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Attempts toward the expression in transgenic *Arabidopsis thaliana* of *Esi47*, a salt stress-induced gene encoding a protein kinase from *Lophopyrum elongatum*

Marc-François Pelletier

Thesis

in

The Department

of

Biology

Presented in Partial Fulfilment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

September, 1996

CONCORDIA UNIVERSITY

School of graduate studies

This is to certify that the thesis prepared

By: Marc-François Pelletier

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Master of Science (Biology)

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ABSTRACT

Attempts toward the expression in transgenic *Arabidopsis thaliana* of *Esi47*, a salt stress-induced gene encoding a kinase homologue from *Lophopyrum elongatum*

Marc-François Pelletier

Plant transformation vectors for constitutive gene expression were prepared to study the role of *Esi47*, a gene that encodes a protein kinase, which normally is induced as part of the salt-stress response in *Lophopyrum elongatum*. *Agrobacterium*-mediated transformation of *A. thaliana*, by tissue-culture and vacuum infiltration, were both attempted. A number of plant transformation constructs were made for gene promoter analysis. Expression constructs were also made and tested for the production of Esi47 protein in *E. coli* using the pATH11 *trpE* fusion vector.

The tissue-culture method was partially successful. Callus cultures were established for the *Arabidopsis* Col-2 genotype, embryogenesis was observed in Col-2, and ten regenerated seedlings arose directly from RLD-1 genotype explants grown on shoot inducing medium (SIM) containing 100 μg/ml kanamycin. Col-2 callus was maintained for three passages on SIM with antibiotics, while the RLD-1 regenerated seedlings were plated directly on hormone free growth medium (GM) with 100 μg/ml kanamycin for three weeks. All developed roots and were transferred to the greenhouse, but rapidly wilted and died upon transfer. Attempts were made to regenerate from *de novo* meristem with Col-2 callus tissue by subculturing on B5 medium with various combinations of benzylaminopurine (BAP) and naphthalene acetic acid (NAA), but the

callus remained unresponsive. Five series of transformations by vacuum infiltration were attempted, but proved unsuccessful.

The *trpE::Esi47* fusion gene constructs, one with the full protein kinase open reading frame, and the other with the carboxylic-terminus of *Esi47*, were successfully expressed in *E. coli*. The TrpE fusion proteins were found in the insoluble fraction of lysed cells. Resolubilized fusion proteins derived from the two expression constructs had apparent molecular masses of 75 and 45 kDa, respectively.

ACKNOWLEDGMENTS

I would like to thank Dr. Patrick Gulick for his guidance and encouragement throughout this project, and for introducing me to the world of plant molecular genetics. I would also like to thank Drs. Jennifer Saleeba, Jacques Grandmaison, and Antonin Gauthier, as well as Susan Aiken and Wei Shen for their invaluable technical support, and for answering the endless "how do you ... DNA?" questions. Special acknowledgments go to Drs. Paul Joyce and Ragai Ibrahim for sitting on my committee, and again to Dr. Ibrahim for the use of his tissue-culture facilities, protein electrophoresis materials, and incubator used to grow the transgenic seedlings. Dr. Narinder Kapoor's photographic expertise was greatly appreciated, as well as Hervé de la Fourchardières' horticultural know-how.

I thank Maria Koutroumanis for her valuable help in the preparation of this thesis and for her encouragement throughout all my endeavors. I'd like to thank Amani and Dave for being positive and keeping the moral high in the lab throughout the year. And finally, a special thanks goes out to my parents and brother who have supported, put up with, and encouraged me from the beginning.

