank data base search showed that the clone has high sequence similarity with a cDNA clone from *Triticum aestivum* and moderate with similarity genes from other species (Table 2). The previously identified genes are referred to as actin binding or actin depolymerizing proteins. The ESI28 gene sequence had the highest degree of sequence similarity to the *T. aestivum* Wcor719 gene, a cold stress regulated gene (Table 2).

The 5' upstream region between nucleotide 15 and 21 (Figure 3) contains the sequence ACACGTA which is similar to the ABRE found in many ABA-responsive genes. The ESI28 gene has a putative TATA-box (TATAAAAT) which starts at nucleotide 79, and potential translation initiation methionine codon at nucleotide 219. Surprisingly, this methionine is immediately followed by a putative intronic sequence. A polydenylation signal sequence, AAATAAT, is found at position 788 (Heidecker and Messing, 1986).

This gene sequence contains a single open reading frame (ORF), which is 429 bp in length. The predicted protein from the translation of the ORF contains 143 amino acids residues with a predicted molecular weight of 15,953 kDa. The most frequently occurring amino acids are Asp (20%) followed by Ala (18%) and

Clones 17 & 21



Clone 20

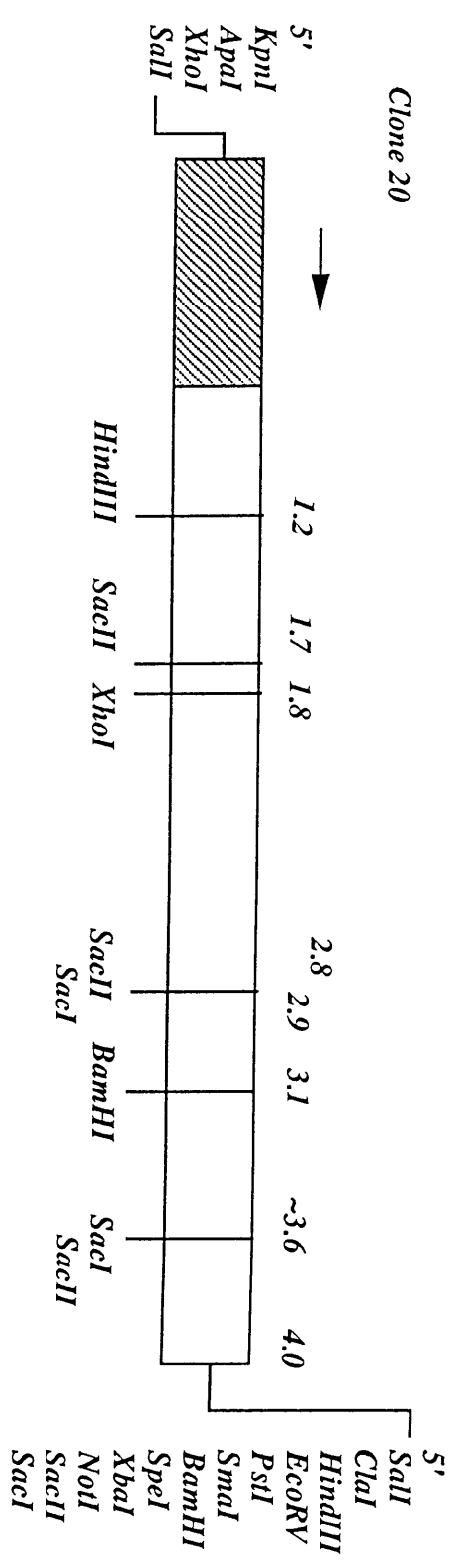


Figure 2:

Restriction map of three genomic subclones cloned in pBluescript KS⁻.

The entire coding region of ESI28 was included in a 4 kb *SalI* fragment of the lambda clone ESI28-G-5. Ligation of the 4- kb fragment to pBluescript yielded three clones (17 and 21 in the same orientation within the vector and 20 in the opposite orientation). The hatched box indicates the portion of the clone which was sequenced and the arrow indicates the direction of transcription. The list of restriction sites flanking the boxes represent the multiple cloning site of the vector.

Figure 3:

Nucleotide sequence of *ESI28* and the deduced amino acid sequence.

The first box indicates a ABRE-like element and the second box shows the putative TATA box. The intronic region is indicated in lowercase letters. The arrows indicate the beginning and end of the partial length cDNA clone that was used as a probe to isolate the genomic clone. The amino acid sequence for the actin binding domain is underlined, and the PIP₂ binding domain is double underlined.

