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Characterization of Two Members of a Multigene Family in the Wheatgrass, *Lophopyrum elongatum*

Hongyan An

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in the Department

of

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ABSTRACT

Characterization of two members of a multigene family in the wheatgrass, *Lophopyrum elongatum*

Hongyan An M.Sc Biology

Several genes are known to be induced by NaCl treatment to high levels of expression in the roots in *Lophopyrum elongatum*, a highly salt tolerant wild relative of cultivated wheat. The genes are also induced by abscisic acid treatment and by osmotic shock with mannitol. Presented here are one full length cDNA sequence and another partial length cDNA sequence of members of a salt-stress-induced gene family from *L. elongatum*. The polypeptide sequence as deduced from the nucleotide sequence of the cDNA clone *ESI*35 reveals a protein with a molecular weight of 27 kDa. This polypeptide has a repeated lysine-rich amino acid sequence found in dehydrins from barley and other plant species. Southern blot analysis showed that there is only one gene copy of *ESI*35 in the *L. elongatum* genome.

Clone *ESI*18-1, a 1.1 Kb partial length cDNA also has regions of high similarity to rice and barley dehydrins. Its derived amino acid sequence has four repeated sequences: lysine-rich motifs which occur only twice in genes reported from barley and rice. This lysine-rich motifs present in *ESI*18-1 share 80% sequence similarity to those of barley, rice and corn dehydrins. Southern blot analysis using probes from *ESI*18-1 as well as other *ESI*18 family members, showed that there are four or more copies of this gene in the *L. elongatum* genome. Western blot analysis revealed that the *ESI*18-1 protein was immunologically cross-reactive with the antibodies to the dehydrins and the cold-stress related proteins.

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1. LITERATURE REVIEW

1.1. Variety of Environmental Stress Faced by Plants

Plants are subjected to numerous environmental stresses. In what has become a classic treatise in the field of plant stress, Levitt (1980) classifies environmental stresses into two broad categories: (1) biotic stresses which include infection or competition by other organisms and (2) physiochemical stresses which include (a) high or low temperature; (b) excess or deficiency of water; (c) radiation stresses including infrared, visible, ultraviolet, ionizing radiation; (d) chemical stresses (eg. salt, ions, gases, heavy metals, herbicides); and (e) mechanical and electromagnetic stresses. A stress on a biological system may be defined as any environmental factor capable of inducing a potentially injurious strain in a living organism (Levitt, 1980). Those various stresses may affect normal growth and reproduction or even result in severe injury or death to a plant. The biological strain is the reaction of the organisms to the imposed stress and may result in physical change (eg. a change in dimension) or biochemical change (eg. a shift in metabolism). Those various stresses may affect normal growth and reproduction or even result in severe injury or death to a plant.

Many plant species have evolved responses which mitigate the effect of various stresses. Much work has been conducted in the areas of: (1) pathogen stress; (2) responsiveness to heavy metals; (3) oxidative stress; (4) anaerobic stress; (5) heat shock; (6) drought and water stress; (7) low temperature stress; and (8) salt stress.

This review addresses preliminary work on the salt stress response and salt tolerance as well as osmotic stress and low temperature stress responses since both the osmotic and low temperature stress responses appear to have common physiologic and genetic components with the salt stress response. The analysis of stress response and stress tolerance includes a number of similar approaches to

understand and to modify these complex traits in cultivated species. Such analysis has included the physiological characterization of the stress response, the attempt to decipher key metabolic pathways involved in stress response and the study of genetic variation of tolerance and of physiological and metabolic components of the stress response. In the last decade, advances in molecular genetics and recombinant DNA techniques have generated an intense interest in the investigation of stress related genes. These genes have included both those of enzymes of previously characterized biosynthetic pathways associated with the stress response and those of genes of unknown function that have been selected by nature of their stress regulated gene expression. The investigation of this kind of gene expression in plants, in response to salt stress as well as osmotic stress and cold stress, offers great potential to expand the understanding of the mechanisms of tolerance, and in the long run to be able to use a broader base of germplasm for crop improvement via recombinant DNA technologies.

1.2. Water or Osmotic Stress

Water deficit results in drought or water stress. With most stresses, two resistance strategies have evolved in plants; avoidance or tolerance. Avoidance of osmotic stress is achieved through specialized adaptations involving root and shoot architecture and phenology (Paleg and Aspinal, 1981). Tolerance involves more subtle biochemical changes. The phytohormone abscisic acid (ABA) is involved in mediating various developmental and physiological events including response to dehydration or osmotic stress. While even a mild water deficit may be injurious to a plant at some stages of development, at other stages of its life cycle, desiccation is a natural event and facilitates seed survival during a dormant period. Desiccated seeds are extremely resistant to many environmental stresses. ABA has been implicated in

the control of many of the events during embryogenesis and seed maturation, including tolerance to desiccation (Kermode and Bewley, 1987).

Dehydration of plants has been shown to trigger a rise in endogenous ABA levels of up to 50-fold (Hensen and Quarrie, 1981) due to *de novo* biosynthesis of the phytohormone (Milborrow and Robinson, 1973; Zeevaart, 1980). A loss of cell turgor (Pierce and Raschke, 1980), or cell membrane perturbation associated with pressure potentials approaching zero are believed to be key factors in triggering ABA accumulation (Akerson and Radin, 1983). ABA accumulation in dehydrated plants requires transcription of nuclear genes since pretreatment of excised pea plants with transcriptional inhibitors prior to plant dehydration inhibits the synthesis of ABA (Guerrero and Mullet, 1986).

The ABA-deficient tomato mutant, *flacca* (*Lycopersicon esculentum* Mill, cv Ailsa Craig), does not synthesize ABA in response to drought stress (Neill and Horgan, 1985). This mutant is sensitive to water stress, and readily looses leaf turgor under drought conditions. This mutant has been used to distinguish polypeptides and *in vitro* translation products that are synthesized during drought stress in response to increased levels of ABA from those that are apparently not controled by ABA levels (Bray, 1988). This study indicates that many of the polypeptides and mRNAs synthesized during drought are regulated by changes in ABA concentration.

Reduction of turgor induced rapid changes in leaf translatable RNA in pea (*Pisum stativum*) (Guerrero and Mullet, 1988). Analysis using 2D-PAGE of radiolabeled translation products of poly $(A)^+$ RNA from wilted versus control leaves leads to the identification of several messages that increased in wilted plants. Most of these did not accumulate in response to heat shock or exogenously applied ABA despite elevated endogenous ABA levels in water stressed leaves. Differential screening of a λgt10 cDNA library constructed from poly $(A)^+$ RNA from wilted shoots identified four clones corresponding to genes induced in wilted shoots. The sequences of three

of these clones were reported by Guerrero (1990). Clone 7a encoded a 289 amino acid protein with low similarity to soybean nodulin-26, was abundant in roots and induced in shoots by dehydration, heat shock and to a lesser extent by ABA. The protein is predicated to have six potential membrane spanning domains similar to those proteins that form ion channels. Clone 15a encoded a 363 amino acid protein with homology to cysteine proteases and was more abundant in roots than in shoots of control plants. Transcript levels were induced by dehydration but not by heat shock. Clone 26g encoded a 508 amino acid protein with sequence similarity to several aldehyde dehydrogenases. Transcript levels were induced by dehydration of shoots but not of roots. ABA and heat shock failed to increase levels of this message. Rehydration of wilted shoots caused levels of mRNA hybridizing to cDNA 26g to decline to pre-stress levels within 2 h. Nuclear run-on experiments showed that transcription of the three genes was induced within 30 min following reduction of turgor pressure. Together, the results indicate that plant cells respond to changes in cell turgor by rapidly increasing transcription of several genes.

Although desiccation stress is injurous at other phases of a plant's life cycle, it is a normal part of seed formation. The accumulation of LEA proteins (Late Embryogenesis Abundant) is characteristic of seed desiccation. LEA proteins are not found in abundance in either other plant organs or in embryonic tissues of seeds in earlier stages of development (Dure et al., 1989). Levels of LEA proteins remain constant in resting dry seeds but disappear at the onset of germination. First described in cotton (Galau et al., 1986), subsequent reports suggest that LEA proteins are widely distributed in higher plant species (Scherer and Potts, 1989). LEA genes may also be induced to high levels of expression in other tissues and at other times of ontogeny by ABA and/or desiccation or osmotic stress (Baker et al., 1988; Galau et al., 1986).

Approximately 18 *Lea* genes have been cloned from cotton (Galau et al., 1992). Homologues of the cotton *Lea* genes have been identified as stress related genes in a number of species, these include: (a) *rab* (responsive to ABA) genes in rice seedlings exposed to ABA or salt (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1989) and maize embryos and calli exposed to ABA (Vilardell et al., 1990); (b) *Dhn* (dehydrins) in maize and barley seedlings exposed to water stress (Close et al., 1989); (c) RSLEA2 in dry seeds of radish (Raynal et al., 1990); (d) TAS14 in tomato seedling exposed to high leaves of osmoticum or ABA and (e) Wcs120 in wheat subjected to low temperature (Godoy et al., 1990; Houde et al., 1992).

Sequence analysis of different *Lea* genes showed several of these proteins to be extremely hydrophilic and revealed the presence of conserved amino acid, repeated motifs, which likely exist as amphiphillic helices (Dure et al., 1989). The dehydrins are also extremely hydrophillic. Dehydrins which are similar to *lea*-D11 proteins are glycine-rich and possess repeated lysine rich motifs. These repeats occur twice: once at the carboxyl terminus; and again near the middle of the polypeptide. The protein also contains a series of consecutive serine residues. Both the repeating units and the adjacent flanking stretch of serines are highly conserved with minimal variation among the dehydrins studied so far (Close et al., 1989). This structural organization is found in rab21 from rice (Mundy and Chua, 1988) and dehydrins from Barley and maize (M3) (Close et al., 1989), and in cotton LEA protein D11 (Baker et al., 1988). These conserved domains are thought to be functionally important in desiccation protection (Dure et al., 1989).

LEA proteins (Table 1) are believed to be desiccation protectants. It has been postulated that cellular proteins are stabilized during desiccation through interactions with LEA proteins (Delham, 1986) as well as by interaction with free amino acid proline (Csonka, 1989). The LEA proteins are predicted to form amphiphillic helices which are predicted to form intramolecular helical bundles and to present a globular

surface for the binding of ions and the formation of salt-bridges. This would prevent crystallization due to increased ion concentration caused by desiccation. Other LEA proteins which have an unusual abundance of glycyl residues which allows free rotation around the peptide bond are predicted to exist as random coils.

Table1. LEA proteins of known sequence

Species	Derivation	Clone name	Reference
Group 1			
Wheat	complete cDNA	Em Protein	Litts et al. (1987)
Cotton	complete cDNA	D 19	Baker et al. (1988)
Group 2			
Rice	genomic DNA	rab 21	Mundy and Chua (1988)
Cotton	complete cDNA	D 11	Baker et al. (1988)
Group 3			
Cotton	composite cDNA /genomic DNA	D 7	Bake et al. (1988)
Barley	complete cDNA	pHva 1	Hong et al. (1988)
Rape	complete cDNA	PLEA 76	Choi et al (1987)

Data from Dure III et al., 1989.

ABA has been demonstrated to play an important role in the response of vegetative tissue in osmotic stress (Singh et al., 1987; Zeevaart and Creelman, 1988).

Current models suggest the following chain of events. Water deficit results in dehydration which leads to a loss of turgor pressure. ABA levels increase in the cytosol and apoplast due to *de novo* synthesis as well as the release of the hormone from internal stores sequestered in organelles (Zeevaart and Creelman, 1988). The increase in free ABA is dependent on *de novo* transcription. Increased levels of ABA induce the expression of genes of the *lea/rab*/dehydrin family and other genes (Table 2). The pattern of gene expression suggests that the expression of *lea/rab/dehydrin* genes may lead to an increase in osmotic stress tolerance, however this has not been demonstrated experimentally. Glycophyte plants and cells respond to high osmoticum by changes in the composition of cell wall proteins and polysaccharides (Iraki et al., 1989), by accumulating osmoprotectants such as proline (Rhodes et al., 1986) and by accumulating stress related proteins (Ramagopal, 1987; Singh et al., 1987). These changes are observed in both cell cultures and in plants which are acclimated to high salt (Bressan et al., 1987).

A recent study focused on the signal transduction connecting osmosensation with changes in gene expression reported that ion channels and active transport are involved in osmoregulation and signalling in plant cells (Schroeder and Hedrich, 1989). Lynch et al. (1989) also reported that rapid increases in intracellular Ca^{2+} levels after osmotic stress in roots may be mediated by phosphoinositides. This result is supported by extensive evidence including Ca^{2+} mobilization studies (Rincon and Boss, 1987), phosphatidylinositol turnover and signalling (Morse et al.,1989).

Characterizing the ABA receptors may elucidate the events mediating ABA-responsive gene expression. The maize *Viviparous*-1 locus, isolated via transposon tagging, may encode a regulator of ABA reception or a component of an ABA transduction pathway (McCarty et al., 1989). The 5' upstream sequences of several of the *rab* and *lea* genes listed in Table 2, Marcotte et al. (1989) suggested that the conserved sequences may be ABA-responsive upstream regulatory elements. Mundy et al (1990) also reported that *in vitro* footprinting and gel retardation experiments have demonstrated nuclear proteins binding to motifs I and II of the rice *rab* 16A gene promoter. A similar sequence motif in the promoter of an ABA-regulated wheat gene, Em, was found to interact with a leucine zipper protein (Guiltinan et al., 1990), and mutation of that sequence abolished ABA responsiveness of the promoter when it was transiently expressed in rice suspension culture protoplasts (Giltinan et al., 1990). Skriver et al. (1991) reported that the conserved sequence from *rab-16A*, when placed as a tandem repeat of six units upstream from a minimal promoter, mediated increased transcription by ABA in barley aleurone protoplasts. Above all, these results indicate that in the promoter of a hormonally regulated gene *cis*-acting DNA sequences were responsible for mediating the effects of that hormone on transcription.

Table 2. ABA-Responsive Genes

Clone Name	Stress induced	Species	Organ Specific	Reference
pHVA1	?	Barley	?	Hong et al. (1988)
PLea 76	D	Rape	?	Harada et al. (2989)
LEA D7	?	Cotton	?	Baker et al. (1988)
Rab16	O.D.C	Rice	-	Mundy and Chua (1988)
RAB21	O,D,C, W	Maize		Vilardell et al. (1990)
LEA D11	?	Cotton	?	Baker et al. (1988)
Dehydrin	D	Barley	-	Close et al. (1989)
p8B6	D	Radish	?	Raynal et al. (1989)
Em	D	Wheat	-	Marcotte et al. (1988)
LEA D19	?	Cotton	?	Baker et al. (1988)
pN24	O	Tomato	-	King et al. (1988)
Osmotin	O	Tobacco	-	Singh et al. (1989)
LEA D34	?	Cotton	?	Baker et al. (1988)
LEA D113	?	Cotton	?	Baker et al. (1988)
salT	O,D	Rice	st	Claes et al. (1990)
Glb1	?	Maize	sd	Kriz et al. (1990)
p511	?	Wheat	sd	Williamason (1988)
Napin	D	Rape	sd	Finkelstein et al. (1988)
Longlycinin	?	Soybean	sd	Bray and Beachy (1985)
WGA	O	Wheat	-	Cammue et al. (1989)
pMAH9	D,W	Maize	-	Gomez et al. (1988)
ASI	D	Barley	sd	Leah and Mundy (1989)
PI-2	W	Potato	-	Pena-Cortes et al. (1990)
Hs 70	O,D,H,W	Maize	-	Heikkila et al. (1984)

Data are from Skrivre et al., 1990. O = high osmoticum (PEG or salt), D = desiccation, C = cold, W = wounding, H = heat, ? = untested or unknown. Sd = seed, St = stem, Pase = protease; ASI = -amylase/subtilisin inhibitor, WGA = wheat germ agglutinin, RNP = ribonuclear protein; - denotes not organ specific.

1.3. Iron Stress

Recent studies conducted by Lobreaux (1993) indicate that iron induces ferritin mRNA accumulation in maize (*Zea mays*, var. M017) coincidently with abscisic acid (ABA) accumulation. Ferritin mRNA also accumulates in response to exogenous ABA. In this work, synergistic experiments demonstrated that the ABA and iron responses are linked, although full expression of the ferritin genes cannot be entirely explained by an increase in ABA concentration. This results strongly support the hypothesis that ABA is involved as an hormonal relay during iron-induced ferritin biosynthesis and proved that ABA is essential for maximum synthesis of ferritin induced by iron. Iron stress is not likely to be related to osmotic stress (Mundy and Chua, 1988; Skriver and Mundy, 1990) because of the micromolar range of iron salt used. It is more likely to be related to an oxidative stress because of the role metals, such as iron, can reducing oxygen into hydroxyl radicals, through Haber-weiss reaction (Halliwell, 1987).