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LIST OF ABBREVIATIONS

ABA	=	Abscisic acid
aba	=	*Arabidopsis* mutant deficient in abscisic acid biosynthesis
abi	=	*Arabidopsis* mutant which is insensitive to abscisic acid
BAP	=	Benzylaminopurine
CIM	=	Callus inducing medium
ESI	=	Early salt-stress induced
GM	=	Plant growth medium
NAA	=	Naphthalene acetic acid
RAB	=	Responsive to abscisic acid
SP	=	Selection plates

INTRODUCTION

There have been many recent sudies in plant molecular biology, that have focused on cellular dehydration. Whether the stresses incurred are the result of cold, drought, or salt, a number of the cellular responses are similar. These studies have identified genes whose expression is modified by dehydration stress and are thought to play a role in transducing cellular signals, and maintaining cellular integrity. Such findings have contributed to the formulation of models for stress response which may be utilized in engineering crops that can withstand adverse environmental conditions (Bray, 1993; Bohnert *et al.,* 1995).

A plant's response to the stress of dehydration, whether it is caused by drought, salt, or cold, is complex. The plant exposed to dehydration stress will undergo changes in metabolism and gene expression, and will exhibit modifications in cellular structure. All plants can adapt, although with varying ability, to environmental change. Most cultivated species have close wild relatives that exhibit excellent environmental stress tolerances.

Throughout the plant kindom, there are consistent ways plants counteract dehydration. First among these similarities are the biochemical processes that lead to the accumulation of organic metabolites upon dehydration stress. Plants exposed to dehydration stress have been observed to accumulate a range of sugar and amino acid derivatives including sucrose, mannitol, sorbitol, proline, and glycine betaine as well as inorganic ions including Na^+ and K^+ (for a review see Delauney and Verma, 1993).

Similar organic osmolites have been found to accumulate in vascular and non-vascular plants, as well as in algae, fungi and even bacteria (Csonka, 1989; Potts, 1994).

A wide variety of plant species express similar genes when stressed, such as the responsive to abscissic acid (RAB) related genes and those encoding dehydrins (Skiver and Mundy, 1990). Moreover, nearly all plants express genes throughout seed development that mediate tolerance to the normal desication process associated with seed maturation (Bray, 1993). This implies that non-tolerant plants have genes similar to those required for dehydration stress responses, but that they are developmentally regulated and are expressed primarily during seed maturation. Recent evidence also demonstrates the functional conservation of stress-induced gene promoters across the span of the plant kingdom. Knight *et al.* (1995) found the stress-induced Em promoter elements from wheat to be inducible in moss by using promoter-reporter analysis in transgenic moss.

An overview will be presented here, that will examine the response mechanisms that plants share to withstand dehydration-related stresses. There will be a review of the genetic studies of the salt-tolerant wheat grass, *Lophopyrum elongatum,* signal transduction in plant stress responses will be examined, and a synopsis of the work involving transgenic *Arabidopsis thaliana* in the characterization of a stress-induced protein kinase.

1. Genetic studies on *Lophopyrum elongatum*, a salt tolerant wheat grass.

Lophopyrum Löve (syn. *Elytrigia* Desv.) species grow naturally in saline environments, either in the littoral zone or in saline soils, and are more tolerant to salinity than their related genus *Triticum* L. (Dvorak *et al.*, 1988). Cytogenetic studies with hybrids from *Lophopyrum* (2n = 2x) and the salt-sensitive wheat, *Triticum aestivum* L. (2n = 6x = 42), involving the addition or substitution of chromosomes into a salt-sensitive wheat background lead to a better understanding of the adaptation to growth under saline stress (Dvorak *et al.*, 1988; for review Dvorak *et al.*, 1991). An octoploid amphiploid of *T. aestivum* cv. Chinese Spring x *Lophopyrum elongatum* demonstrated that the adaptation of *L. elongatum* to saline environments was expressed dominantly in a salt-sensitive wheat genetic background. Moreover, individual disomic additions and substitutions in Chinese Spring for the homologous chromosomes 2E, 3E, 4E, and 8E, from *L. elongatum* displayed enhanced dry matter production and seed yield. It could be concluded from this work that the adaptation to growth in saline environments evolved from the accumulation of a few alleles at a number of loci, each independently enhancing salt-tolerance, and acting codominantly to the original alleles (Dvorak *et al.*, 1988). It was also suggested that the loci controlling tolerance may also be present in the sensitive wheat, but in different allelic states (Dvorak *et al.*, 1988).