GACGTCCCAGCGCCTACACGTAGTGTACACCCCGCTCCTCCCTGACTTTATCTCGCCG 60
ACATGGTCACGGCCCTCTATAAAATCACCACACCAGACACTGCTAAATCAACAACACTC 120
ATCACCGGCAACTAGACCAGCTTTCAATCCAGCTACCAGTTCCCGTCTCAGCATCAGCAC 180
*
ACAGATTTTTCCTCGGGCCAACCTCCAGATCAACCAACAATGgtgagtactattacgacc 240
M 1
tgctctgctttctttaacctgcttgctctgttcgagctgtttcagacaactgcaagtctg 300
ttcatcattgatcctggtggattttggttggtgtgctgcagGCGAACTCTGTGTCTGGGGTG 360
A N S V S G V 8
GCGGTGAACGAGGAGTGCGTGAAGGTGTTCCAGGAGCTGCGGGCGGAGCGCAAGCACCGG 420
A V N E E C V K V F O E L R A E R K H R 28
TTCGTGGTGTACAAGATGGACGACGACGAGGACGCGCAGCAGGTGGTGGTGGACAAGGTG 480
F V V Y K M D D D E D A Q Q V V V D K V 48
GGCGGCTCGACGCCACCTTCGACGACCTGGCGGGCGGCGATGCCCCGCCGACGACTGCCGC 540
G A L D A T F D D L A A A M P A D D C R 68
TACGCGGTGTACGACCTGGACTTCGTGTGCGGAGGACTCGGCGGGGGACACGCCGCGGAGC 600
Y A V Y D L D F V S E D S A G D T P R S 88
AAGATCTTCTTCATCCACTGGTTCGCCCCGAGGCGGCGGACGCGAGGAGCAAGATGGTGTAC 660
K I F F I H W S P E A A D A R S K M V Y 108
GCCAGCTCCACGGAGGGGCTCAAGAAGGAGCTGGACGGCGTGCAAATCGACGTGCAGGCC 720
A S S T E G L K K E L D G V Q I D V Q A 128
ACCGACGCCAGCGAGCTCACCTCGACATCCTCAAGGACTACACCACCTAATCAGCTAAT 780
T D A S E L T L D I L K D Y T T 144
CCATCCTGTACGTTTGCTCTTGATGTGCGATGTGTGGGATCGCCCTTCCTGCTTGAAT 840
TGGATCGCCTGGATCCAAATAATGTTTTTCCATGTGTGAATGATTGAGTGATTTTTACCT 900
TCGAGATTGATAATTGAGTGCTTGATTTATTCCTTACGAGAAAAAGAACTTCTACTGCC 960
AGCAATCGATCTTTTTGATTGACGTTGCGCCAATTCTTACAACATTAGACGTTTCATTG 1020
GTCAGAATAATGACATCAGTAGAAGATTCTGCACTTCATTGATTTGAATAATCATCATAC 1080
GTGTTAACTGTAAACTTGCCAATAAGGACATCTTTTCAACGGCAACCCGCAAATACGGG 1140
AGACTGCAATCCAACGCGGTCAACAATTCAAATAATTGGACGAAATTCGATCAAACCGG 1200
GCAGATTTTCATTGAAGTTCGGACATAATTTAATAAAAAGGTCGATATTTGCGCCTTTAAC 1260
NAAAGNAAAACTAAACCCTATACGCGTGGCGCCCTGCCCTGCTGCACCGCCGTCTCCGT 1320
CGGTGTCGTCTTCGTGTCGTGGTGGTCC 1349

Figure 4:

Sequencing strategy for the ESI28 clone.

ESI28 full sequence was obtained by the primer walking method. Vector flanking primers and sequence specific primers were synthesized and used in the sequencing strategy. Arrows indicate the direction of individual sequencing runs; the hatched box represents the position of the original partial length cDNA probe used for screening. The open box represents the intronic sequence which begins immediately after the start codon (ATG). The location of the ORF and TATA box in the putative promoter region are indicated.