1.4. Low Temperature Stress

Low temperature stress can be divided into two categories (Levitt, 1980); chilling stress, and freezing stress. Chilling stress occurs at temperatures between 15°C and

0°C; that is, at low, non-freezing temperatures. Freezing stress occurs at subzero temperatures and usually involves the formation of ice. During the period of cold acclimation, numerous biochemical, physiological and metabolic functions are altered in plants (Cattivelli and Bartels, 1990; Christie et al., 1991; Danyluk and Sarhan, 1990). In general, total RNA increases during cold acclimation and precedes the period of increased freezing tolerance. In potato (Chen and Li, 1980) and wheat (Sarhan and Chevrier, 1985) the increase in total RNA content was positively correlated with the low temperature induction of freezing tolerance. The activities of chromatin-bound RNA polymerases I and II increase during acclimation (Sarhan and Chevrier, 1985). Similar studies with alfalfa also show an increase in RNA content during cold acclimation (Mohapatra et al., 1987a). In alfalfa increases in poly (A)+ and poly (A)- RNA during cold acclimation were reported (Mohapatra et al., 1987a, b). A marked increase in RNA content was observed after only 2 days in the less resistant cultivar (*Medicago sativa* cv. Saranac). Two days were also sufficient for maximal hardiness. In the more freezing-resistant cultivar (*Medicago falcata* cv. Anik), a more gradual increase in RNA content was observed which closely paralleled the more gradual increase in freezing tolerance. Drought stress, which also increases freezing tolerance, was correlated with an increase in RNA content in red osier dogwood (*Cornus stolonifera*) after an initial decline in RNA content during the first 3 days of increased tolerance. Most studies report an increase in ribosomal RNA (Johnson-Flanagan and Singh, 1988) and indeed the rate of rRNA synthesis increased at low temperature in freezing-resistant winter wheat but not in freezing-sensitive spring wheat (Sarhan and D'Aoust, 1975; Paldi and Devay, 1977).

Increase in soluble protein content during acclimation is a nearly universal observation. A statistical analysis of the linear regression of freezing tolerance and soluble protein content in potato yielded a correlation coefficient greater than 0.97 (Chen and Li, 1980). Studies with alfalfa seedlings showed a similar trend (Mohapatra

et al., 1987a, b). This increase in due at least in part to increased rates of protein synthesis during the cold acclimation period (Siminovitch et al., 1968; Guy et al., 1985; Mohapatra et al, 1987a, b). Protein synthesis inhibitors, (if applied during the first week of acclimation), block the low-temperature induction of freezing tolerance in winter wheat (Zvereva and Trunova, 1985). After 7 days, cycloheximide addition had no effect on the development of freezing tolerance. A similar result was reported for potato-stem cultures (Chen et al., 1983). These data lend credence to the idea that *de novo* protein synthesis during cold acclimation is causally related to the development of freezing tolerance.

Although not all ABA-inducible genes may be involved in the hardening proess, expression of some of these genes could be required for development of frost tolerance and be responsible for the numerous physiological and biochemical changes detected during hardening (Levitt, 1980). Using differential hybridization, several cold-regulated cDNAs have been identified. An accumulation of the corresponding mRNAs for some of these cDNAs is also regulated by water stress and ABA (Mohapatra et al., 1988). Similar results were reported for the enrichment of a 42 kDa protein in both ABA- and low temperature-treated cell suspension cultures of *Medicago sativa* (Robertson and Gusta, 1985). ABA and cold treatments also induced several proteins in cell suspension cultures of *Brassica napus* (Johnson-Flanagan and Singh, 1987) and *Bromus inermis* (Robertson et al., 1987).

Desiccation often accompanies cold acclimation and freezing stress (Guy, 1990). In other words, freezing stress is considered a form of desiccation stress, because extracellular freezing causes cellular water to move outside the cell (Levitt, 1980). Therefore, it is not surprising that genes responsive to water deficit and ABA are also induced during cold acclimation. Recently published analysis of a partial-length cDNA clone (Gilmour et al., 1992) and a full length cDNA clone, *WCS*120 (Houde et al., 1991), showed the predicted polypeptide product to share sequence homology

with the group II class of LEA proteins which are synthesized by many plants in embryo tissue undergoing desiccation (Baker et al., 1988). As discussed above, several of the genes for *LEA* group II proteins are induced by drought and ABA in rice (Mundy and Chua, 1988) and by cold in maize (Christie et al., 1991). *Lea* group II includes a set of genes encoding dehydrins which are induced by drought in barley and maize (Close et al., 1989), a tomato salt-induced polypeptide (Godoy et al., 1990), and the *ESI*18 gene family which is induced by salt stress in *Lophopyrum elongatum* (Gulick and Dvorak, 1990). Desiccation is known to induce accumulation of ABA in plant cells (Wright, 1977; Terry et al., 1988). Since low temperature also leads to an increase on endogenous ABA levels in many species (Chen et al., 1983; Kacperska-palacz, 1978; Chen and Gusta, 1983), it has been proposed that the increase in chilling and freezing tolerance by desiccation may be mediated by ABA.

1.5. Salt Stress

Salinity is an important limitation to crop production in many agricultural areas. In Canada, salt toxicity affects as much as 2 million hectares, however, it has been difficult to gauge its worldwide effect due to the dearth of information about the areas of land involved. The total area in the countries so far mapped is vast (some 3×10^6 km^2), and while maps of the saline land in the remaining areas of the world have yet not been completed, estimates indicate there to be at least another million km^2, not including the areas of many of the major deserts (Flowers, 1977).

During the past 40 years considerable attention has been given to the effect of NaCl on plant growth, since soil salinity is becoming an increasingly serious worldwide problem affecting productivity of several agricultural crops (Epstein et al., 1977). In addition to the osmotic stress, accumulation of Na^+ and Cl^- ions in the plant is toxic and is considered to be one of the main causes for growth inhibition

induced by salinity (Yeo and Flowers, 1986). Some varieties and ecotypes within a single species, exhibit very different levels of tolerance towards NaCl and increased inhibition of growth is highly correlated with increased salt accumulation in plant tissue (Hodson et al., 1985). Overall, the extent of the effect of salt stress on growth is dependent on the species of plants, the salinity level and the ionic composition.

A number of physiological mechanisms have been implicated in the adaptation of plants to growth under osmotic or salt-stress. Plants adapted to salt-stress have been shown to accumulate Na^+ and other ions as osmotica in the vacuole (Binzel et al., 1988). In addition to ions, certain low-molecular-weight organic solutes are accumulated or degraded in response to changes of salinity (Ben-Amots and Avron, 1983; Kauss, 1977, 1986; Wegmann, 1986). The particular organic molecules that organisms accumulate in the presence of saline environments varies widely among different organisms, but these compounds include: polyols such as glycerol in fungi (*Asteromyces cruciatus*; *Chaetomium globosum*) (Troke, 1976), and in microalgae (*Dunaliella* spp.) (Ginzburg, 1987); mannitol in fungi (*Chaetomium globosum*) (Troke, 1976) and in algae (*Platymonas* spp.) (Mariani et al., 1985; Maykut, 1985); sorbitol, the amino acid proline (most diatoms) (Liu and Hellebust, 1976); and sucrose (Kirst and Bisson, 1979; Kirst et al., 1988). Several solutes derived from the quaternary type ammonium compounds such as glycine-betaine (Dickson and Kirst, 1986; Wyn Jones and Gorham, 1983). These compounds may have a protective function, for example, polyols, especially glycerol, may act as "water-like" substances that mimic the water structure and maintain an artificial water sphere around macromolecules and act as small amphiphillic molecules to protect the hydrophobic parts of proteins, which are first affected when water potential is lowered. Hellebust (1976) reported that the accumulation of proline and glycerol allows growth at salinities close to saturation of NaCl for the green flagellate *Platymonas suecica*.

Among agricultural plant species, barley has relatively high salt-stress tolerance (Epstein and Norlyn, 1977). Its ability to tolerate relatively high concentrations of NaCl is correlated with the maintenance of a high ratio of K^+/Na^+ in the cytoplasm, a high ratio of Na^+/K^+ in the vacuole, exclusion of Na^+ at the plasma membrane, accumulation of Na^+ in mature leaves as opposed to rapidly expanding young leaves, and redistribution of K+ from mature to young leaves (Pitman, 1984). The selective transport of K^+ and Na^+ undoubtedly involves the PM and the tonoplast. Hurkman (1987) reported that NaCl altered polypeptide synthesis in barley roots and some of these changes occurred in polypeptides located in the microsomal membrane fraction. Salt stress has been shown to cause quantitative changes in a relatively small set of proteins in barley (Ramagopal, 1987a. b; Hurkman and Tanaka, 1987, 1988) and in cell cultures of tobacco (Singh et al., 1985, 1987) and tomato (King et al., 1986). The induction of a number of polypeptides in bromegrass cell suspension cultures (*Bromus inermis*) by treatment with abscisic acid is known to play an important role in the increased resistance of the culture cells to salt as well as low temperate stress (Robertson et al., 1987, 1988). Hasegawa et al. (1987) found that when cultured tobacco cells are exposed to gradually increasing concentrations of NaCl, they undergo phenotypic adaptation and exhibit dramatically increased tolerance to NaCl characteristic of halophytic plant species. The adapted tobacco cells exhibit a number of physiological changes including the accumulation of considerable amounts of osmotic solutes (Binzel, 1985, 1987). The treatment of roots of tomato (*Lycopersicon esculentum*) with NaCl showed the induction of organ-specific accumulation of a pair of polypeptides (Chen, 1991). In the halophytic plant *Mesembryan themum crystallium*, NaCl induced a set of proteins, some of which corresponded to crassulacean acid metabolism enzymes (Michalowski et al., 1988). When this photosynthetic pathway is engaged, the plants open their stomata primarily during the night and thus are able to conserve water. Winicov and Seeman (1990)

reported that the salt tolerant alfalfa cell line HG2-N1, in contrast to its salt sensitive parent Line HG2, possesses the capacity for a salt-stimulated induction of mRNA and protein accumulation from nuclear and chloroplast genes coding for chloroplast proteins involved in photosynthesis. This increased photosynthetic capacity in the presence of salt has been suggested to be important in osmo-regulation in response to salt stress, for example, for betaine synthesis and accumulation (Weigel et al., 1988), and may contribute to osmoprotectant synthesis by alfalfa (Sethi and Carew, 1974).

1.6. Salt-tolerance and salt-stress response of *Lophopyrum elongatum*

Though the mechanisms of salt tolerance are only superficially understood at the physiological, biochemical, molecular or genetic level, it is clear that the trait is complex and that there is wide genetic variation for salt tolerance among plants. The introgression of genes promoting salt tolerance from wild, salt-tolerance species into salt sensitive crop species has the potential of advancing both our understanding of this trait and in alleviating an immensely important agricultural problem.

Dewey (1960) investigated the relative salt-tolerance potential of wild wheat grasses and found wide differences among 14 species; strains of tall wheat grass *Lophopyrum elongatum* Love [syn *Elytrigia elongata* (Podp) Holubc = *Agropyron elongatum* (Host) P.B., 2n = 10X = 70], were among the most tolerant. Subsequently, tall wheat grass has repeatedly been shown to be highly salt tolerant (Elzam and Epstein, 1969; Moxley et al., 1978; Shannon, 1978). Genetic variability for salt tolerance has been demonstrated within the following species: *L. pontica* (Dewey, 1960; Shannon, 1978), *L. intermedia* (Host) Nevski [= *Agropyron intermedium* (Host) P.B.] (Dewey, 1960; Hunt, 1965), and *A. cristatum* (L.) P.B. (Dewey, 1962).

Although lines of *Triticum aestivum* L. em. Thell. have been found which possess tolerance to salinity of 50% seawater (Epstein et al., 1979), this tolerance level is considerably lower than that found in *L. pontica*. Since *L. pontica* and its close allies can be crossed with common wheat, they are possible sources of salt tolerance genes for *T. aestivum* . The techniques that have been used in interspecific gene transfer involving wheat have all employed genes for disease resistance, a character that has simple dominant inheritance and is largely unaffected by the environment (Knott and Dvorak, 1976). Dvorak (1981 a) reported that *L. elongata and L. scirpea* have a genome, designated E, in common with *L. disticha*. *L. diae* also has modified versions of this basic genome. Thus these tested species that have the E genome show high salt tolerance. This suggests that the ancestral diploid that possessed this genome was probably adapted to a saline environment.

The facultatively halophytic *Lophopyrum elongatum* Love (syn. *Agropyron elongatum* Geart., *Elytrigia elongata* Nevski; 2n = 2x =14, genome EE) offers a unique opportunity to examine the molecular basis of the adaptation of a halophyte to salt stress. *L. elongatum* grows naturally in salt marshes in the Mediterranean region. It was hybridized with the closely related cultivated wheat, *T. aestivum* (2n = 6x = 42, genomes AABBDD). The octaploid amphiploid from the cross is more tolerant of salinity than the parental wheat cultivar 'Chinese Spring' indicating that the salt tolerance of *L. elongatum* is partially expressed in the genetic background of the relatively salt-sensitive wheat (Dvorak and Ross, 1986; Dvorak et al., 1988; Omielan et al., 1991). Dvorak (1991) reported that *L. elongatum*, wheat and their amphiploid were shown to have better survival on exposure to salt stress if they were acclimated with sptepwise increases of NaCl treatments rather than given a sudden shock. This suggests that the capacity of salt-tolerance may require a period of acclimation to the sait stress which may be associated with changes in gene expression. Indeed, the changes in the expression of a number of genes in the roots of wheat and the

amphiploid were detected by comparison of *in vitro* translation of mRNAs isolated prior to and after acclimation to 250 mM NaCl (Gulick and Dvorak, 1987); in the same studies no striking changes were found in samples extracted from leaves of treated plants.

1.7. Molecular Cloning of Salt-Stress-Induced Genes

In recent years there have been a number of reports of the isolation of cDNA clones of salt-stress-induced genes. Zhao et al. (1989) reported that three cDNA clones pDZ1.3, pDZ2.8, and pDZ6.2, from salt-stress induced genes in *Distichlis spicata* . Transcripts of pDZ2.8 increased approximately 2-fold at 4 and 8 h after stress and decreased substantially (4-fold) by 24 hr after initiation of NaCl treatment. In contrast, the level of pDZ6.2 mRNA increased 10-fold by 24 h. These results indicate that these two transcripts have different kinetics of accumulation, although both mRNAs are up-regulated transcripts. Claes et al. (1990) isolated a salt stress induced cDNA clone, *salT*, which was found to contain an open reading frame encoding a protein of 145 amino acid residues. SalT mRNA accumulates very rapidly in sheaths and roots from mature plants and seedlings upon treatment with salt. The organ-specific response of *salT* can be correlated with the pattern of Na^+ accumulation during salt stress. Two members of the PEPCase gene family from the common ice plant, *Mesembryanthemum crystallinum* have been isolated and characterized by Cushman (1989). Upon salt stress, steady-state transcript levels of one member of the PEPCase gene family (Ppc1) increase about 30-fold in leaves within 5 days of salt stress. In contrast, steady-state levels of Ppc2 transcripts decrease slightly in leaf tissue over the same stress period. Wereticnyk and Hanson (1990) reported that a cDNA clone for betaine-aldehyde dehydrogenase (BADH), a nuclear-encoded chloroplastic enzyme, was isolated from leaves of salt-stressed

spinach (*Spinacia aleracea* L.). This clone hybridized to a 1.9-Kb mRNA, which was more abundant in salt-stressed plants than in unstressed controls. This is consistent with the known salt induction of BADH activity. The amino acid sequence deduced from the BADH cDNA sequence showed substantial similarities to those for nonspecific aldehyde dehydrogenase from several sources (Wereticnyk and Hanson, 1990). Bostock and Quatrano (1992) characterized the expression of the *Em* gene in rice cell suspension cultures after exposure of cells to various combinations of ABA and NaCl, and they found that gene expression was induced by NaCl and ABA. The authors also note additive effects with simultanious treatments of ABA and NaCl. The abscisic acid induced gene family, *rab*16, which was cloned from rice by Mundy and Chua (1988) and Yamaguchi-Shinozaki et al. (1989), is also induced by salt-treatment (Hahn and Walbot, 1989). Recently, one group working with *Arabidopsis thaliana* reported the isolation of a cDNA, RD29, which is induced by high-salt conditions (Yamaguchi-shinozaki and Shinozaki, 1993).

Gulick and Dvorak (1990) isolated a number of partial length cDNA clones of genes with enhanced expression following salt-stress from an enriched cDNA library prepared from mRNA isolated from the roots of *L. elongatum* six hr after exposure to 250 mM NaCl. DNA sequence comparisons indicate that they corresponded to 11 different genes which do not show more than 55% sequence similarity in pairwise comparisons. They were designated ESI, *e*arly *s*alt-stress *i*nduced genes (Gulick and Dvorak, 1990). The timing of induction of transcripts of these genes by salt stress was similar. Northern blot analysis showed low or undetectable levels of mRNAs in the roots of plants grown in the absence of NaCl, the message levels increased after 2 h salt treatment and reached peak expression after 6 h salt treatment (Gulick and Dvorak, 1992). The gene response was almost entirely confined to the roots, except for ESI15, which also exhibited increased expression in the leaves of salt stressed *L. elongatum* plants (Galvez et al., 1993). Since roots are the organ which is in

immediate contact with the osmoticum, it is not surprising that a set of genes have expression particularly associated with this tissue. Although nucleotide sequences of 10 of these initial partial length clones did not show similirity to sequences in GenBank data base, Gulick and Dvorak (1992) reported that the clone *ESI*18 contains a repeated conserved region for 39 amino acids; 32 of these 39 amino acids were identical with the carboxyl-terminal portion of the protein coded by the barley dehydrin clone *dhn*3 and had similar sequence identity with other members of this gene family as well as with the *rab*16 gene family isolated by Mundy and Chua (1988). *ESI*18 detected three bands on northern blot (Gulick and Dvorak, 1992). The larger two messages, 2.1 and 1.3 kb, appeared by 2 h, and the 1.1-kb message was apparent after 6 h of stress. The genes corresponding to *ESI*18 were the most strongly expressed among all 11 ESI genes identified.