A study by Omielan (in Dvorak *et al.* 1991) demonstrated that grain yield and biomass of plants exposed to saline stress correlated with K^+/Na^+ ratios in Chinese Spring, the *Lophopyrum* x Chinese Spring amphiploid, and the set of disomic substitution

lines. These high correlations are suggestive that exclusion of Na^+ and accumulation of K^+ are related to its salt-tolerance and dominantly controlled by loci on the *Lophopyrum* chromosomes (Dvorak *et al.*, 1991). The importance of high K^+/Na^+ ratios for salt tolerance was further demonstrated by Dvorak *et al.* (1994). A single locus which controls Na^+ exclusion and K^+ accumulation, *Kna1* from Chinese Spring, was shown to contribute to higher grain yields upon salt stress. This was made evident when *Kna1* was transferred to Durum wheat through homologous recombination, improving its tolerance to salt-stress. This provided further evidence that K^+/Na^+ is related to the tolerance of saline stress and that tolerance can be transferred between species.

With the tools for comparison of the genetic response to salt stress becoming available, studies on changes in gene expression after exposure of plants to salt stress revealed different mRNA profiles for the salt sensitive Chinese Spring wheat and salt-tolerant Chinese Spring x *L. elongatum* amphiploid. This was found using two-dimensional gel electrophoresis of the *in vitro* translated products as a basis for comparison. This provided direct evidence, on the one hand, of a change in gene expression upon salt-stress, and on the other, of differential expression of these genes in salt-sensitive and -tolerant related species. Additionally, the study showed the roots to be the major site of change in gene expression (Gulick and Dvorak, 1987).

Using a selective enrichment and differential hybridization approach, eleven unique genes or gene families that were expressed in an early response to saline stress were cloned (Gulick and Dvorak, 1990; Gulick and Dvorak, 1992). Using these clones as probes in northern analysis, gene expression was found to start at 2 h and to peak after

6 h returning to basal levels within 24 h to 7 days. Thus these genes have been called early salt-stress induced (*Esi*). The expression of *Esi47* is exceptional however, although it is induced within 2 hours of stress, it maintains relatively high levels for at least 3 days (Gulick and Dvorak, 1992).

When a comparison of the expression of the *Esi* genes was made between the salt-tolerant *L. elongatum*, salt-sensitive wheat, and their amphiploid, the difference in genetic response was confined to the first phase of induction, during which the accumulation of mRNA occurred rapidly, within a few hours of the onset of stress. For most of the genes, the level of salt-stress induction was greater in the salt tolerant genotypes than in the sensitive genotypes. This genetic response was also found to be elicited by abscisic acid, and was primarily biphasic. When the levels of expression were compared, *L. elongatum*'s genetic contribution to the amphiploid correlated with the enhanced expression of the salt-stress-induced genes (Galvez *et al.*, 1993). This molecular evidence supports the additive model for salt tolerance that was demonstrated in earlier studies with the disomic addition and substitution lines of earlier studies. This indicates that salt tolerance is proportionally transferred depending on the genetic contribution of *L. elongatum* to Chinese Spring (Dvorak *et al.*, 1988; Gulick and Dvorak, 1987).

Among the eleven early salt-stress induced genes cloned by Gulick and Dvorak (1990) from salt stressed *Lophopyrum*, one gene had a temporaly unique expression pattern. This gene, *Esi47*, as was mentioned earlier, maintained relatively high levels of expression for three days. In addition, *Esi47* was shown by sequence comparison to encode a serine/threonine protein kinase (Shen and Gulick, unpublished). Furthermore,

like the other Esi clones, it appeared to be inducible by saline stress and ABA (Gulick and Dvorak, 1992; Galvez *et al.*, 1993). Considering its inducibility by ABA, it is plausible that *Esi47* mediates one or more ABA-dependent stress responses

2. Signal perception and transduction of stress responses in plants

Signal perception of dehydration related stresses is presently unclear. Plants most probably use many signal receptors to generate an array of signals required to compensate for the intricate changes in environmental conditions. Bray (1993) suggests that stretch-activated channels (Ding and Pickard, 1993) cause alterations in the cell wall-plasma membrane continuum, triggering signal transduction pathways inducing gene expression.