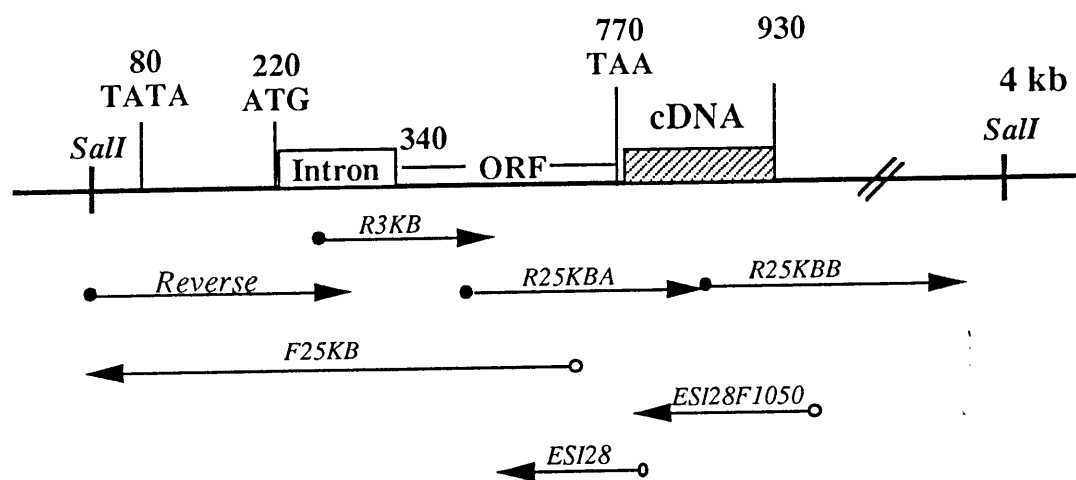


Table 2:

Amino acid sequence identity between ESI28 and selected actin-binding proteins.

Organism	Gene	Identity	Reference
<i>Triticum aestivum</i>	Wcor719 ABP	97%	Danyluk <i>et al</i> , 1996.
<i>Arabidopsis thaliana</i>	AtADF1	52%	Newman <i>et al</i> , 1994
<i>Arabidopsis thaliana</i>	AtADF2	52%	Newman <i>et al</i> , 1994
<i>Zea mays</i>	ZmABP1 ADF	50%	Rozycka <i>et al</i> , 1995
<i>Lillium longiflorum</i>	LLADF	49%	An, 1993
<i>Zea mays</i>	ZmABP2 ADF	48%	Lopez <i>et al</i> , 1996
<i>Zea mays</i>	ZMABP3	48%	Lopez <i>et al</i> , 1996
<i>Brassica napus</i>	BNADF	43%	Kim <i>et al</i> , 1993
<i>Schizosaccharomyces pombe</i>	COFILIN	43%	Conner and Churcher
<i>Drosophila melanogaster</i>	TWINST	31%	Edwards <i>et al</i> , 1994

Val (16%). The amino acid sequence of the predicted ESI28 protein is shown in (Figure 3).

DISCUSSION:

ESI28 ENCODES A PUTATIVE ACTIN DEPOLYMERIZATION FACTOR

L. elongatum has been used in several studies as a model for plants tolerant to salt-stress (Dvorak *et al.*, 1988, Gulick and Dvorak, 1990; Omielan *et al.*, 1991 and Dubcovsky *et al.*, 1994). Such studies aimed to map, isolate and characterize genes that are involved in salt tolerance. The identification and characterization of such genes will facilitate the understanding of the physiological and the molecular pathways of salt tolerance in crop plants.

ESI28 is an early salt-stress induced gene. In an initial attempt to isolate salt stress regulated genes a partial cDNA for ESI28 was isolated from a differential cDNA library constructed from *L. elongatum* roots (Gulick and Dvorak, 1990). In the present work, the genomic clone of the ESI28 gene from *L. elongatum* has been isolated. The isolation of the genomic sequence would save time to get the regulatory region (promoter) and to analyze the intron/exon organization of the gene. The ESI28 genomic sequence included a partial putative promoter region in the 5'-upstream sequence flanking the ATG methionine codon. This sequence contains a putative ABRE, and a TATA box.

The ESI28 deduced amino acid sequence was analyzed and protein databases were searched for sequence homology. The *T. aestivum* Wcor719 gene has 97% amino acid identity to the protein sequence deduced from ESI28 (Table 2; Figure 6). Since *L. elongatum* and *T. aestivum* are phylogenetically related, it may be concluded that these two genes are orthologs. In addition to the wheat Wcor719, three maize ABP-coding genes (ZmAB1, ZmAB2 and ZmAB3) were analyzed for their amino acid sequence similarity to ESI28 (Lopez *et al.*, 1996). All of these proteins show more than 48% amino acid sequence identity to ESI28. These structural similarities include two conserved domains that are usually found in ADFs, an actin-binding domain and phosphatidylinositol 4,5-biphosphate

(PIP₂) binding domain which are conserved in members of the cofilin family, and a potential phosphorylation site at Ser3 of the actin-binding domain which is also conserved among ADFs (Figure 3).