Among 11 ESI genes, mRNA levels for the *ESI*35 clone were significantly more enhanced in the salt stressed *L. elongatum* than in salt stressed wheat. Also, *ESI*35 mRNA levels responded significantly more strongly to ABA treatment in *L. elongatum* than in wheat (Galvez et al., 1993). Thus, it appears that this gene, involved in the physiological response to osmotic stress, evolved regulation in *L. elongatum* that was significantly different from the regulation in wheat. Clone *ESI*35 hybridized to messages of 1.2 kb. By sequence comparison of the partial length *ESI*35 cDNA sequence to sequences in GenBank, showed no significant similarity to any genes. This is not surprising considering that relatively few salt stress-induced genes had been reported, and some of these are from quiet different biological systems. Since the results reported by Gulick and Dvorak (1992) were based on the partial cDNA sequence that is likely to contain largely 3'-nontranslated sequences such comparison can be considered incomplete. The objectives of this work are to focus on the characterization of *ESI*35 and *ESI*18-1, one member of the *ESI*18 gene

family. A brief descrption of the cloning and sequencing of *ESI*35 which was part of the work included in this thesis has been recently published (Gulick and An, 1993).

2. Materials and Methods

2.1. Chemicals, Reagents and Equipments

Unless specifically listed, most of the chemicals used in this study were purchased from ACP Chemical Inc. Montreal, Quebec. Restriction endonucleases and DNA modifying enzymes, Taq polymerase from Bio/Can Scientific Inc., Pharmacia; DNA molecular size standards, from Gibco/BRL Canada, Burlington, Ontario; dithiothreitol (DTT), ethidium bromide (EtBr), ethylenediamine tetraacetate (EDTA), formamide, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), dextran sulfate; from Sigma Chemical Co., St Louis, MO, USA; Gene/Clean from Bio 101 Inc., La Jolla, CA, USA; Agarose from Schwarz/Mann Biotech, Division of ICN Biomedicals Inc., Cleveland, Ohio; Hybond-N+ nylon, from Amersham Canada Ltd., Oakville, Ontario; ^{35}S-dATP, from Du Pont Canada Inc., Pointe Claire, Quebec. ^{32}P-dCTP, from ICN Biomedicals Canada Ltd., Montreal, Quebec; T-7 DNA polymerase sequencing kits (Sequenase Tm version 1.0), from United States Biochemical, Cleveland, Ohio, USA; bacteriological media from Difco laboratories, Detroit, Michigan, USA; sequi-gen nucleic acid sequencing cell, from Bio-Rad laboratories Canada, Ltd., Mississauga, Ontario.

2.2. Plant Materials

The accessions of *Lophopyrum elongatum* and *Triticum* species, used in this study along with their genome designations are listed in Table 3. Seeds of the different species were surfaced-sterilized in a solution of 1 % hypochlorite, 0.01% SDS for 30 min, rinsed thoroughly in sterile dd$H_2$0. Seeds were then placed on slant boards on a sterile filter paper moistened with sterile dd$H_2$0 in a glass tray containing sterile

distilled water and put at 4°C for 2 days, and then transferred into greenhouse. When the seedlings were 10 cm long, they were transferred into 15-liter solution culture tanks (=30 plants per tank). After 30 days, plants were transferred to 150-liter solution culture tanks. Seedling were grown in a greenhouse in modified Hoagland solution (Hoagland and Arnon, 1950) containing 2 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 5.5 mM $Ca(NO_3)_2$, 50 mg of Fe-EDTA per liter (10% Ion), 25 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, and 2 μM H_2MoO_4.

Table 3. Species used in the Study

Species	Chromosome constitution	Genome formula	Sources
Lophopyrum			J. Dvorak, University
elongatum (Host) love	7	E	of California, Davis
Triticum uratu (Iz)	7	A	Agri. Canada, Ottawa
T. tauschii	7	D	Agri. Canada, Ottawa
T. monococcum	7	A	Agri. Canada, Ottawa
T. boeticum	7	?	Agri. Canada, Ottawa

2.3. Preparation of Plasmid

Plasmid DNA was prepared on a large-scale by a modification of the procedure of Zhang. Single colony containing plasmid was inoculated to 25 ml liquid LB medium with 40 mg ampicillin/L in a 50 ml flask and incubated overnight at 37°C. The 25 ml of overnight culture was transferred into 1 L LB medium with 40 mg ampicillin/L in a 2 L flask and continued to grow with continuous shaking at 37°C. After 5 hours, 170

mg/L chloramophenicol was added to the culture which was grown with vigorous shaking at 37°C for overnight. The bacterial cultures were transferred into two 500 ml centrifuge bottles and centrifuged at 6,370 x g, 4°C, for 10 min. The cells were suspended in 100 ml TE buffer by vigorous shaking and combined into one centrifuge bottle then centrifuged at 4.420 x g, 4°C, for 10 min. Then the cells were resuspended in 24 ml SET (100 mM Tris-Cl (pH 8.0), 5 mM EDTA, 0.1% SDS) buffer, and transferred into a 250 ml flask. 5 ml lysozyme solution (20 mg/L) was added slowly to this cell suspension solution with shaking at 37°C for 15 min. 6 ml of 0.25 M EDTA (pH 8) was added into this mixture and incubated on ice for 5 min. The cells were lysed by adding 36 ml of a 0.2 N NaOH - 1% SDS solution. After 5 min on ice, during which time it was shaken gentle several times, the cell suspension was incubated 15 min at room temperature with gently shaking. The lysate was centrifuged for 1 hour at 1,880 x g at 4°C, the supernatant was saved and extracted with 40 ml phenol, after 5 min incubation at room temperature, 40 ml chloroform was added also with shaking and centrifuged at 3,840 x g, 16°C for 15 min. Then, the upper aqueous phase was transferred into a plastic graduate cylinder and 1/25 volume of 5 M NaCl and 1.1 volume of ice-cold isopropanol was added. After 1 hour incubation at -20°C, the solution with plasmid DNA was centrifuged at 9,800 x g, 4°C for 25 min. The supernatant was discarded and the plasmid DNA (pellet) was resuspended in 3 ml of TE buffer (pH 7.6).

2.4. CsCl Density Gradient Centrifugation

After the plasmid DNA pellet was dissolved in 3 ml TE buffer (pH 7.6), 3 gram of CsCl was added to this solution, then 0.8 ml of a 10 mg/ml ethidium bromide solution was added for each 10 ml of this solution. The resulting solution density was adjusted to 1.55 g/ml and pipetted into polyallomer Quick-Seal tubes (5 ml volume). The

tubes were capped and centrifuged in a Beckman LB-70 Ultracentrifuge (Beckman instruments, Canada, Inc., Mississauga, Ontario) at 60,000 rpm for 16 hours at 20°C in a NVT-90 rotor. When the tube was visualized under UV light, the plasmid DNA was seen as the lower band stained by EtBr. It was collected by piercing the tube just below the band with a #19 needle, bevel side up, attached to a 1 ml syringe which was used to remove the DNA. The DNA solution after collection was extracted repeatedly with iso-amyl-alcohol to remove the EtBr, and then divided into two Eppendorf microcentrifuge tubes. The solution was made 0.3 M with ammonium acetate, and the DNA precipitated with 2 volumes of cold ethanol. After chilling at -20°C for at least 1 hour, the DNA was pelleted at 13.000 rpm in a Eppendorf microcentrifuge for 30 min at 4°C. The DNA pellet was washed in cold 70% ethanol, drained, dried for 10 min under vacuum at room temperature, and dissolved in 1 ml TE buffer (pH 7.6). The DNA concentration was determined from the 260/280 absorbance value using a UV visible recording spectrophotometer (Model MD 825-24, Shimadzu Corporation, Japan) and its purity estimated by the 260/280 absorbance ratio, and by its appearance in an agarose gel after electrophoretic separation.

2.5. Plasmid Mini - Preparation

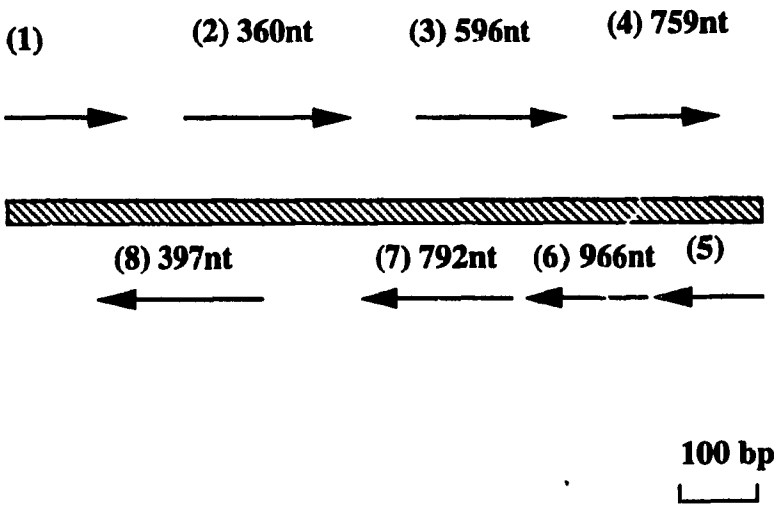
Small scale preparation of the plasmid DNA were performed by the boiling method (Maniatis et al., 1982). One ml of overnight cultures in LB liquid medium with ampicillin (40 mg/l) in a test tube was transferred into an Eppendorf microcentrifuge tube and centrifuged at 13,000 rpm at 4°C for 3 min. The cell pellet was completely resuspended in 300 ml of 0.1 M NaCl - 10 mM Tris-HCl (pH 8.0) - 1 mM EDTA - 5% Triton X-100, and 10 µl of lysozyme solution (20 mg/ml) was added. The tube was capped and placed in a boiling water bath, or a heating blok at 98°C, for 30 sec, and centrifuged at 13,000 rpm at 4°C for 15 min. The supernatant was transferred

into a fresh tube and the DNA recovered by precipitation with ethanol as previously described. The pellet, after washing with 70% ethanol, was dissolved in 20 ul sterile distilled water. The DNA was further purified by adding 2 ul RNase (10 mg/ml) incubating for 30 min at 37°C, following by incubation for 30 min at 37°C with proteinase K (final enzyme concentration = 400 μg/ml). The incubation mixture was then extracted once with an equal volume of phenol/chloroform (1:1), and twice with an equal volume of chloroform, and the DNA recovered by ethanol precipitation as described above. The pellet, after washing in 70% ethanol, was dried and dissolved in a volume of TE buffer (pH 7.6) appropriate for the use to which the DNA was to be applied.

2.6. DNA Sequencing

The DNA sequence of the cDNA clones was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) as adopted for the USB Sequenase kit. Double-stranded templates for sequencing were prepared by the mini preparation as previously described. The cDNAs were sequenced in both directions in their entirety using a nested deletion series for *ESI*18-1 and synthetic oligonucleotide primers for *ESI*35. The strategy for sequencing of the cDNA clone, *ESI*35, is presented in Fig. 1. The series of deletions of *ESI*18-1 were created using exonuclease III and mung bean exonuclease digestions (Henikpft. 1984); the Erase - a - Base system (Promega corporation) was used for this procedure. All the clones whose sequences are presented were sequenced a second time with a nucleotide mix containing dITP in the place of dGTP according to the supplier's (USB) instruction.

Figure 1. The strategy for sequencing of cDNA clone *ESI*35.
The arrows represent the synthetic oligo-nucleotides. 1. reverse primer; 2. 5'-1 (5' AGAGGAGGAGGTGATCG 3'), 3. 5'-2 (5' GGCTTCATGGAAAAGAT 3'); 4. 5'-3 (5' AGATCATGGACAAGCTG 3'); 5. Universal primer; 6. 3'-1 (5' CCGGACCTTCAGAGACG 3'); 7. 3'-2 (5' GTCTTGTGGTAACCAGG 3'); 8. 3'-3 (5' GATCACCTCGCCGTTGT 3'); The numbers on the arrow indicate the nucleotide position of the first base at the 3' end of the primer. Sequences derived from each primer overlapped with sequence from the adjacent primer.


(1)
(2) 360nt
(3) 596nt
(4) 759nt
(8) 397nt
(7) 792nt
(6) 966nt
(5)
100 bp

2.7. Computer Analysis

The SOAP programme, based on the method of Kyte and Doolittle (1982) from the PC/Gene analysis package (Intelligenetics Inc.), was used for estimating the hydrophilicity of the predicated proteins. Searches for sequence similarity in the GeneBank data base were performed using the BlastN and BlastP programme from the National Center for Biotechnology Information (USA). The COMPARE programme based on the method of wilbur and Lipman (1983), from the GCG Sequence Analysis Software Package (Madison, Wisconcin) was used for dot matrix comparison of amino acid sequence similarity between *ESI*35 and barley dehydrin clone Dhn3 and between *ESI*18-1 and Dhn3. An analysis for dot matrix comparisons used a window of 20 amino acids and various levels of stringency.

2.8. Genomic DNA Isolation

Genomic DNAs were isolated from single plants using the methods developed by Dvorak et al. (1988). 50 g plant tissue was cut into 1-2 cm pieces with scissors. Tissue was added to 100 ml ice cold extraction buffer containing 0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA, 0.08 M KCl, 1 mM spermidine, 1 mM spermine, 0.1 M sucrose and 0.1% (v/v) β-mercaptoethanol was added, and ground in a blender for 30 seconds at speed 4. The mixture was filtered through 3 layers of cheesecloth into a 250 ml centrifuge bottle. 5 ml of extraction buffer as described above but with 20% Triton X-100 stock added immediately to the filtered solution to a final Triton concentration of 0.5%. The solution was held on ice for 20 min, and centrifuged 20 min at 1,880 x g. The supernatant was discarded and the pellet was resuspended gently in two steps using 30 mls of extraction buffer with 0.5% Triton X-100 and transferred into a 30 ml Teflon centrifuge tube. Then, samples were centrifuged for

15 min at 1,380 x g. The supernatant was discarded and the pellet was resuspended gently in 1 ml ice cold PDIB buffer (0.1 M NaCl-0.1 M EDTA-0.05 M Tris-HCl (pH 8.0) and 0.5 ml freshly made proteinase K solution (2.0 mg/ml)). After 10 min of incubation on ice, the tube containing the genomic DNA solution was put into the 65°C water bath for a few seconds, then 15 mls of PDIB buffer and 0.5% SDS preheated to 65°C were added to the samples. After 1 hour of incubation in 65°C water bath, samples were extracted with 15 ml of 1:1 phenol-chloroform and centrifuged for 5 min at 1,380 x g. The supernatant was transferred into a 30 ml polypropylene centrifuge tube. Samples were centrifuged for 1 hour at 12,100 x g at room temperature. Immediately, the colourless and moderately viscous supernatant was removed to another tube. The genomic DNA was precipitated with 2 volumes of -20°C 95% ethanol. The DNA was collected with a sterile toothpick, washed 20 min in a solution of cold 70% ethanol. After the alcohol was evaporated, the genomic DNA was dissolved in 10 ml of TE buffer. The concentration of genomic DNA was determined by UV absorbance as described above.

2.9. Isolation of cDNA Inserts

Plasmid DNA was prepared by either CsCl density gradient or the boiling methods, as previously described. The insert DNA was cut from each of these plasmids by successive digestion with *Eco* RI and *Bam* HI. Fragments from these preparative scale double digests were separated by agarose (1%) gel electrophoresis, using 40 mM Tris-HCl (pH 7.8)-20 mM sodium acetate-1.8 mM EDTA electrophoresis buffer (TAE). Molecular size marker was 1 Kb DNA ladder (Gibco/BRL). Insert DNA was obtained by electrophoresis of the desired fragment into troughs filled with LM agarose, removal of the LM agarose pieces, and recovery

of the DNA from the LM agarose using the GENE/CLEAN kit, according to the manufacturer's directions.

2.10. Amplification of a Desired DNA Fragment by PCR

Primers for amplification of a 121 bp fragment of I1*ESI*18 -1 which includes terminal glycine and lysine rich repeated sequences were synthesized by Dalton Chemical Lab., North York, Ontario. Two primers were used as following:

ES18GKREP-5'

5' GGCACCTACGGGCAGCA 3'. This corresponds to nts 11 to 28 on the *ESI*18 sequence (counting 10 initial C's) or to nts 745-762 on sequence *ESI*18-1, or 121 bases 5' to the putative stop codon for *ESI*18 and *ESI*18-1. The primer can be used for extension of DNA synthesis toward the 3' end of the gene. Full length cDNA clones are not available for these genes, so numbering with reference to the start of transcription is not possible.

ES18GKREP-3'

5' AGTGCTGTCCAGGCAGC 3'. This corresponds to the terminal 17 bases of coding sequence ending on the T of the TAA stop codon. The primer is the reverse complement of the coding sequence in order to prime toward the 5' end to the gene.

Template for amplification of the repeated sequence is the insert of *ESI*18 in pJET2 vector (Gulick and Dvorak, 1990). The PCR reaction was conducted using Taq polymerise (Bio/Can). The *ESI*18 plasmid DNA was prepared as previously described. Total reaction solution was in 100 µl, which contained 10 ng of plasmid DNA, 10 µg of BSA, 10 µl of 10x Taq buffer (500 mM KCl-100 mM Tris-HCl-15 mM $MgCl_2$-1% Triton X-100), 2 µl of dNTP's mix (this means each base was 2.5 mM), 2 µl of each primer (10 pmol/µl), 0.5 µl of Taq polymerase (2 units). Reaction was overlayed with 75 µl of mineral oil and the synthesis was run with the following:

5 cycles of 95°C, 30 sec/56°, 30 sec/72°C, 2 min; 30 cycles of 95°, 30 sec/51°C, 30 sec/72°C, 2 min; 72°C, 10 min. The PCR products were checked on 2% agarose gel using 10 µl of reaction solution. After the electrophoresis, the PCR products were extracted from 2% agarose gel using GENE/CLEAN as previously described.