Abscisic acid (ABA), a ubiquitous phytohormone, discovered in the 1960s by Wareing and his collegues, has been shown to be implicated in a range of physiological responses including plant adaptation to environmental stress, leaf abscission, and geotropism (Bidwell, 1979; and for a review, Giraudat *et al.*, 1994). More recently, support for the role of ABA in the regulation of the dehydration stress response has been confirmed with observations of stress-induced increases in endogenous ABA levels (Cohen and Bray, 1990; Stewart and Coetberg, 1985, Chen *et al.*, 1983). Moreover, exogenously applied ABA has been shown to mimic dehydration stress-related physiological (Vartanian *et al.*, 1994) and molecular responses in plants (Bostock and Quatrano 1992; Moons *et al.*, 1994; Galvez *et al.*, 1993). The involvment of ABA in stress response was clearly demonstrated by Koorneef *et al.* (1984) in the characterization of the *A. thaliana* ABA-insensitive mutants. They found that water-stress tolerance was

enhanced by applications of 10 μM ABA, and that ABA-insensitive mutants were unable to benefit from these ABA applications.

Schroeder (1995) speculates that slow anion channels that maintain depolarisation in guard cells may be involved in the signal propagation of ABA-induced stress responses. These channels, which are permeable to Cl^- and other halides, as well as physiologically occuring anions such as malate and nitrate, were shown to suppress stomatal closing by ABA and malate treatments when blocked by slow anion channel blockers (Schroeder *et al.*, 1993; Schroeder, 1995). In vegetative tissue, similar anion channels have been found in root cortical cells and xylem parenchyma. Their activity appears to be enhanced by extracelluar Cl^- and Ca^{2+}. This leads to the hypothesis that these anion channels, involved in membrane potential regulation and Cl^- uptake in response to salt stress (Skerret and Tyerman, 1994), may aid in the generation of ABA-induced stress responses.

Gene expression and morphological response studies in *Arabidopsis*, using differential responses of ABA-biosynthetic and ABA-insensitive mutants (aba and abi, respectively), as well as the characterization of stress-induced regulatory elements of gene promoters, have not only been informative in further defining the role of ABA in mediating stress responses (Giraudat *et al.*, 1994) but also have supported the notion of two signal transduction pathways: one ABA-dependent and the other ABA-independent.

Genes capable of being induced in both the ABA biosynthetic mutant and in the wild-type plants, use the ABA-independent pathway. Drought-induced genes that are not expressed in the biosynthetic mutants are referred to as ABA-requiring genes (Bray,

1993). However, many genes have been identified that respond to, but do not require, ABA for expression. Some of these genes possess two differently responding cis-elements that coordinate their expression (Yamagushi-Shinozaki and Shinozaki, 1994). It is thought that these may lead to the accumulation of ABA, which in turn induces ABA-requiring genes. Stress-induced genes which have been cloned and are non-ABA-responsive include *Lti30, Cor47*, and *ATCDPK1-2* (Welin *et al.*, 1994; Ureo *et al.*, 1994). Stress-induced genes that are ABA-responsive include *rd29a*, *lti65*, and *lti78* (Nordin *et al.*, 1991; Nordin *et al.*, 1993; Yamagushi-Shinozaki *et al.*, 1992; Yamagushi-Shinozaki and Shinozaki, 1994). Those which require ABA for gene expression include *rab18* (Welin *et al.*, 1994; Yamagushi-Shinozaki and Shinozaki, 1993; Yamagushi-Shinozaki and Shinozaki, 1994).