This significant amino acid sequence similarity between ESI28 and the four other proteins, including their conserved binding domains, suggests that ESI28 is functionally similar to Wcor719, ZmAB1, ZmAB2 and ZmAB3.

The phylogenetic analysis revealed that the ESI28 gene and the other four genes from maize and wheat are evolutionarily conserved suggesting a functionally important role through evolution. A number of genes that code for ABP (ADFs) have been analyzed and their amino acid sequences were aligned in (Figures 5 and 6). Therefore, ESI28 is likely to be an actin-binding protein that is involved in actin depolymerization (Cofilin/ADF), and can be probably involved in the regulation of different cellular processes resulting from, actin depolymerization, such as cytokinesis or cytoplasmic streaming.

In vitro analysis of bacterially expressed ZmABP3 protein showed that this protein is able to bind G-actin and F-actin indicating that it is an ABP. Moreover, it has the ability to decrease the viscosity of polymerized actin solutions indicating its actin depolymerizing function (Lopez *et al.*, 1996).

Northern analysis showed that Wcor719 transcript is rapidly accumulated and upregulated by low temperature in cold-tolerant plants, and this transcript accumulation is not tissue specific (Danyluk *et al.*, 1996). Other stress conditions applied either induced a low level of transcript accumulation (water stress) or did not induce its accumulation (exogenous ABA, heat shock, salinity, and wounding). This study of Wcor719 raises an important question: how to reconcile the fact that ESI28 and Wcor719 are structurally highly similar, yet they differ in their response to salt stress? Northern analysis of the Wcor719 gene was done on transcripts isolated from leaf tissue only and not roots; root expression is a

Figure: 5

Multiple alignment of *L. elongatum* ESI 28 with similar genes from other species.

ESI 28, *L. elongatum*; Wcor719, *T. aestivum*; AtADF1 and AtADF2, *A. thaliana*;; ZmABP2, ZmABP1 and ZmABP3, *Z. mays*; LIADF, *L. longiflorum*; BnADF, *B. napus*.

	*** ** *** * * * * *	*** *	
ESI28	MANSVSGVAVNEECVKVFQELRAERKHRFVVYKMDDDEDAQQQVVVDKVG	50	
Wcor719	MANSVSGVAVNEECVKVFQELRAERKHRFVVYKMDD--DAQQVVVDKVG	48	
AtADF1	MANAASGMAVHDDCKLRFLELKAKRTHRFIVYKIEEKQ--KQVVVEKVGQ	48	
AtADF2	MANAASGMAVHDDCKLRFLELKAKRTHRFIVYKIEDKQ----VIVEKLGE	46	
ZmABP2	MANSSSGLAVSDECKVKFRDLKARRSFRFIVFRIDDKD--MEIKVDRLGE	48	
ZmABP1	MANSSSGLAVNDECKVKFRELKSRRTFRFIVFRIDDTD--MEIKVDRLGE	48	
LlADF	MANSSSGMAVDDECKLRFLELKAKRNFRFIVFKIEEKV--QQVTVERLGQ	48	
BnADF	-----EDNCKLKFLELK-KRIFRFIIFRIDG----QQVVVEKLG	35	
ZmABP3	MANARSGVAVNDECMLKFGELQSKRLHRFITFKMDDKF--KEIVVDQVGD	48	

	* *** * *** * ** * *	
ESI28	LDATFDDLAAAMPADDCRYAVYDLDFVSEDSAGDTPRSKIFFIHWSPEAA	100
Wcor719	LDATFDDLAAAMPADDCRYAVYDLDFVSEDSAGDTPRSKIFFIHWSPEA	98
AtADF1	PIQTYEEFAACLPADDCRYAIYDFDFVTAE---NCQKSKIFFIAWCPDIA	95
AtADF2	PEQSYDDFAASLPADDCRYCIYDFDFVTAE---NCQKSKIFFIAWSPDTA	93
ZmABP2	PNQGYGDFTDLSLPADDCRYAIYDLDFTTVE---NCQKSKIFFISWSPDTA	95
ZmABP1	PNQGYGDFTDLSLPANECRYAIYDLDFTTIE---NCQKSKIFFISWSPDTA	95
LlADF	PNESYDDFTECLPPNECRYAVFDFDFVTDE---NCQKSKIFFISWSPDTS	95
BnADF	PQETYDDFTASLPADDCRYAVFDFDFTTNE---NCQKSKIFFIAWSPDSS	82
ZmABP3	RATSYDDFTNSLPENDCRYAIYDFDFVTAE---DVQKSRIFYILWSPSSA	95