2.11. Random Primed Labelling

Approximately 25 ng of cDNA insert from each clone was labelled by random primer synthesis with radioisotope, whereas fragments of the repeated sequence obtained by PCR were labelled by the two specific primers used for amplification with a radioisotope. The ^{32}P-dCTP was incorporated into the DNA, using the random primer synthesis kit of Amersham according to the manufacture's instructions for 30 min at 37°C. The labelled DNA fragments were separated from unincorporated nucleotides by passage through a 1 ml Sephadex G-50 column. The activity of the probe was measured by a scintillation counter (LKB, Wallac 1217 RACKBETA). The probes generally obtained a specific activity of 1 X 10^9 cpm/µg of DNA template. As required, labelled DNA was denatured by heat (2 min; 100°C), chilled on ice (5 min) and then used for hybridization as described below.

2.12. Southern Blots

10 µg of sample genomic DNA of *Lophopyrum elongatum* were digested individually with *Bam* HI, *Eco* RI, *Eco* RV, *Hind* III, *Hae* III, *Hinf* I, and *Xba* I. 10 µg of genomic DNA of other species were digested with *Bam*H I, *Eco*RI, and *Hind*III. The DNA fragments were separated by gel electrophoresis in a 15 cm 1% agarose gel in 3 X TAE buffer, run for 18 hours at 20 V. After completion of electrophoresis, gels were soaked in distilled water for 5 min, followed by

denaturation with the solution of 1.5 M NaCl-0.5 M NaOH for 30 min, then placed in distilled water 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) - 0.001 M EDTA. The DNA fragments were transferred to a Hybond-N^+ nylon membrane (Amersham) by the capillary transfer method with 20 X standard saline citrate (SSC) (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The DNA was fixed to the surface of the nylon membrane by UV irradiation of 254 nm for 2 min. Prehybridization was carried out in a rotisserie hybridization oven (HYBAID, HB-OV-BL. Bio/Can) in the solution containing: 50% deionized formamide, 5 X SSC, 1 X Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinyl pyrolidone), 10% dextran sulfate, sonicated and denatured salmon sperm DNA (100 μg/ml), and 50 mM sodium phosphate buffer (pH 6.5). Filters were prehybridized for at least 4 hours at 42° and, the solution was replaced with hybridization solution containing freshly-denatured ^{32}P-dCTP-labelled DNA probes, and incubation continued at 42°C for 16-24 hours. The membrane was washed twice for 10 min at 42°C with 2 X SSC, 1% SDS, 0.1% sodium pyrophosphate, twice for 10 min at 42°C with 1 X SSC, 1% SDS, 0.1% sodium pyrophosphate, and finally for 15 min at 68°C with 0.5 X SSC, 1% SDS, 0.1% sodium pyrophosphate. The filters were covered with plastic wrap, then placed in a cassette fitted with Lightning Plus (Dupont) intensifying screens with X-ray film. The cassette was placed at -80°C for one to seven days and autoradiographs were subsequently developed.

2.13. cDNA Clones Used as Probes for Southern Blots

In addition to the cDNA clones of *ESI*35 and *ESI*18-1, several other members of *ESI*18 family were partially sequenced by other students in the lab. These partial cDNA clones have similar gene structure and conserved sequences.

Table 4. cDNA clones used in the genomic organization analysis

Clone	Length (bp)	Lysine rich repeat
*ESI*35	1152	3
*ESI*18-1	1122	4
*ESI*18-2	672	3
*ESI*18-10	679	3
*ESI*18-14	490	2

2.14. Expression of *ESI*18-1 and *ESI*35 in *E. coli*

cDNA clone *ESI*18-1 used for expression was unmodified. The insert is between the *Eco* RI and *Xho* I cloning sites of pBluescript (SK⁻) vector. The coding region of *ESI*18-1 is in frame with the ATG start codon of *Lac* Z. cDNA clone *ESI*35 in pBluescript (SK⁻) as originally isolated from cDNA libary was modified for expression. A restriction enzyme, *Cla*I was used to digest the clone 9 bp upstream of the ATG start translation codon. Mung bean exonuclease was used to remove the single strand overhang region and thus remove 2 bp from the sequence. The plasmid was religated and the resulting clone has an ORF in frame with the *Lac* Z ATG start translation codon. This ORF includes both the 5' UTR (Untranslated Region) and the coding region of *ESI*35. 100 µl of overnight cultures in LB medium with ampicillin (40 mg/l) was transferred into a test tube with 1 ml LB medium containing ampicillin (40 mg/l). After 1 h of incubation at 37°C, 1 mM IPTG was added to the bacterial suspension, and 3 h later the bacteria were collected by centrifugation and resuspended in 1 ml of TE buffer (pH 8.0). After one freeze-thaw cycle, the cells were disrupted by sonication and the lysate was centrifuged at 15,000g for 15 min. Protein

concentrations were determined by a dye-binding assay (Bradford, 1976) using bovine gamma globulin (BioRad 500-0005) as a standard.

2.15. Protein Extraction From *L. elongatum* Embryos

Crude protein was extracted from embryo by grinding in a precooled mortar with TRIS buffer [30 mM Tris-HCl, pH 7.6 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% β-Mercaptolethanol]. The extract was immediately centrifuged for 5 min at 15,000g for 10 min to remove debris. In some cases the supernatant was further clarified by centrifugation at 15,000 g for 15 min. The supernatant was boiled for 10 min and then centrifuged at 15,000 g for 20 min to eliminate high temperature insoluble proteins. Protein concentrations were determined as previously described by a dye-binding assay.

2.16. Protein Extraction From *L. elongatum* Roots

Seedlings and plants older than 90 days were transferred from Hoagland solution to fresh solution. After 24 hr, NaCl was added to the solution to the final concentration of 250 mM. Crude protein was extracted from the plant roots after 6, 9, 12, 24, and 48 hr of treatment with NaCl and from non-treated control plants by grinding the roots with a precooled mortar in the presence of Tris buffer as described above in 2.15. Protein concentrations were determined also as previously described by dye-binding assay.

2.17. Immunoblots

The soluble proteins were separated by electrophoresis on 12% polyacrylamide-SDS gels (SDS-PAGE) using Mini-Protean II Electrophoresis Cells (BioRad), then transferred electrophoretically in Mini Trans-Blot Cells (BioRad) to nitrocellulose. After blocking with 3% gelatin in TBS (50 mM Tris pH 7.8, 1 mM $MgCl_2$, 150 mM NaCl), the blot was incubated with a 1:1000 dilution of the purified WCS120 antibody (Houde, et al., 1992) or 1:600 dilution of the antibody against dehydrins (Close et al., 1993). After washing with TTBS (TBS plus 0.1% Tween 20), the blot was incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Fisher OB1400-AlPH). Secondary antibody was detected using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Pre-stained protein standards (BioRad) included the following proteins (with their apparent modified molecular weight): rabbit muscle phosphorylase B (110 kDa), bovine serum albumin (84 kDa), hen egg white ovalbumin (47 kDa), bovine carbonic anhydrase (33 kDa), soybean trypsin inhibitor (24 kDa), hen egg white lysozyme (16 kDa).

2.18. Antibodies

The two antibodies used in this study are anti-dehydrin antibody and anti-WCS120 antibody. Dehydrins are a family of plant desiccation related proteins that are characterized by the consensus KIKEKLPG amino acid sequence domain found at the carboxy terminus, and usually repeated from one to many times within the complete protein. The anti-dehydrins were produced by the coupling of a synthetic pepetide covering this consensus sequence to activated BSA and injecting into rabbits (Close et al., 1993). Anti-WCS120 antibody was raised against the gene product of *Wcs*120 (Wheat cold stress). This gene also belongs to the dehydrin family based on the gene structure since its protein sequence has the conserved regions found in all dehydrins (Houde et al., 1992).

3. RESULTS

3.1. The DNA Sequence of cDNA Clone *ESI*35

The nucleotide sequence of the cDNA clone *ESI*35 is presented in Fig. 2. In this 1131 bp sequence, the first ATG occurs at position 86 and is followed by a 759 bp open reading frame (ORF) encoding 253 amino acid polypeptide. The ORF is followed by 287 bp of a 3' non-coding region. Similar to many other plant genes, the context sequence of the first ATG has A at the -3 position and G at the +4 position (Joshi, 1987). Other nucleotide bases in the context sequence are not similar to the plant consensus TAAACAATGGCT. The 3' end of this cDNA insert is characterized by a 21-bp long poly (A) tail and absence of any consensus AATAAA-like polyadenylation signal which usually occures between 9-23 bp upstream from the polyadenylation site (Joshi, 1987). However, a similar polyadenylation signal, ATATTAAA, occurs at position 1093.

Figure 2. The nucleotide and deduced amino acid sequences of cDNA clone *ESI*35. The nucleic acid sequence is presented on the top line with the derived amino acid sequence below. The DNA sequence was obtained on both strands by the chain termination method. "*" indicates the positions of the stop codon. The characteristic serine run and semi-conserved lysine-rich repeats are underlined.

```
AAGCCACAAGCCAAGAACCAATACTTGATCTGTTGTTTTCTTTAGTTCCCGGAAGACTT   59
TTTAGCTGCACCGATCGATCTCGATC  ATG GAG GAT GAG AGG AGC ACC CAG  109
                            M   E   D   E   R   S   R   Q     8
TCA TAC CAG GGA GGT GAG GCC GCC GAG CAG GTG GAG GTG ACG GAT   54
S   Y   Q   G   G   E   A   A   E   Q   V   E   V   T   D    23
AGG GGC CTC CTC GGC AAC CTC CTC GGC AAG AAG GAG GAG GAG GAG  199
R   G   L   L   G   N   L   L   G   K   K   E   E   E   E    38
GAC AAG CAG AAG GAG GAG GAG CTG GTC ACC GGC ATG GAG AAG GTC  244
D   K   Q   K   E   E   E   L   V   T   G   M   E   K   V    53
TCC GTG GAA GAG CCC GAG GTT AAG AAG GAG GAG CAC GAG GAT GGC  289
S   V   E   E   P   E   V   K   K   E   E   H   E   D   G    68
GAG AAG AAG GAG ACC CTC TTC TCC AAG CTG CAC CGA TCC AGC TCC  334
E   K   K   E   T   L   F   S   K   L   H   R   S   S   S    83
AGC TCC AGC TCG TGT AGT GAC GAG GAA GAG GAG GAG GTG ATC GAC  379
S   S   S   S   S   S   D   E   E   E   E   E   V   I   D    98
GAC AAC GGC GAG GTG ATC AAG AGG AAG AAG AAG AAG GGG CTC AAG  424
D   N   G   E   V   I   K   R   K   K   K   K   G   L   K   113
GAG AAG CTG CCC GGC CAC AAG GAC AAC GAG GGT GAG CAC GTG ACG  469
E   K   L   P   G   H   K   D   N   E   G   E   H   V   T   128
GGC CTA CCC GAC CCG GCG GCC CCC GCG TCT GTG CAG ACC CAC CAT  514
G   L   P   A   P   A   A   P   A   S   V   Q   T   H   H   143
GAC ACC GAC GTC GTC GTC GAG AAG ATC GAC GGT GAC GTG AAG ACA  559
D   T   D   V   V   V   E   K   I   D   G   D   V   K   T   158
GAG GCG ACA CCT CCA GTG CCC GAG GAG GAG AAG AAA GGC TTC ATG  604
E   A   T   P   P   V   P   E   E   E   K   K   G   F   M   173
GAA AAG ATC AAG GAG AAG CTG CCC GGC GGC CAC AAG AAG CCG GAG  649
E   K   I   K   E   K   L   P   G   G   H   K   K   P   E   188
GAC GTT GCT GCG GTG CCC GTC ACG CAC GCT GCC CCA GCA CCG GTG  694
D   V   A   A   V   P   V   T   H   A   A   P   A   P   V   203
CAC GCG CCG GCC GCC GAG GAA GTG AGC AGC CCT GAC GCG AAG GAG  739
H   A   P   A   A   E   E   V   S   S   P   D   A   K   E   218
AAG AAG GGC TTG CTG CCG AAG ATC ATG GAC AAG CTG CCT GGT TAC  784
K   K   G   L   L   G   K   I   M   D   K   L   P   G   Y   233
CAC AAG ACA GGG GAG GAG GAC AAG GCC GCC GCC CCT GCA GGC GAG  829
H   K   T   G   E   E   D   K   A   A   A   P   A   G   E   248
CAC AAG CCC AGC GCT TAA TCGCCGCCGTGCCCGAGACTCGTGACCGGAGCTCG  882
H   K   P   S   A   *                                        253
ATTGAATTGTTGGCGTGTGTTGTGTTTGCTTTACGTCTAAGTTGGTGTCAAGGTGGGA  941
GGGGTTGATCGTCTCTGAAGGTCCGGTCTGTGAAGCCCGTTCAGTGACGGATGTTTGT 1000
GTTTCAGTTTGGTTCAGAGTCAGGTCCTGGATGTTGTTAAGTTTGTTTACTTATGGGC 1059
ACTTGTGTATTGGTTTATTGCTGGGCATTATGCCTTGATATTAAAGATTTCCGCCCAG 1118
ATTTGCTTATTGTTGCAAAAAAAAAAAAAAAAAAA                        1152
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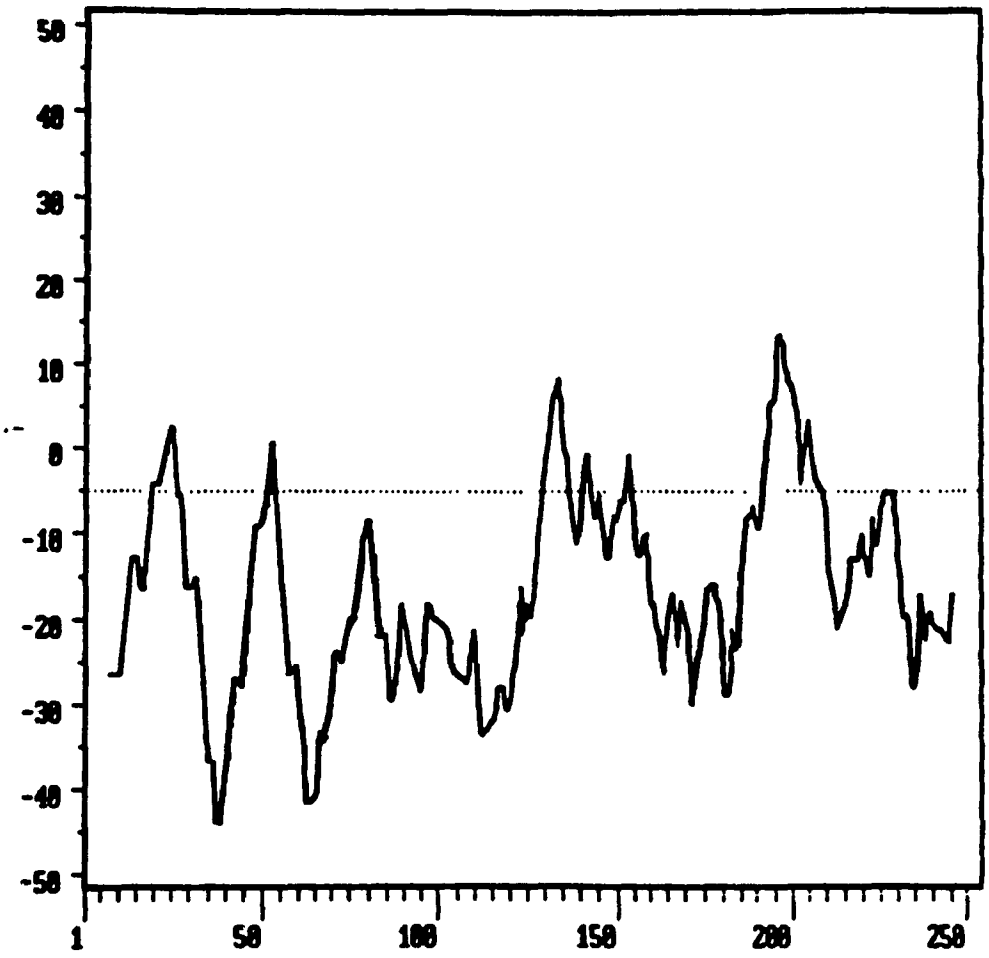
3.2. The Protein Sequence of cDNA Clone *ESI*35

The deduced amino acid sequence of the clone *ESI*35 is shown in Fig. 2. The predicted relative molecular mass of the derived polypeptide is 27,452 with an isoelectric point of 4.8. Figure 3 shows the hydropathy plot of the deduced amino acid sequence of *ESI*35 calculated according to method of Kyte and Doolittle (1982) using a window of 15 residues (Kyte and Doolittle, 1982). This predicted protein has a pronounced hydrophilicity throughout its length. The predicted protein contains a run of 9 serines and a repeated lysine-rich amino acid sequence. The lysine-rich amino acid sequence is repeated three times in the ORF (underlined). Two lysine rich repeats are present between amino acid positions 169 and 184 and 219 and 234. The third lysine rich repeat is present between amino acid position 105 and 120.

Table 5. Mole percent composition of *ESI*35 amino acid sequence

Amino Acid	Mole Percentage	Amino Acid	Mole-Percentage
Ala (A)	8	Met (M)	2
Cys (C)	0	Asn (N)	1
Asp (D)	6	Pro (P)	7
Glu (E)	17	Gln (Q)	2
Phe (F)	1	Arg (R)	2
Gly (G)	8	Ser (S)	7
His (H)	4	Thr (T)	4
Ile (I)	2	Val (V)	8
Lys (K)	14	Trp (W)	0
Leu (L)	6	Tyr (Y)	1

Figure 3. Hydropathy profile of *ESI*35 protein generated according to procedure of Kyte and Doolittle (1982) using a 15-amino acid averaging window.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10
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-10
-20
-30
-40
-50
1
50
100
150
200
250

3.3. The DNA Sequence of cDNA Clone *ESI*18-1

The DNA sequence of partial cDNA clone *ESI*18-1 is shown in Fig. 5. The 1 kb clone, a partial length cDNA clone (the full length mRNA from this gene being estimated to be 2.1 Kb), contains a 795 bp open reading frame. This open reading frame is terminated by the stop codon, TAA, at position 797 followed by 206 bp of a 3' nontranslated region. The 3' end of this cDNA insert has an 18-bp long poly $(A)^+$ tail and the presence of the AATAAG seqence at positions between 837 and 842 which is similar to the concensus AATAAA polyadenylation signal in most of the plant genes (Joshi, 1987).

3.4. The Protein Sequence of cDNA Clone *ESI*18-1

The deduced amino acid sequence of the cDNA clone *ESI*18-1 is presented in Fig. 5. The deduced amino acid sequence of *ESI*18-1 was used to generate a hydropathy plot (Fig. 6). The *ESI*18-1 predicted protein has pronounced hydrophilicity throughout its length, calculated by the method of Kyte and Doolittle using a window of 15 aa residues.

The four lysine rich repeats which are present between amino acid positions 55 and 70, 119 and 134, 183 and 198, 250 and 265 respectively, are almost identical with the consensus sequence of KKGI(K/M)(E/D)KIKEKLPG(G/Q)H (Close *et al.*, 1989).

The intervening region between these lysine-rich repeats is characterized by 6 imperfect repeats of a glycine rich repeating sequences which are inturn composed of 2 subrepeats of 20 glycine rich amino acids with the following consensus sequence: TGGTYGQHGHTGVTGTGMHG. These repeats occur at positions 9-28, 32-51, 96-115, 160-179, 204-223 and 224-243 followed by a portion of the repeat with the sequence TDGTGG at positions 244-249.

Figure 4. Nucleotide sequence and the deduced amino acid sequence of partial cDNA clone *ESI*18-1. The sequence was obtained on both strands by dideoxy chain termination reaction.