	** *** ** * ***** *	
ESI28	DARSKMVIYASSTEGLKKELDGVQIDVQATDASELTLDILKDYTT	144
Wcor719	DARNKMLYASSTEGLKKELDGVQIDVQATDASELTNLILKDYTT	142
AtADF1	KVRSKMIYASSKDRFKRELDGIQVELQATDPTEMGLDVFRSRAN	139
AtADF2	KVRDKMIYASSKDRFKRELDGIQVELQATDPTEMGLDVFKSRTN	137
ZmABP2	RTRSKMLYASSKDRFRRELDGIQCEIQATDPSEMSLDIVKSRTN	139
ZmABP1	RTRSKMLYASSKDRFRRELDGIQCEIQATDPSEMSLDIVRSRTN	139
LlADF	RVRSKMLYASTKDRFKRELDGIQVELQATDPSEMSMDIIKARAF	139
BnADF	RVRMKMVIYASSKDRFKRELDGIQVELQATDPSEMSFDIIKSRAL	126
ZmABP3	KVKSKMLYASSNQKFKSGLNGIQVELQATDASEISLDEIKDRAR	139

Figure: 6

Dendrogram showing the relative degree of amino acid sequence similarity between ESI28 and 10 genes from other species.

The protein sequences are ESI 28, *L. elongatum*; Wcor719, *T. aestivum*; AtADF1 and AtADF2, *A. thaliana*; ZmABP2, ZmABP1 and ZmABP3, *Z. mays*; LIADF, *L. longiflorum*; BnADF, *B. napus*; Cofilin, Pig; TWINST, *D. melangaster*. The comparisons were carried out using PC/Gene using the Align program, Based on the method of Higgens and Sharp (1988).

11

1000



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1000

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possibility. ESI28 expression, on the other hand, is root-specific and it is induced by treatments with exogenous ABA and salinity (Galvez *et al.*, 1993) suggesting that the induction of these genes involves different signaling pathway(s) and/or different regulatory sequences that differentiate between leaf and root expression. This question requires further study since the stress treatments of these reports are not strictly comparable.

Potential regulatory sequence in the promoter of the ESI28 gene

ABRE is a cis-acting DNA regulatory element whose function has been characterized for ABA-regulated gene expression in plants such as maize, tobacco, barley, rice and *Arabidopsis*. The element has the consensus sequence of RYACGTGGYR; where R and Y represent purines and pyrimidines, respectively. It was first defined in the wheat Em gene. The element has a core sequence of ACGT followed by a G which is a typical "G box" - an element found in many genes in different species with very different types of regulation (Bray, 1997 and references therein). The ABRE has been shown to be bound by bZIP type DNA binding protein which is referred to as ABRE-BP (Bray, 1997 and references therein). The presence of ABRE alone is not sufficient for the induction of ABA-regulated genes, in some genes such as the HVA22 gene in barley, the ABRE element is associated with a coupling element CE1 (Bray, 1997 and references therein). These two elements together are sufficient for ABA gene induction. Data from this study also show a similar core element (ACACGTAC) in the 5'-upstream flanking region of ESI28 gene.

Conventional regulatory sequences are usually located in the 5' flanking region upstream of the start of transcription, however, an enormous body of evidence has demonstrated that sequences downstream of the start of transcription can play a major role in regulating gene expression. These

sequences be located in coding regions, introns and 5' or 3' transcribed but untranslated region (Rose and Last, 1997). The positive effect of introns on gene expression has been recognized in several plant genes, especially in monocot species. Plant reporter genes have been expressed in transgenic plants under the control of various maize Adh1, Sh1, Bz1 or Act promoter sequences. The reporter expression is increased up to several hundred fold when the first intron sequence was included (Vasil *et al.*, 1989; Rose and Last, 1997). Three polyubiquitin-encoding genes isolated from the dicot specie *A. thaliana* were shown to contain conserved regulatory intronic sequences (Norris *et al.*, 1993).

Immediately after the start codon of ESI28 there is an intronic sequence of 117 bp is present. This observation is based on several observations. First, the alignment between ESI28 and the cDNA sequences of Wcor719 created a gap at this position in Wcor719. Second, this sequence is bordered by a GT and an AG at the 5' and the 3' -ends, respectively, which is consistent with the splicing sequences found in monocot plants (Simpson *et al.*). Third, the sequence is AT rich (54%), which is characteristic of monocot introns (Norris *et al.*, 1993). The position of the intron immediately after the start codon is an unusual observation in genomic sequences.

In the slime mold *Dictyostelium discoideum*, two genes were identified that code for cofilin (DCOF1 and DCOF2). The DCOF1 gene contains a predicted intron just after the start codon methionine, but nothing is known about the function of this intron (Aizawa *et al.*, 1995). Interestingly, DCOF2 lacks the intronic sequence and is also not expressed under any condition examined.

In *A. thaliana* the At gene which encodes the elongation factor 1 β (1 β eEF-1 β), was found to contain an enhancer-like element in its first intron sequence (Gidekel *et al.*, 1996). Transgenic tobacco plants, expressing chimeric genes fusing the promoter and 5' untranslated region of the At-eEF-1 β gene

upstream of the *gus* reporter gene showed that the first intron sequence was required for high levels of expression. Electrophoresis mobility shift assays showed that this intron interacts with putative transcription factors present in nuclear protein extracts indicating that the involvement of this sequence in high level gene expression may be through interaction with transcription factors.

Other genes which contain essential regulatory sequences in their first introns include Act-1 from rice, Adh-1 and Sh-1 from maize and the cat-1 from castor bean (Gidekel *et al.*, 1996). In some cases, the extent of activation by the intronic sequences is variable depending on the promoting sequence, the plant species and the cell line. For instance, Callis *et al.* (1987) showed that *Adh1* first intron increased reporter gene expression in cultured maize cells 16- to 112 fold when the *Adh1* promoter was used, but only increased expression 5- to 22-fold in constructs containing the constitutive 35S promoter. Vasil *et al.* (1989) compared the effect of incorporation of the first intron sequence of the maize Shrunken-1 (*Sh1*) locus in reporter constructs in different species. Gene expression was increased in the different cell lines including *Panicum maximum*, *Pennisetum purpureum* and *Zea mays* but increased expression was more pronounced in *Z. mays* cells, suggesting species specific activity. Also, the intronic sequence caused a higher gene expression when incorporated with the *Sh1* promoter versus the 35S promoter.

The *A. thaliana* gene that encodes the tryptophan biosynthetic enzyme phosphoribosylanthranilate transferase (PAT1) contains two introns which were tested for their regulatory properties (Rose and Last, 1997). In *A. thaliana* lines stably transformed with a single copy of a fusion construct, composed of the entire plastid transit peptide and the first two introns of PAT1, showed a 30-fold increase in GUS enzymatic activity compared to plants transformed with GUS constructs lacking the two introns. The authors suggest that these intronic

sequences greatly upregulate the fusion protein expression through posttranscriptional mechanism by increasing the steady-state level of mRNA .

Intronic sequences are likely to enhance gene expression by one of two mechanisms: first, through a transcriptional mechanism by interacting with certain nuclear factors which are involved in enhancing transcription; second, through a posttranscriptional mechanism which stabilizes mRNAs thus increasing steady-state levels.

ESI28 and Water Deficit: Possible Mechanisms of Induction

The ESI28 gene is induced by salt in *L. elongatum*, *Chinese Spring* wheat and the amphiploid derived from *L. elongatum* and *Chinese Spring*. The induction of ESI28 expression is higher in *L. elongatum* than in the amphiploid and it is higher in the amphiploid than in *Chinese Spring* (Galvez *et al.*, 1993). The ABA-enhanced signal transduction pathway that lead to the induction of these genes is not known. The fact that all the ESI genes are co-expressed under salt-stress or ABA treatment may indicate that a common regulatory mechanism is shared among these genes.

Although ESI28 is inducible by ABA treatment, this induction is less than that caused by NaCl (Galvez *et al.*, 1993). This may suggest that salt-stress may initiate a signal transduction pathway that involves other factors necessary for the induction of ESI28. This involvement could either be due to the biosynthesis of new factors or inhibition of others. The ABA signal transduction pathway, on the other hand, may not require all of these factors (if any) for the induction of ESI28. It would be interesting to study the possible synergistic effect of both ABA and NaCl. The addition of ABA synthesis inhibitors in the presence of salt-stress may help to reveal if the salt induction pathway is related to ABA. Further

sequencing of the 5' upstream flanking region of ESI28 may elucidate further regulatory elements that are crucial for the ESI28 induction.