```
 CT GGA GGC CAC GGT GAC CAC CAG CAG AGC GGT GGC ACC TAC GGG  44
    G   G   H   G   K   H   Q   Q   S   G   G   T   Y   G   14
CAC CAG GAA GAC ACC GAG ATA ACT GGC ATG GGG ATG CAT AGC ACC  89
H   Q   E   D   T   E   I   T   G   M   G   M   H   S   T   29
CCG GCC ACC GGC GGC GCC TAT AGG CAG CAT GAA CAC ACC ACA GTG 134
P   A   T   G   G   A   Y   R   Q   H   E   H   T   T   V   44
ACC GGC ACG GGA ATG CAC GGC ACC GAC GAG AAG AAG GGT GTC ATG 179
T   G   T   G   M   H   G   T   D   E   K   K   G   V   M   59
GAG AAC ATA AAG GAG AAG CTT CCT GGT CCG CAT GTT GAC CAC CAG 224
E   N   I   K   E   K   L   P   G   G   H   V   D   H   Q   74
CAG ACC GCA GGC TCC TAC GGG CAG CAG GGA CAC GTC GAC ACG GGG 269
Q   T   A   G   S   Y   G   Q   Q   G   H   V   D   T   G   89
ACC CAT GGC ACG CCG GCC ACC GGC GGC ACC TAC GGG CAG CAC GGG 314
T   H   G   T   P   A   T   G   G   T   Y   G   Q   H   G  104
CAC ACC GGA GTG ACC GGC ACG GGA ATG CAC GGC ACC GGC GAG AAG 359
H   T   G   V   T   G   T   G   M   H   G   T   G   E   K  119
AAG GGT GTC ATG GAG AAC ATA AAG GAG AAG CTT CCC GGT GGC CAT 404
K   G   V   M   E   N   I   K   E   K   L   P   G   G   H  134
GCT GAC CAC CAC CAG ACC GCA GGC TCG TAC GGG CAG CAG GGA GAC 449
A   D   H   H   Q   T   A   G   S   Y   G   Q   Q   G   D  149
GTC GAC ACG GGG ACG CAT GAC ACG CCG GCC ACC GAC AAC ACC AAT 494
V   D   T   G   T   H   D   T   P   A   T   D   N   T   N  164
GGG CAG CAC GGA CAC ACC GGA GTG ACC GGC ACT GGT ACA CAT GGC 539
G   Q   H   G   H   T   G   V   T   G   T   G   T   H   G  179
ACC GGC GAG AAG AAG GGT GTC ATG GAG AAC ATC AAG GAG AAG CTC 584
T   G   E   K   K   G   V   M   E   N   I   K   E   K   L  194
CCC GGT GGT CAC AAT GAC CAT CAT CTG ACC ACT GAC ACC TAC GGA 629
P   G   G   H   N   D   H   H   L   T   T   D   T   Y   G  209
CAG CAT GGA CAC ACC GGA GTG ACC GGC ACA GAG ACG CAT GGC ACC 674
Q   H   G   H   T   G   V   T   G   T   E   T   H   G   T  224
GGT GGC ACC TAC GGG CAG CAG GCA CAC ACC GGC ACG ACC GGC ACT 719
G   G   T   Y   G   Q   Q   A   H   T   G   T   T   G   T  239
GGG ACA CAC GGC ACC GAC GGC ACC GGG GGG AAG AAG AGC CTC ATG 764
G   T   H   G   T   D   G   T   G   G   K   K   S   L   M  254
GAC AAG ATC AAG GAG AAG CTG CCT GGA CAG CAC TAA GTCCGGTCCGA 811
D   K   I   K   E   K   L   P   G   Q   H   *              265
CCCGGCAGCGGCTGCTACCTCTTCAGAATAAGATGGCGAACTTCCACCGTATACACATT 870
CACGGATTCACCTAGCTCACTTGGTCGTTGGAGGAGCGAATGTATCTTGGCTTAAGTTT 929
TCACTGACAACAGTATGTTGACAGTTTTCGTTTATTTACAGCCTGCAGACTGTTGTGCA 988
AATTTCCTTTTGGTTCAAAAAAAAAAAAAAAAA
1022
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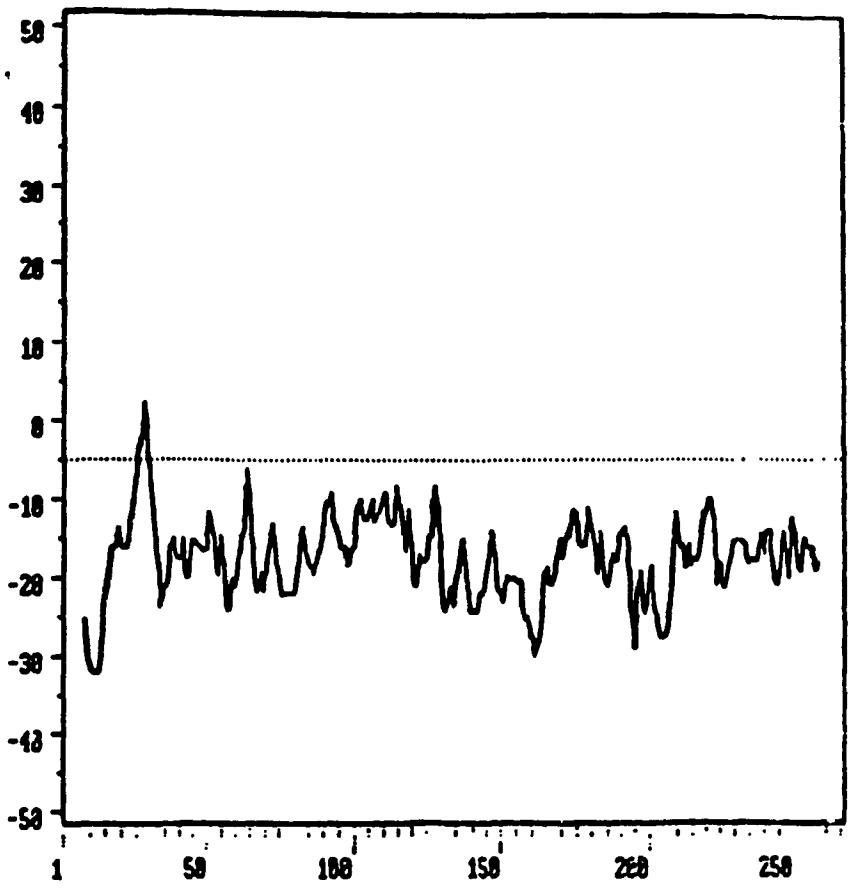
Figure 5. Hydropathy profile of *ESI*18-1 protein generated according to procedure of Kyte and Doolittle (1982) using a 15-amino acid averaging window.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3.5. Genomic Southern Analysis

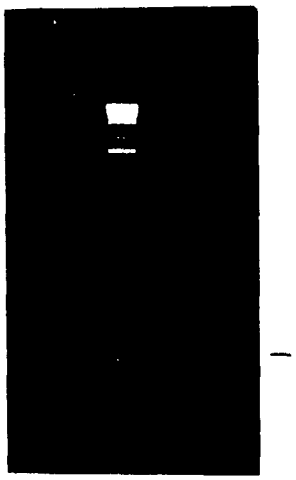
The cDNA clones, used as probes in this study are listed in Table 4. The PCR product which contains a 121 bp fragment of *ESI*18 including one carboxy terminal lysine-rich repeat was also used as a probe in the Southern analysis. Fig. 6a shows the 121 bp amplified fragment on 2% agarose gel by electrophoresis. A diagram showing the portion of *ESI*18-1 used as PCR generated probe is shown in Fig. 6b.

Previous studies have indicated that genes encoding RAB/DHN/LEA proteins belong to small families in higher plants (Villardell et al., 1990; Piatowski et al., 1990; Yamaguchi-Shinozaki et al., 1989). To determine the complexity of the *ESI* gene family in diploid *L. elongatum*, the genomic Southern analysis was performed. Genomic DNA, digested to completion with *Bam* HI, *Eco* RI, *Eco* RV, *Hind* III, *Xho* I, *Hae* III, or *Hinf* I, was analyzed by Southern blots. The blots hybridized with the entire *ESI*35 cDNA probe showed one strong hybridization signal in each digest with the exception of the *BamHI* digest in which two bands appeared (Fig. 7). On the contrary, hybridization using probes from the various *ESI*18 family members (listed in Table 4) and the PCR product from the repeated region of the *ESI*18 gene showed a multitude of bands in each digest (Fig.8, Fig. 9, Fig. 10, Fig 11, Fig. 12). This suggests that presence of multigene *ESI*18 family in *L. elongatum*. The 1 kb cDNA *ESI*18-1 probe strongly hybridized to three *Bam* HI fragments, four *Eco* RI fragments, four *Eco* RV fragments, four *Hind* III fragments, four *Xho I* fragments, and three *Hinf* I fragments (Fig. 8). The 0.7 kb cDNA *ESI*18-10 probe strongly hybridized to two fragments in each digest, and several weak hybridization signals are also visible (Fig. 9). The 121 bp PCR probe hybridized to three *Bam HI* fragments, four fragments of *Eco* RI, *Eco* RV, and *Hind* III separately, and three *Hinf* I fragments (Fig. 10). The 0.7 kb cDNA *ESI*18-2 probe strongly hybridized to three Bam HI fragments, three *Eco* RI fragments, four *Eco* RV fragments, two *Xho* I

fragments, and two *Hae* III fragments (Fig. 11). The 0.5 kb cDNA *ESI*18-14 probe strongly hybridized to three fragments of *Bam* HI, *Eco* RV, and two fragments of *Eco* RI digests (Fig. 12).

The diplid *Triticum* spp. are closely related to tetraploid and hexaploid wheat. Southern blot analysis was conducted to determine the presence and approximate gene copy number. Genomic DNA from four diploid *Triticum* spp. (*T. uratu, T. tauschii, T. monococcum, T. boeticum*) were isolated and digested with restriction enzymes of *Bam* HI, *Eco* RI, and *Hind* III. For the *T. tauschii*, there are more hybridization bands apparent than in any other species. For these three restriction enzyme digestions, there are at least three hybridization bands present in Southern blots for all species.

Figure 6. a. The amplified 121 bp fragment containing terminal glycine and lysine rich repeated sequences. b. The deduced amino acid sequence is shown schematically. K represents the lysine-rich repeat and G represents the glycine repeat.



A

ESH8-1
265 aa

40 aa

B

Figure 7. Southern blot analysis of genomic DNA. *L. elongatum* DNA (10 μg) was degested with 7 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with *ESI*35 Lane 1, *Bam* HI; Lane 2, *Eco* RI; Lane 3, *Eco* RV; Lane 4, *Hind* III; Lane 5, *Xho* I; Lane 6, *Hae* III; Lane 7, *Hinf* I.

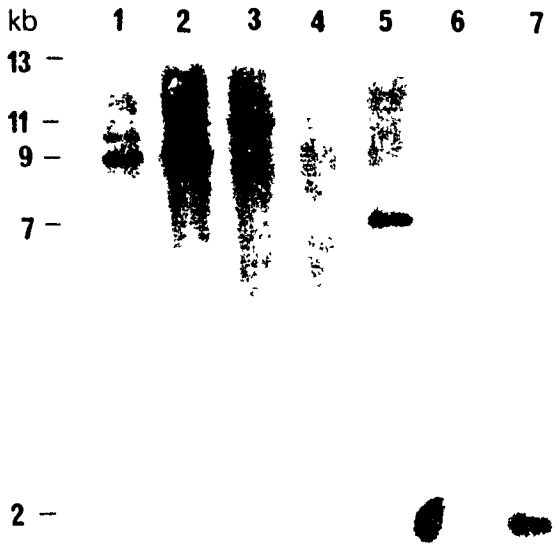

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Figure 8. Southern blot analysis of genomic DNA. *L. elongatum* DNA (10 μg) was digested with 7 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with *ESI*18-1. Lane 1, *Bam* HI; Lane 2, *Eco* RI; Lane 3, *Eco* RV; Lane 4, *Hind* III; Lane 5, *Xho* I; Lane 6, *Hae* III; Lane 7, *Hinf* I.

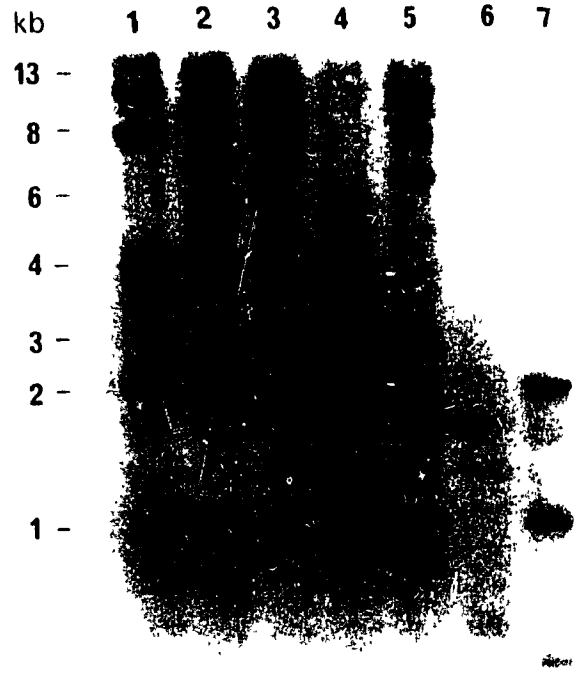
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Figure 9. Southern blot analysis of genomic DNA. *L. elongatum* DNA (10 μg) was digested with 7 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with *ESI*18-10. Lane 1, *Bam* HI; Lane 2, *Eco* RI; Lane 3, *Eco* RV; Lane 4, *Hind* III; Lane 5, *Xho* I; Lane 6, *Hae* III; Lane 7, *Hinf* I.