So, how could the ESI28 gene product (a putative ADF/Cofilin) fit in the process of plant tolerance to salt stress? Plant cells under salt stress undergo several mechanisms to adapt to higher salt levels and to minimize water loss. These include a cytoplasmic response and gene regulation, and both of these events are coordinated through signaling pathways (Sarhan *et al.*, 1997). Danyluk *et al.*, (1996) showed that in wheat low temperature causes microtubule depolymerization and that the level of depolymerization was related to the degree of cold tolerance.

ADF/Cofilins are important for the acceleration of actin filament turnover during cell motility, cell growth and cytokinesis which is the movement of sister chromatids to the spindle poles followed by the physical separation of the daughter cells (Carrier *et al.*, 1997). When cytokinesis starts, the actin filaments and myosin become concentrated in a cortical band midway between the two spindle poles. The disruption of the actin-myosin will result in deficient cytokinesis (Eckley *et al.*, 1997). The ADF/Cofilins plays an important role in cytokinesis in which the cofilin is localized at the cleavage furrow in several mammalian cell lines and in *Xenopus* embryos. The presence of the filaments in the cleavage furrow is important for furrow function. In *Drosophila*, cofilin is the product of the twinstar gene which is required for cytokinesis. The mutated gene showed defects in the cytokinesis and in centrosome migration. There were unusually large foci of actin filaments in their cells which means that the cells that lack sufficient levels of cofilin can assemble actin filaments for cleavage furrow formation but cannot disassemble them in a timely manner (Gunsalus *et al.*, 1995).

Plant cells contain both actin-based and microtubule-based motors that are involved in intracellular motility. The actin filaments that are found in the dividing

plant cells and in the microtubules at the interphase play an important role in the regulation of the division plane and cell morphogenesis in general (Asada and Collings,1997). A highly organized structural system is involved in repetitive cytoplasmic streaming, cytokinesis and mitosis. The bulk movement of organelles in interphase cells and in the process of cytoplasmic streaming (the intracellular movement of organelles in non-dividing plant cells) is also dependent on the interaction of the organelles with actin filaments (Asada and Collings,1997).

The mechanism of plant intracellular motility is not clear. However, studies on animal and fungal cells revealed that this mechanism involves two types of molecular motors; actin-based motors and microtubule-based motors. These enzymatic molecules generate a mechanical force along actin filaments or microtubules by liberating the free energy stored in ATP molecules (Asada and Collings,1997). Interestingly, organelles isolated from *Lilium* pollen tubes move along characean actin bundles indicating that in higher plants, cytoplasmic streaming is also facilitated by actin-based motors. The streaming is inhibited by the increase in the intracellular Ca^{+2} concentration which causes the inactivation of actin-based motors. This slow unidirectional movement is also dependent on actin filaments and the process of pollen tube elongation is inhibited when actin cytoskeleton is disrupted by cytochalasin. On the other hand, depolymerization of microtubulin does not affect tube growth, streaming or organellar motility indicating that these process are actin dependent (Asada and Collings, 1997).

Earlier studies have shown that actin filaments anchor the nucleus in the center of dividing vacuolated cells (Staiger and Lloyd, 1991 and references therein). The formation of the preprophase band (PPB) by the bunching-up of cortical microtubules was accompanied with the reorganization of the nucleus-radiating actin filaments into the same plants. The transition period between mitotic divisions is relatively long and involves the attachment of microfilaments

to the nucleus and the migration of the nucleus into the center of vacuolated cells. The second stage of premitotic phase involves chromatin condensation, microtubule polymerization from perinuclear regions reorganization and/or depolymerization of cortical microtubules and the formation of PPB. This is followed by a narrowing of the PPB and the formation of the phragmosomal raft. This organization of phragmosome is composed of both microtubules and actins. By metaphase, microtubules disappear; the actin component PPB remains little longer, but the cytoplasmic actin remains throughout cell division in the nucleus. When prometaphase is reached, the nuclear envelope breaks down, the cytoplasmic microtubules depolymerize, the spindle forms and cytoplasmic streaming in the residual actin strands is ceased. Streaming starts again during cytokinesis and the formation of nuclear envelope (Staiger and Lloyd, 1991 and references therein).