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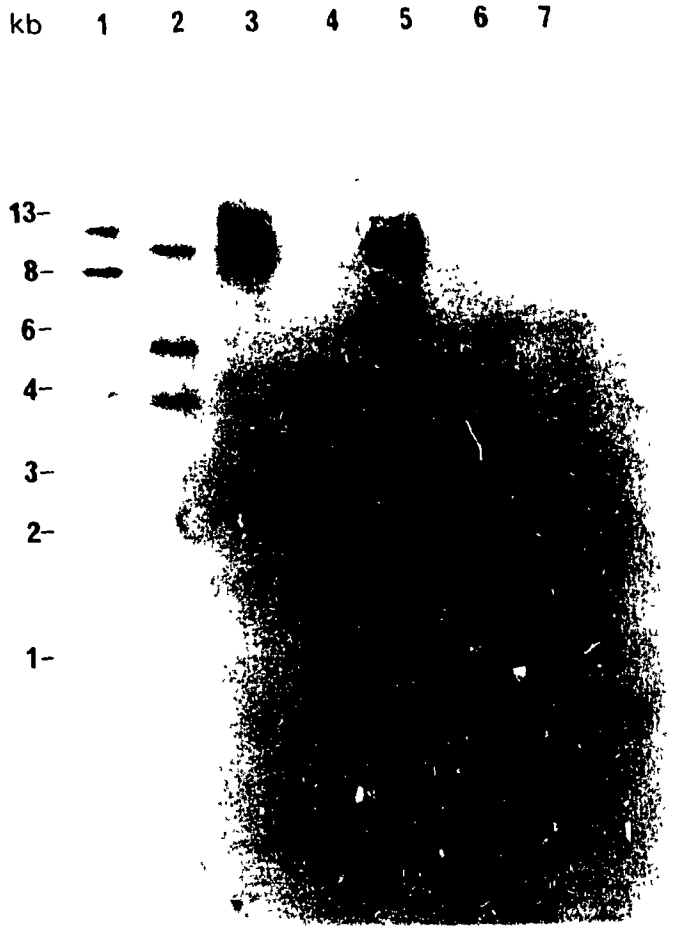
Figure 10. Southern blot analysis of genomic DNA. *L. elongatum* DNA (10 μg) was digested with 7 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with PCR product. Lane 1, *Bam* HI; Lane 2, *Eco* RI; Lane 3, *Eco* RV; Lane 4, *Hind* III; Lane 5, *Xho* I; Lane 6, *Hae* III; Lane 7, *Hinf* I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2–
1–

Figure 11. Southern blot analysis of genomic DNA. *L. elongatum* DNA (10 μg) was digested with 7 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with *ESI*18-2. Lane 1, *Bam* HI; Lane 2, *Eco* RI; Lane 3, *Eco* RV; Lane 4, *Hind* III; Lane 5, *Xho* I; Lane 6, *Hae* III; Lane 7, *Hinf* I.


Kb
1
2
3
4
5
6
7
13
11
8
2

Figure 12. Southern blot analysis of genomic DNA. *L. elongatum* DNA (10 µg) was digested with 7 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with *ESI*18-14. Lane 1, *Bam* HI; Lane 2, *Eco* RI; Lane 3, *Eco* RV; Lane 4, *Hind* III; Lane 5, *Xho* I; Lane 6, *Hae* III; Lane 7, *Hinf* I

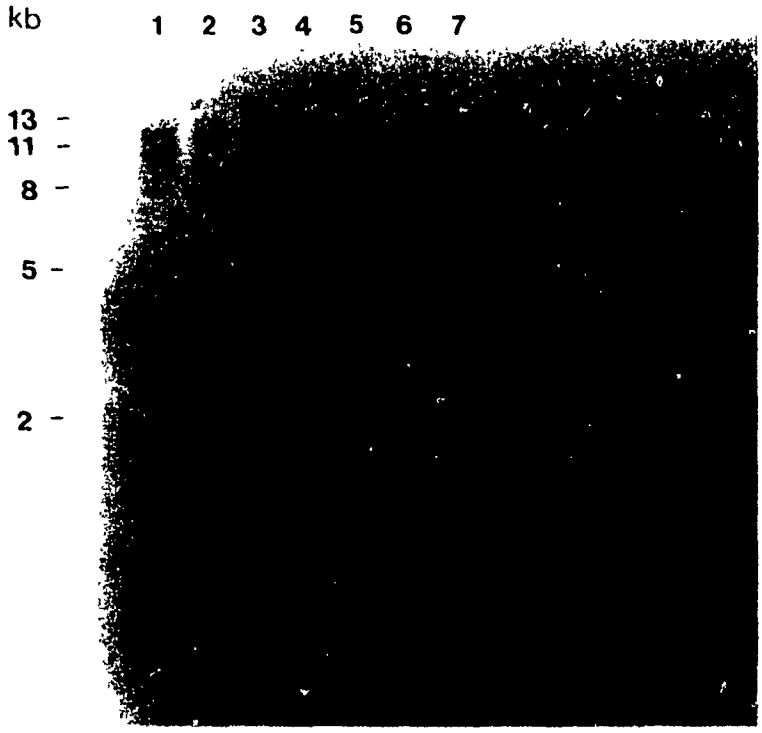
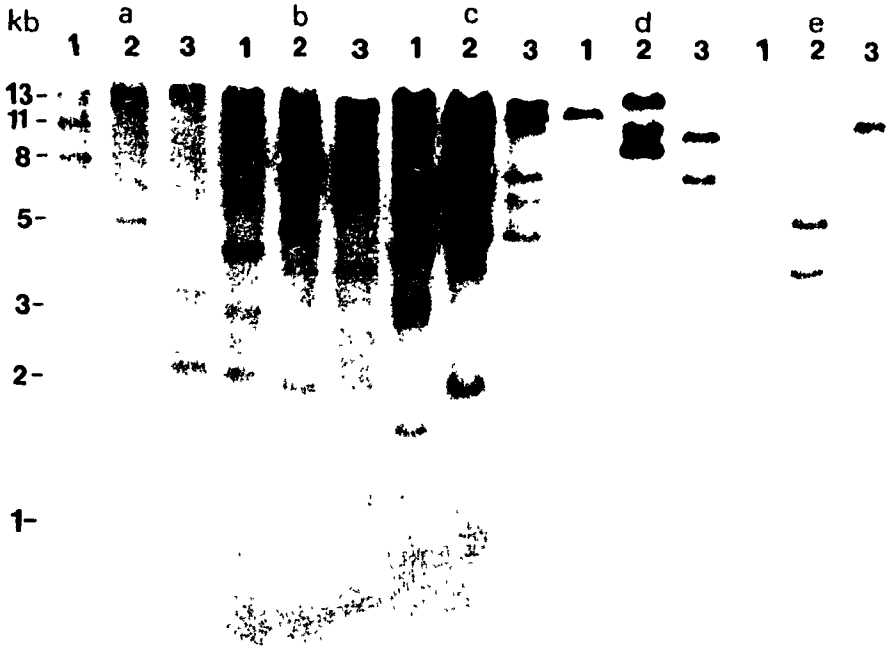

kb
1 2 3 4 5 6 7
13
11
8
5
2

Figure 13. Southern blot analysis of genomic DNA. Genomic DNA (10 μg) from four *Triticum* species, a, *L. elongatum*; b, *T. uratu*; c, *T. tauschii*; d, *T. monococcum*; e, *T. boeticum* digested with three different restriction enzymes, separated by the agarose gel electrophoresis, transferred to nitrocellulose, and then probed with the 121 bp fragment. Lane 1, *Bam* HI; Lane 2, *Eco* R1; Lane 3, *Hind* III.


kb
a
b
c
d
e
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
13-
11-
8-
5-
3-
2-
1-

3.6. Identification of the Polypeptides Encoded by *ESI*18-1 and *ESI*35

To identify the encoded proteins, the *ESI*18-1 ORF and *ESI*35 ORF were expressed in *E. coli*. Figure 14 shows that the polypeptide encoded by *ESI*18-1 reacted strongly with the anti-dehydrin antibody and the anti-WCS120 antibody but not with pre-immune serum. In contrast, the polypeptide extracted from *ESI*35 clone was not detected by either of this two antibodies.

3.7. Detection of Proteins in *L. elongatum* Embryos and roots by Anti-dehydrin antibody and anti-WCS120 antibody

The result of immunoblotting for the protein extracted from embryos using anti-dehydrin antibody and the anti-WCS120 antibody is shown in Figure 15. The antibodies recognized three proteins ranging in apparent molecular weight from about 25 to 70 kDa. The profile of boiled samples and the unboiled samples are the same, ie, the antibodies recognized the same number and size of proteins from both boiled and unboiled protein extracts. There is no protein extracted from salt stressed *L. elongatum* roots recognized by these two antibodies.

Figure 14. Immunoblot analysis of *ESI*18-1 and *ESI*35 proteins isolated from *E. coli*. A, anti-dehydrin antibody; B, Pre-immune serum; C, Purified anti-WCS120 antibody; D, Pre-immune serum. a, plasmid (pBluescript) without insert (IPTG); b, *ESI*35 (IPTG); c, *ESI*35 (no IPTG); d, *ESI*18-1 (IPTG); e, *ESI*18-1 (no IPTG)

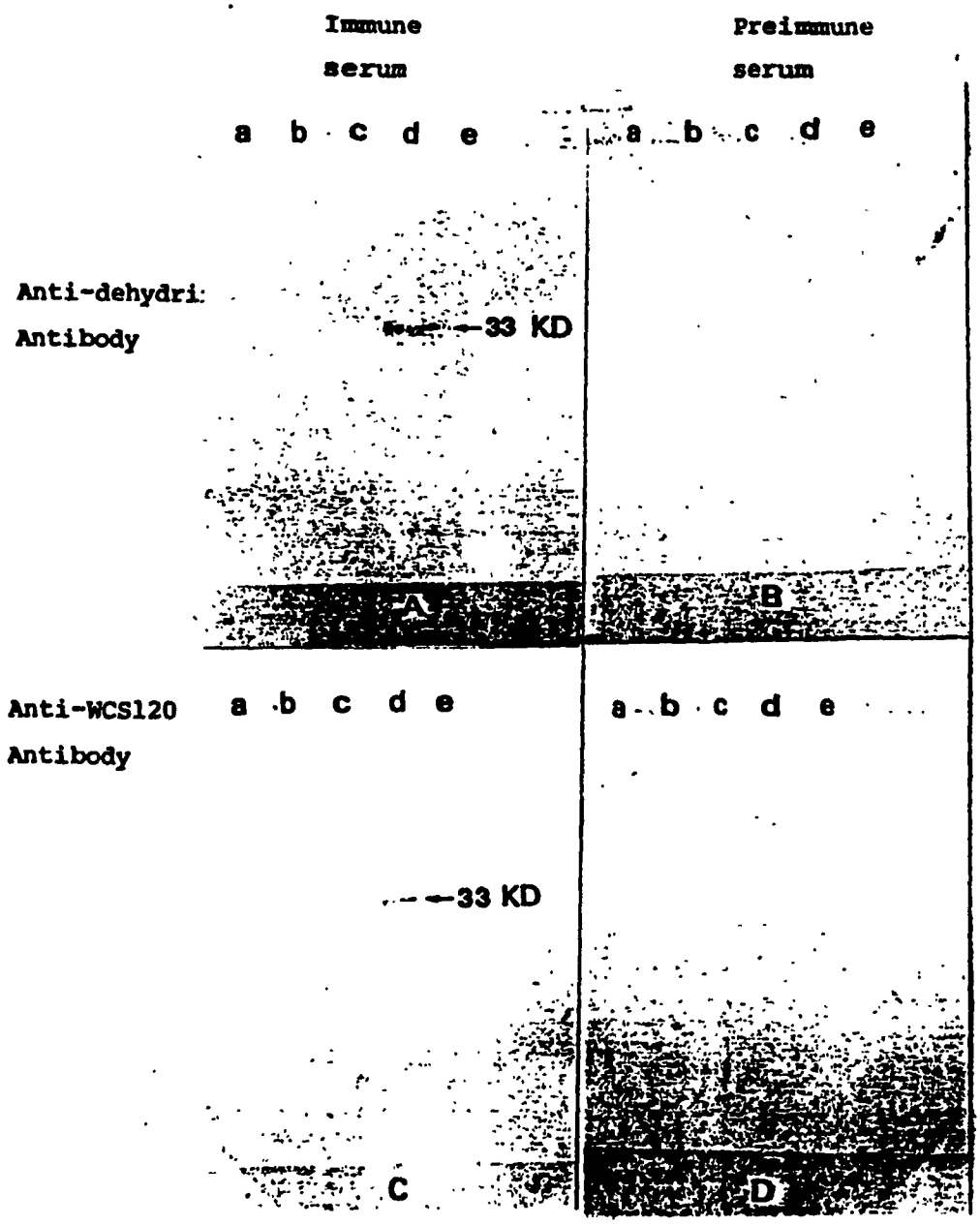
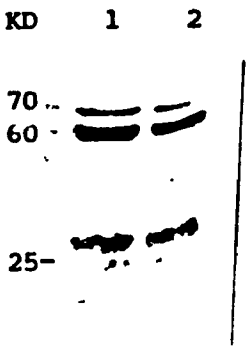
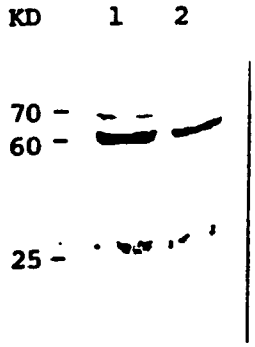

Immune serum
Preimmune serum
a b c d e
a b c d e
Anti-dehydri:
Antibody
33 KD
A
B
Anti-WCS120
Antibody
a b c d e
a b c d e
33 KD
C
D

Figure 15. Immunoblot analysis of proteins isolated from *L. elongatum* embryos. A, Anti-dehydrin antibody, Lane 1, unboiled sample; Lane 2, boiled sample. B, Anti-WCS120 antibody, Lane 1, unboiled sample; Lane 2, boiled sample



A



B

4. DISCUSSION

The predicted MW of the protein encoded by *ESI*35 is 27 kDa. The encoded polypeptide is extremely hydrophilic. The presence of an in-frame stop codon before the initial ATG indicates that the clone includes the entire coding region of the cDNA. In addition, the cDNA of 1152 bp is in agreement with the size of a full length cDNA as predicted by northern blot analysis (Gulick and Dvorak, 1992).

Figure 17 shows the dot matrix comparison of amino acid sequences for *ESI*35 and Dhn3. Small fragments of a diagonal line indicating regions of high similarity are visible. It is apparent from the plots that tracts of high similarity exist only in the regions of the lysine rich motif and the serine run. Regions containing 50% or higher sequence identity between the proteins comprise only 13% of the *ESI*35 aa sequence.

Though *ESI*35 does not show significant overall sequence similarity to these dehydrin gene sequences, it has subregions that strongly suggest it is homologous to known dehydrin-like genes, and the conservation of these sequences indicates a functional importance for these regions. In spite of these recognizable features, *ESI*35 has other sequence characteristics that make it the most atypical dehydrin-like gene among the more than 20 sequences reported to date. Unlike nearly all other dehydrins, the *ESI*35 encoded protein does not have a lysine rich repeat at its carboxyl terminus. Its lysine rich repeat closest to its carboxyl terminus is followed by 19 amino acids. There is one other reported dehydrin like sequence D11, which lacks a lysine rich carboxyl terminal repeat (Galau and Close, 1992), however that sequence appears to have a different origin than *ESI*35. It is apparent from its cDNA sequence that the D11 gene has aquired a stop codon mutation between the two lysine rich repeats, thus eliminating one lysine rich repeat and leaving 11 amino acids at the COOH end of the remaining lysine repeat in the ORF. The gene is hypothesized to be nonfunctional (Galau and Close, 1992).

*ESI*35 has 3 degenerate lysine rich repeats and no evidence in the sequence of its 3'UTR of a mutation giving rise to premature truncation of the ORF. Furthermore, *ESI*35 homologues have been mapped in *L. elongatum*, wheat and barley by Southern blot analysis with the *L. elongatum* cDNA as a probe indicating that this gene sequence has been conserved over a evolutionary time, suggesting that it is a functional gene. Since *ESI*35 and its closely related homologues occur in a single copy per haploid genome in *L. elongatum* as well as in wheat & barley, it appears that such analysis is gene specific, any cross hybridization to more typical dehydrin genes would be expected to reveal multiple gene copies. A cDNA clone of the *ESI*35 homologue from wheat, Wcor410, has recently been isolated (Danyluk *et al.*, 1994). The deduced amino acid sequence of Wcor410 has 95.6% identity with that of *ESI*35. The authors have shown that its corresponding mRNA levels are induced by cold acclamation and that there is a positive correlation between the level of mRNA accumulation and the level of freezing tolerance among wheat genotypes (Danyluk *et al.*, 1994).

A structural comparison of *ESI*35 to the typical dehydrin like protein is presented in a schematicly in Figure 17. The lysine-rich motif and the serine repeats are characteristic of the ABA inducible *rab*16, *dhn* and *lea*-D11 gene families. As mentioned above, previously reported sequences of this family commonly contain two Lys-rich repeats with one occurring at the carboxy terminus of the protein sequence. Conservation of this Lys-rich repeat is relatively high, having about 85% identity among monocotyledonous species. *ESI*35 contains three Lys-rich repeats, however, the repeat located closest to the carboxy terminus is followed by an additional 19 amino acids. This subterminal repeat is also the least conserved of the three in relation to the consensus sequence for this motif. The most conserved Lys-rich repeat, the middle repeat in *ESI*35 has only 65% identity with the consensus sequence. A more conserved subset of this region composed of KEKLPG is found in

only the first two repeats in *ESI*35. The regions which have been conserved in different species most likely have functional significance. In the compositional bias for Gly (8.2%), *ESI*35 is not similar to dehydrin like proteins which have more than 25% Gly residues in their polypeptides. Table 6 shows the carboxyl terminal sequence of *ESI*35 and dehydrin-like proteins. Figure 19 shows the dendrogram of alignment of the K-repeat units of *ESI*35 and the related dehydrin-like amino acid sequences in which the lysine rich repeats are numbered sequentially beginning with the carboxyl terminal repeat. Though the K2 repeat of *ESI*35 has relatively high similarity to the K1 repeats of all the other gene sequences used, the K1 and K3 repeats of *ESI*35 are the most divergent sequences used in this comparison. This again underscores the unique nature of *ESI*35 among dehydrin homologues. Unlike most of these dehydrin-like proteins, *ESI*35 does not contain the Gly-rich repeat between its lysine rich repeats. Seemingly, the sequence between the Lys-rich repeats in *ESI*35 is unrelated to the other genes.