As mentioned earlier, cytoplasmic streaming is based on the active binding of molecules to the cytoskeleton components of microtubules and actin filaments. There is increasing evidence that these binding proteins are the motor machinery of actin and tubulin activity (Asada and Collings, 1997). Studies carried out on characean algal cells showed an actin-based motor composed of a 230 kDa polypeptide supports rapid gliding of actin filaments *in vitro*. This polypeptide cosedimented with phosphatidylserine-containing vesicles. Electronmicroscopy revealed that it has a myosin II size and shape. Another actin-based motor was isolated from the *Lilium* pollen tube and was found to consist of a 170 kDa polypeptide which supports translocation of actin filaments. The biochemical analysis suggests that the 170 kDa polypeptide structure differs from that of the heavy chain of myosin II. Similar myosin-like polypeptides were isolated from *A. thaliana*. Out of the eleven classes of the myosin superfamily, only two classes have been found in plants which may reflect differences in the function and

regulation of myosin or in functions of the actin cytoskeleton (Asada and Collings, 1997).

In general, it is well established that actin filaments in dividing cells and microtubules in interphase cells contribute in the regulation of the division plane and cell morphogenesis, respectively. These mechanisms involve cytoskeletal motion which is generated by motor molecules and maybe other cytoskeleton-binding molecules. These structural proteins are, therefore, primary participants in plant-cell responses to internal or external stimuli which trigger cell division/cytoplasmic fluidity. Therefore, how to reconcile the plant cytoskeletal organization as a response to salt stress? What role does ESI28 (ADF) play as a plant response to salt stress?

In addition to the role that actin polymerization and depolymerization plays in cell division, cytoplasmic streaming, and intracellular motility a recent report (Hwang *et al.*, 1997) implicates actin polymerization in the regulation of K⁺-channels. In studies with guard cells the depolymerization of actin has been shown to activate inward K⁺-channel activities. It is very attractive to hypothesizing that the ESI28 gene product, an actin binding protein, is involved in K⁺-channel regulation, especially in facilitating K⁺ uptake in roots (Hwang *et al.*, 1997). As discussed earlier, the maintenance of a high K⁺/Na⁺ ratio in salt stressed plants is a characteristic of salt tolerant genotypes. Increased levels of K⁺ uptake would be expected to a necessary component of this trait.

CONCLUSION:

In conclusion, the cloned ESI28 gene of *L. elongatum* codes for a putative actin depolymerizing factor. The presence of ABRE sequence supports the previous results that the salt-stress response of ESI28 is regulated through ABA signaling. Further work is necessary to understand both the regulation of ESI28 gene expression and to investigate the role of an ABP in the salt stress response. It would be informative to study the tissue localization of ESI28 protein within plant roots, to engineer the overexpression of ESI28 in transgenic plants and further analysis of the 5'-upstream sequence.

Actin has been shown to be involved in cell division. If salt-stress induction of ESI28 is related to changes in cell division, it might be expected to observe a localized change in the level of ESI28 (ADF) in the zone of cell division of the root. On the other hand, if actin is involved in regulating K⁺-channels activity, then it would be expected to observe more dispersed pattern of ESI28 expression. These studies can be done by immunohistochemistry analysis using ESI28 antibody. A second approach could be to construct a fusion of the ESI28 gene, including its promoter with a fluorescent protein tag like green fluorescent protein (GFP) (from Clontech). This construct could be used to generate stably transformed plants, and the distribution of the gene product in stressed plants could be studied histologically.

Overexpression of stress-regulated genes in a plant is a standard method of analysis of such genes. Overexpression of ESI28 in transgenic plant might have two possibilities; either increasing the level of salt tolerance (which alone, is unlikely), or it might be associated with other physiological characteristics that are observed in salt tolerance such as increasing K⁺ uptake. This hypothesis could be tested by comparing ion uptake and distribution in transgenic and control plants. ESI28 overexpression might be done by cloning the gene in front

of either a constitutive promoter like 35SCaMV or a regulated promoter. Transgenic plant can be generated by using *Agrobacterium* transformation or biolistic bombardment gene transfer techniques to transform a salt-sensitive plant like *A. thaliana*.

Related species are known to have ESI homologous which are also salt inducible. However, in a salt-tolerant species like *L. elongatum*, the induction of ESI genes is more rapid and more intense. It would be a useful to elucidate the regulatory element of the ESI28 promoter responsible for this enhanced expression. This can be achieved by, first, obtaining more upstream sequence of the promoter. This can be done by digesting the ESI28-G-5 genomic clone with *HindIII* which generates a (4.5 kb) fragment which would encompass the 5'-upstream sequence of the promoter (Figure 1-b). Gene- expression can be studied by cloning the promoter upstream of a reporter gene like (β -*gus*) and studying gene expression in transformed plants. Gene induction analysis is carried out by analyzing the reporter gene activity under salt stress. Deletion analysis of the promoter sequence should reveal the regulatory element(s) involved in salt-stress induction.

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