The genomic organization analysis of *ESI*35 revealed that there is only a single copy in the *L. elongatum* genome unlike the dehydrin like *ESI*18 gene family which has at least five gene family members. The *Bam* HI digestion has two bands in the Southern blot probably because there is an intron within the *ESI*35 gene which contain a *Bam* HI restriction site. The dissimilarity of *ESI*35 to dehydrins was also shown by western blot analysis. *ESI*35 protein was not immunologically cross-reactive to the antibodies to the dehydrins and the cold-stress induced dehydrin homologue wheat proteins.

In addition, the cDNA clone *ESI*35 contains a motif, characteristic of nuclear-targeting signal sequences (NTS starting at aa 106. This motif shows high homology with essential basic amino acids of other NTS sequences (Table 7). Three of the NTS sequences, *Vir*E2 from *Agrobacterium* (Citovsky et al., 1992), nucleoplasmin from *Xenopus* (Robbins et al., 1991), and O2 from maize (Varagona et al., 1992), have

been demonstrated to be functional by deletion analysis or point mutagenesis. With the exception of a tract of Serine residues and degenerate Lys-rich repeats of the *ESI*35, it has little similarity to the dehydrin-like proteins. These other dehydrin-like proteins do not have sequences resembling this NTS motif. Such sequence analysis raises the possibility that the *ESI*35 protein could encode a dehydrin-like protein with nuclear localization in the *L. elongatum*.

By the comparison between *ESI*18-1, and the related proteins, one extremely conserved repeating motif was found, with a less conserved repeating unit positioned between them. Figure 18 shows the consistent linear order of discrete sequence motifs and their indices of similarity. One highly conserved block among dehydrin like proteins is a tract of 7-9 serine residues; this serine run was not found in *ESI*18-1. However, since cDNA clone *ESI*18-1 is a partial length lacking the 5' end sequence, a tract of serine residues in this region cannot be ruled out. Villardell et al. (1990) have proposed that serine residues are phosphorylated and are likely to be important in the function of these proteins. The second highly conserved block consists of lysine-rich residues in the region after the tract of serine residues with the consensus of EKKG(I/M)MDKIKEKLPG. This repeating unit is seen to be a tract of 19 amino acids that is repeated contiguously. Clearly there is a slight degeneracy in the repeat within a single protein as well as between proteins. In addition, there is another conserved G-repeat unit in the amino acid sequences of *ESI*18-1, and RAB/dehydrins. Furthermore, at the C-terminus of all those proteins (with the exception of *ESI*351) the following identical array of highly conserved amino acids occurs: EKKG(I/V)MDKIKEKLPG(G/-)QH. The regions which have been conserved during evolution are probably due to their functional significance. The deduced polypeptide sequence of *ESI*18-1 has a high degree of sequence similarity to other dehydrin-like gene sequence, though its structure is different from the classical *rab*16/*dhn*/*lea* D11 sequences (Figure 18). The dot matrix comparison of the amino acid sequence of

ESI18-1 and Dhn3 (Figure 21) shows *ESI*18-1 to be much more similar to Dhn3 sequence than is *ESI*35. *ESI*18-1 and Dhn3 do not have colinear structures due different numbers of internal repeating motifs. *ESI*18-1 is composed primary of lysine and glycine repeats and these regions show more than 75% identity to regions in Dhn3. In total, over 90% of the *ESI*18-1 clone sequence shows greater than 75% identity to portions of dehydrins Dhn3 sequence from barley.

Table 6 shows the carboxy-terminal sequence of *ESI*35, *ESI*18-1 and RAB/dehydrin sequences. Figure 19 and 20 show the dendrogram of alignment of the K-repeat units and G-repeat units of *ESI*35, *ESI*18-1, and RAB/dehydrin amino acid sequences. The numbering system used for the dendrogram is the same as that indicated in Fig.18; the carboxyl terminal lysine rich repeat has been numbered 1 and additional repeats are numbered sequentially counting toward the amino terminus of the aa sequence. The dendrogram shows that the *ESI*18-1 carboxyl terminal repeat, K1, is more similar to the K1 repeat of the barley clone Dhn3 than to successive K repeats within *ESI*18-1, suggesting a function for this motif that has been conserved over an evolutionary time scale. *ESI*18-1 repeats K2, K3 and K4 are more similar to each other than to any other lysine rich motif among these genes suggesting that two of these repeats are likely of relatively recent origin and are derived from internal duplication within *ESI*18-1. Since *ESI*18-1 K2, K3, K4 are also quite diverged from K2 of other dehydrin genes, and K2's from those other genes cluster together in the dendrogram, it appears that functional constraints are somewhat different for the *ESI*18-1 internal lysine rich repeats. The most striking differences between the deduced amino acid sequence of cDNA clone *ESI*18-1 and these related proteins is that *ESI*18-1 has 8 glycine rich tandem repeating units. Figure 20 shows the *ESI*18-1 glycine rich repeats, G4 and G6 units are two identical repeats except one amino acid with the consensus of TAGSYGQQGHVDTGTGHTPA which is not found in the other proteins. This supports the hypothesis that two regions containing G4, K3 and

G6, K4 are derived from internal duplication within *ESI*18-1. Since glycine repeat units of *ESI*18-1 are quite divergent from glycine repeat unit of other dehydrines genes, also, the glycine repeat unit of dehydrin genes are quite different from each other, it suggests that the glycine repeat units might not be as functionally important as lysine repeat units for the dehydrin-like genes.

It has been reported that LEA proteins, dehydrins, and several polypeptides induced or accumulated during cold treatment remain soluble during boiling in aqueous solution (Close and Chandler, 1989; Danyluk et al., 1991; Jacobsen and Shaw, 1989; Lin et al., 1990). The large number of hydrophilic residues probably confers a very flexible backbone and this is likely responsible for the boiling stability of those proteins. The high Gly content (25%) of the *ESI*18-1 partial polypeptide may confer high flexibility and mobility. The small size of the Gly molecule and its short side chain gives it a unique function in the structure of proteins. It facilitates the formation of intramolecular hydrogen bonding and thus gives the protein a random coil conformation. Physicochemical characterization of this protein should help to verify this assumption. The significance of those properties is still unclear but the high hydrophilicity may also be important in trapping water inside the cell to prevent local dehydration that may occur during salt stress, freezing or water stress (Greenway and Munns, 1980; Guy, 1990; Steponkus and Lynch, 1989).

In western blot analysis, it is not surprising that the *ESI*18-1 protein was immunologically cross-reactive with the antibodies to the dehydrins and the cold-stress proteins (Fig. 14) since, the *ESI*18-1 polypeptide shares the high aa sequence similarity with those proteins. Moreover, the response of mRNA of *ESI*18 gene to ABA closely approximated the response to NaCl treatment (Galvez et al., 1993). Since ABA plays an important role in stress response, the induction of *ESI*18-1 by ABA suggests that it has a common function in different stresses.

The Southern blot results with *ESI*18-1 revealed that there are at least 5 copies of *ESI*18-1 homologues per genome. Similar results were observed from the hybridization using the probe comprised of one glycine and one lysine rich repeat from *ESI*18 (Fig. 10). We have identified five members of the *ESI*18 gene family from different cDNA clones, and previous northern blot analysis also revealed mRNAs of at least 3 different lengths which hybridized to *ESI*18. Southern blot analysis indicated that *ESI*18 homologues also exist as a multigene family in wild diploid relatives of wheat from the genus *Triticum* (*T. uratu, T. tauschii, T. monococcum, and T. boeticum*). It is perhaps significant that *T. boeticum* and *T. tauschii* have more apparent gene copies than the other species. *T. tauschii* is the progenitor of the D genome of *T. aestivum*, the hexaploid bread wheat (genome ABD). The presence of the D genome distinguishes *T. aestivum* from the related tetraploid pasta wheat *T. durum* (genome AB). *T. aestivum* is considered to be more salt tolerant than *T. durum* and the D genome is thought to be responsible for this greater degree of tolerance. The higher gene copy number observed in the D genome ancestoral species observed by Southern blot analysis of these species provides additional positive correlative data suggesting that *ESI*18 gene family members may be associated with salt-tolerance.

Table 6. Conserved amino acid sequences at the carboxy temini of *ESI*35, *ESI*18-1, RAB16c, Dhn2 and M3

Clone	Amino acid sequence
*ESI*35	KEKKGLLGKIMDKLPGYHKTGEEDKAAAPAGEHKPSA
*ESI*18-1	GGKKSLMDKIKEKLPGQH
RAB16c	GEKKGFMDKIKEKLPGQH
Dhn2	GEKKEIMDKIKEKLPGQH
M3	GEKKGIMEKIKEKLPGQH

Figure 16. Comparison of aa sequences of *ESI*35 and Dhn3 using Dotplot analysis of the COMPARE programme in the GCG analysis package. Window: 20; Stringency: 18; Points: 331

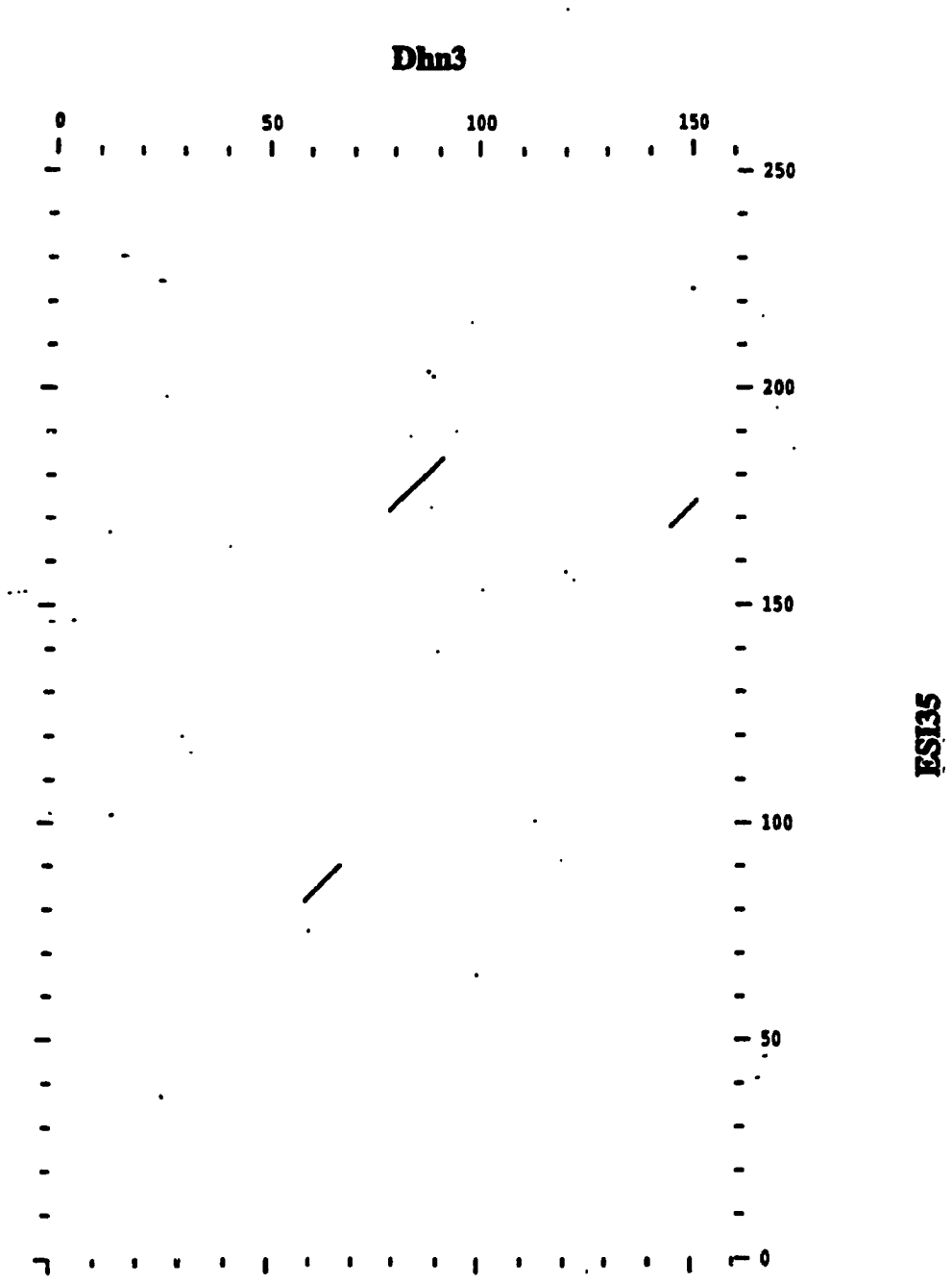

Dhn3
0
50
100
150
250
200
150
100
50
0
ESI35

Figure 17. Conservation of polypeptide sequences. Deduced proteins from *L. elongatum*: *ESI*35, *ESI*18-1; *O. sativa*: RAB16 a, RAB16b, RAB16c, RAB16d; *H. vulgare*: Dhn1, Dhn2, Dhn3, Dhn4, and Maize (M3). Polypeptides are shown schematically. Boxed areas represent open reading frames (polypeptide sizes indicated), and the amount of shading indicates the amount of similarity; darker shading indicates greater similarity. Within the highly similar blocks, S represents a tract of serines, K represents lysine-rich residues, and G represents glycine-rich residues.

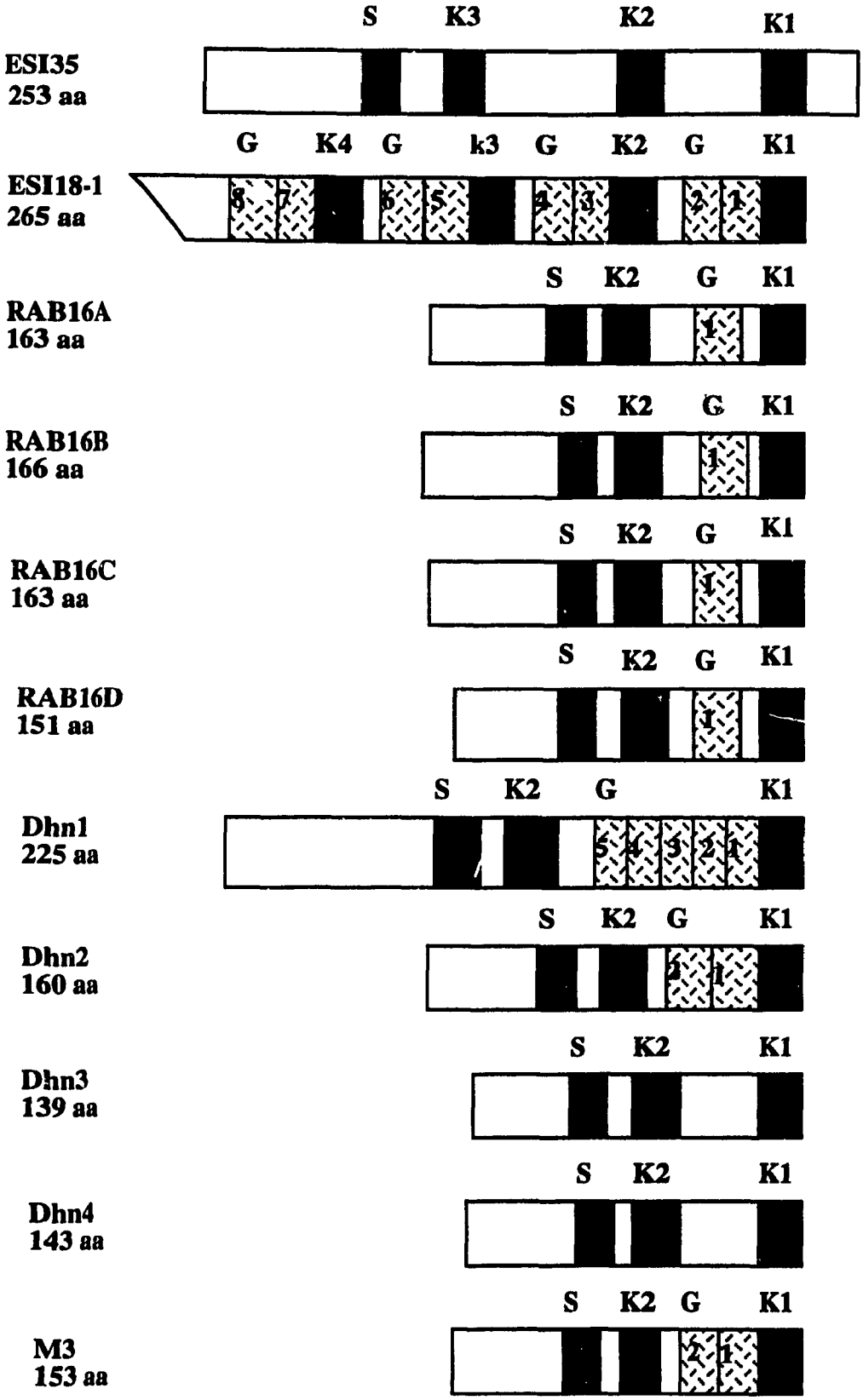

S K3 K2 K1
ESI35
253 aa
G K4 G k3 G K2 G K1
ESI18-1
265 aa
S K2 G K1
RAB16A
163 aa
S K2 G K1
RAB16B
166 aa
S K2 G K1
RAB16C
163 aa
S K2 G K1
RAB16D
151 aa
S K2 G K1
Dhn1
225 aa
S K2 G K1
Dhn2
160 aa
S K2 K1
Dhn3
139 aa
S K2 K1
Dhn4
143 aa
S K2 G K1
M3
153 aa

Figure 18. Comparative evolutionary patterns for the Lysine-rich repeat units of *ESI*35, *ESI*18-1, RAB16a, RAB16b, RAB16c, RAB16d, Dhn1, Dhn2, Dhn3, Dhn4, and M3. The lysine rich repeats are numbered sequentially within each gene sequence begining with the carboxyl terminal repeat K2. The relative location of the numbered repeats within the sequences are shown in Figure 17.

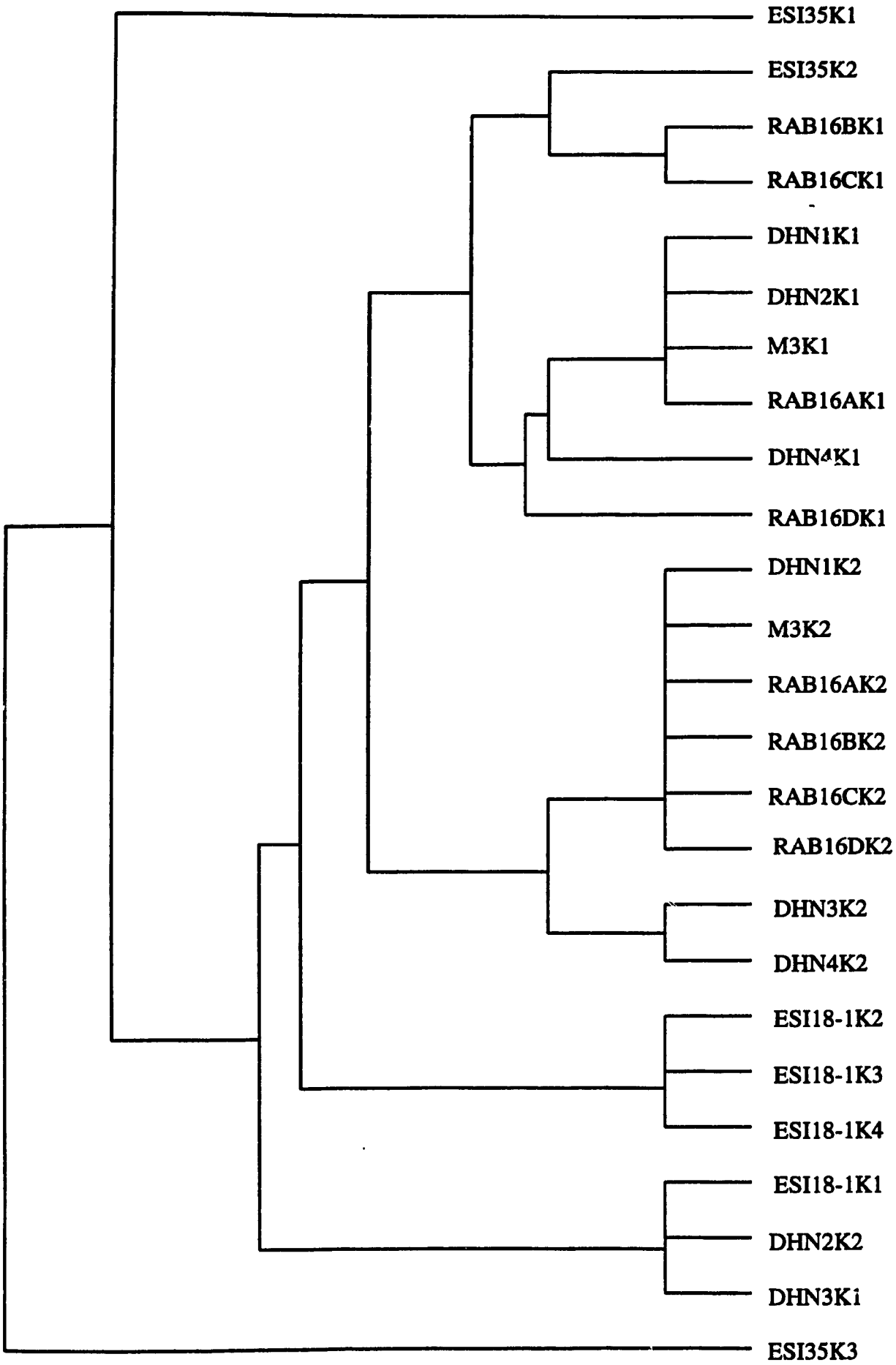

ESI35K1
ESI35K2
RAB16BK1
RAB16CK1
DHN1K1
DHN2K1
M3K1
RAB16AK1
DHN4K1
RAB16DK1
DHN1K2
M3K2
RAB16AK2
RAB16BK2
RAB16CK2
RAB16DK2
DHN3K2
DHN4K2
ESI18-1K2
ESI18-1K3
ESI18-1K4
ESI18-1K1
DHN2K2
DHN3K1
ESI35K3

Figure 19. Comparative evolutionary patterns for the Glycine-rich repeat units of *ESI*35, *ESI*18-1, RAB16a, RAB16b, RAB16c, RAB16d, Dhn1, Dhn2, Dhn3, Dhn4, and M3. AS in figure 19, the glycine repeats are numbered consecutively within individual sequences starting with the repeat close to the carboxyl terminus as G1. The relative location of the numbered repeats within the sequences are shown in Figure 17.

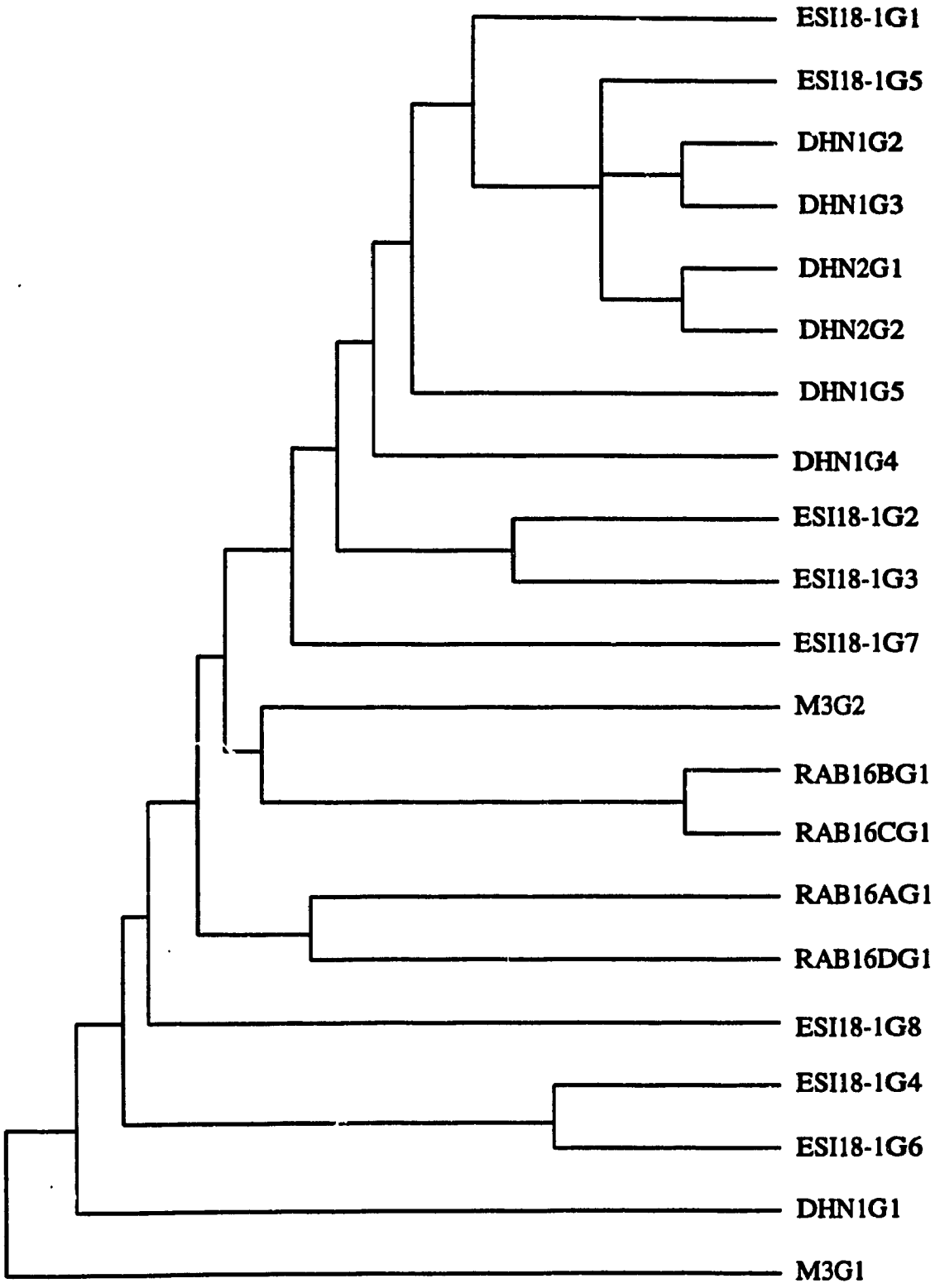
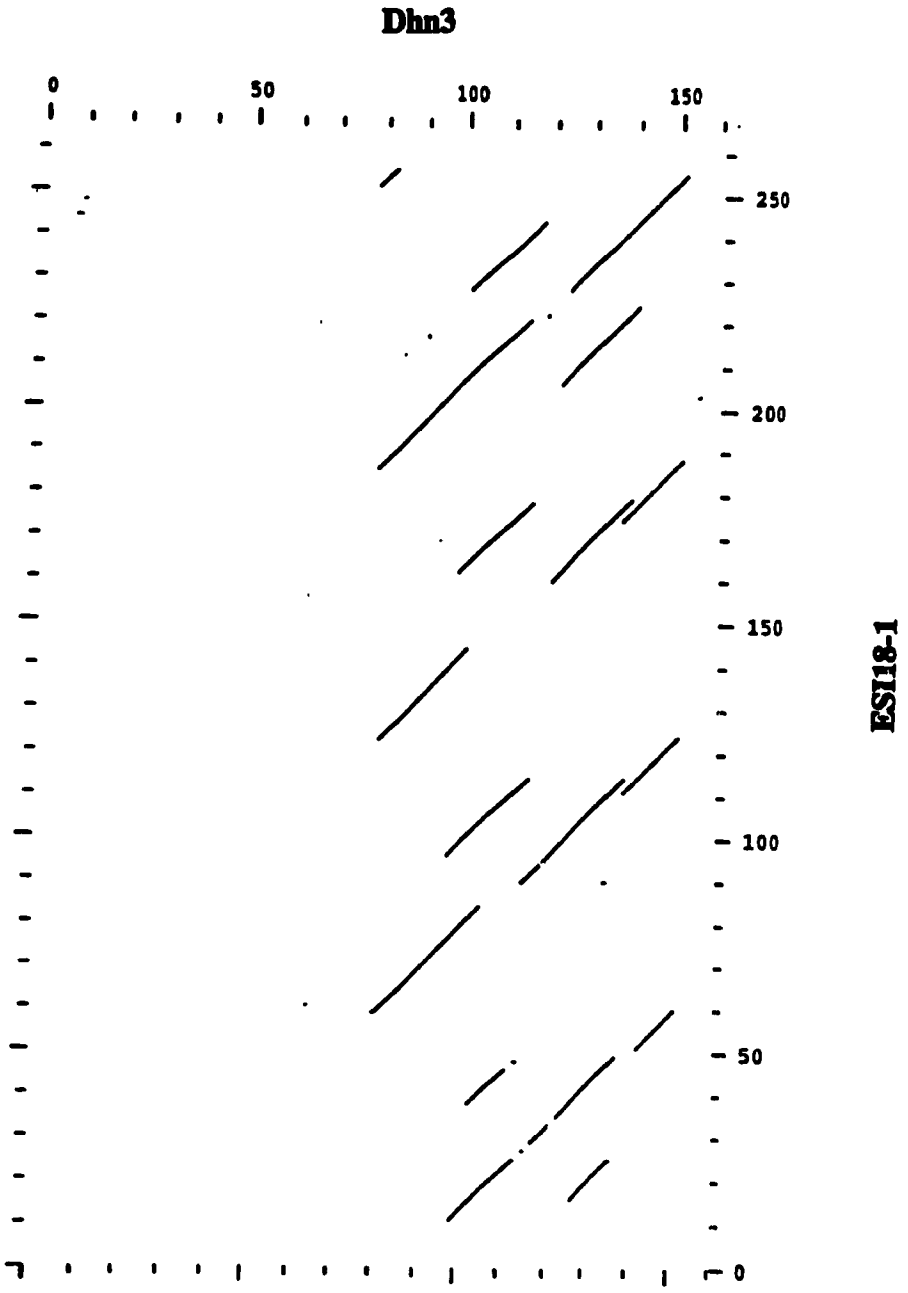

ESI18-1G1
ESI18-1G5
DHN1G2
DHN1G3
DHN2G1
DHN2G2
DHN1G5
DHN1G4
ESI18-1G2
ESI18-1G3
ESI18-1G7
M3G2
RAB16BG1
RAB16CG1
RAB16AG1
RAB16DG1
ESI18-1G8
ESI18-1G4
ESI18-1G6
DHN1G1
M3G1

Table 7. The putative nuclear targeting signal (NTS) sequence present in *ESI*35 protein with six other NTS sequences

Protein	Organism	AA#	NTS sequence
*ESI*35	L. elongatum	106	KRKKKKglK
TGA1a	Tobacco	134	KRKKsse
VirF2	Agrobacterium	296	KIKsKsgi
Top1	Arabidopsis	821	KKiRsle
O2	Maize	237	RRsRyRK
Nucleoplasmin	Xenopus	155	KKKKIdn
N1	Xenopus	534	KdaKKsK

Figure 20. Comparison of aa sequences of *ESI*18-1 and Dhn3 using dotplot analysis of the COMPARE programme in the GCG analysis package. Window: 20; Stringency: 18; Point: 331


Dhn3
0
50
100
150
250
200
150
100
50
0
ES118-1

Immunoblot analysis, showed that the *L. elongatum* embryo may produce proteins of 4 molecular weights which cross react with both anti-dehydrin and anti-WCS120 antibodies. This is consistent with *ESI*18 comprising a multigene family in the *L. elongatum* genome. The low MW protein (25 kDa) has been accounted for by the another *ESI*18 gene member. The 25 and 60 kDa proteins observed in this study are also detected in barley (Close et al., 1989; Close and Chandler, 1990), maize (Close et al., 1989; Vilardell et al., 1990), and rice (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1989). The 75 kDa protein was also detected in freezing tolerance wheat (Houde et al., 1992). This suggested that there are several proteins in *L. elongatum* that are similar to those dehydrins and cold stress proteins. This supports the sequencing which indicates that the *ESI*18 genes are closely related to the dehydrins and the cold stress induced gene *WCS*120. The "heat-stability" of barley and radish proteins detected by the anti-peptide (Close et al., unpublished data) and the wheat protein detected by the anti-WCS120 antibody (Hunde et al., 1992) buttresses the hypothesis that solubility at high temperature may be a general property of dehydrins. Thermostability of α-amylase enzymes in *Bacillus spp.* has been demonstrated to be due to the stabilizing influences of a few specific lysine residues. These residues are believed to participate in salt bridges, thus stabilizing electrostatic interactions and reducing the extent of unfolding at high temperature (Tomazic and Klibanov, 1988). Possibly the formation of thermostabilizing salt bridges is also a general feature of dehydrins.

Although, for the protein extracted from the salt stressed roots of *L. elongatum*, western blotting analysis detected no proteins that cross-react with either antibody to the dehydrins or the anti-WCS120 in this study (data not shown). Since the salt-induced *ESI*18 genes are expressed in the roots of *L. elongatum* after treatment of NaCl, it is possible that protein expression was regulated at the translational or post

translational level. It is also possible that the ESI18 protein accumulates in this tissue in a form in which the antibodies have low affinity for the protein .

Although the *ESI*35 and *ESI*18-1 protein are homologous to the LEA/RAB/dehydrins proteins, the function of the two proteins in salt stress is not known. It has been proposed that the uniform hydrophilic nature of *lea*s suggests that they could provide solvation of cytoplasmic structures during stress. The function of *rab*16/*dhn*/*lea*-D11 type proteins is largely a subject of speculation. They are a subject of keen interest because of their implication in processes in late embryogenesis and in several stress responses. The high degree of localized sequence conservation observed between distantly related plant species suggests a rather precise mode of action what ever that may be.

Both the similarity and the dissimilarity of *ESI*35 and *ESI*18-1 to other dehydrin-like clones emphasizes the possible functional conservation of some other sequence motifs found in this group-especially the serine block and the lysine rich motif. Moreover, the work presented here demonstrates some of the structural variation that exists within the group which implies a corresponding functional variation.

Recently dehydrin homologues have been reported in the cyanobacteria *Anabaena* (Close and Lammers, 1993). This fact and their widespread occurance in vascular plants suggests that genetic analysis via overexpression or anti-sense inhibition of expression could provide additional and perhaps critical insight into their importance as well as function.

5. REFERENCES

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