Two well characterized ABA-responsive promoter elements are Em1a from the *Em* wheat gene (Marcotte *et al.*, 1989) and Motif I from the *rab16A* gene from rice (Mundy *et al.*, 1990). Using gel retardation experiments, these elements were shown to bind transcription factors Em1a (Guiltinan *et al.*, 1990) and TAF-1 (Oeda *et al.*, 1991) respectively. Both elements contain the CACGTG motif, the "G-box" sequence recognized by bZIP proteins (Giulano *et al.*, 1988,; Izawa *et al.*, 1993). Em1a and Motif I elements directly confer ABA-responsivness to their respective genes. These elements alone however, do not account for the full ABA responsivness, which likely relies adjacently encoded information (Marcotte et al., 1989; Mundy et al., 1990). It is important to note that the genes encoding these transcriptional activators that are involved in signal transduction by binding to stress-inducible promoters, are not stress inducible

themselves. In contrast, the gene encoding the stress-induced *myb* transcription factor homologue *Atmyb2* is salt-stress and ABA -inducible (Ureo *et al.*, 1993).

A novel cis-acting promoter element in the *rd29a* gene has recently been characterized. It was shown to be involved in the response to drought, low temperature, or high salt stress. Termed DRE for drought-responsive element, this cis-element (TACCGACAT) is only involved in the rapid response to conditions of dehydration or salt, while an adjacent ABRE element of *rd29a* regulates slow ABA-responsive expression of that gene (Yamagushi-Shinozaki and Shinozaki, 1994).

Another example for a complex salt-responsive promoter is that of Ppc1 from *Mesembryanthemum crystallinum*. *Ppc1* encodes an enzyme involved in crassulacean acid metabolism, a metabolic strategy which allows reduced water loss during drought conditions. Its 5'- flanking region contains several salt-reponsive enhancer regions and one silencer region, reflecting the intricate regulation patterns that may be required for this highly complex adaptive trait (Schaeffer *et al.*, 1995).

3. Plant protein kinases and phosphorylation in signal transduction

Phosphorylation can regulate activity of a protein, and protein phosphorylation has been shown in many eukaryotes to mediate perception and signalling in response to external stimuli. Most likely, plants use protein phosphorylation to mediate cellular responses. Several plant protein kinases have been cloned (Putnam-Evans *et al.*,1990; Harper *et al.*, 1991; Suen and Choi, 1991; Kawaski *et al.*, 1993; Harper *et al*,. 1993). Moreover, many plant proteins have been shown to be phosphorylated (Ureo *et al.*, 1993,

Vilardel *et al.*, 1990). Their role in plant biology though, is for the most part, still speculative and has yet to be fully elucidated (Stone and Walker, 1995; Hwang and Goodman, 1995).

An *Arabidopsis* mutant which is insensitive to the phytohormone ethylene was identified by its etiolated seedling "triple response". Keibler *et al.*, (1993) isolated the gene, *CTR1*, which corresponds to this mutation, and confers a constitutive triple response. Normally upon exposure to ethylene, there is an inhibition of hypocotyl elongation, hypocotyl thickening, and an exaggerated tightening of the apical hook (Bowler and Chua, 1994). The protein encoded by *CTR1* is a Raf-like ser/thr protein kinase, and the recessive nature of the *ctr1*, ethylene insensitive mutant, suggests that its role is to attenuate the signal transduction in the absense of ethylene. By the phosphorylation of its target proteins, it could negatively regulate the ethylene response (Bowler and Chua, 1994).

Another example arises from the the search for a number of *R* genes, which confer resistance to specific plant pathogens in a "gene-for-gene" manner. Upon infection with a pathogen expressing a specific avirulence (*avr*) gene, a host plant that has the corresponding resistance gene (*R*) will undergo a hypersensitive response. This response consists of increased expression of defense-related genes, production of anti-microbial compounds, lignin formation, an oxidative burst and rapid cell death (Zhou *et al.*, 1995). These *R* genes were expected to encode components of signalling pathways that lead to defense response. A map-based cloning strategy was used to clone a gene, *Pto*, that confers resistance to bacterial speck disease in tomato, and as suspected, a

ser/thr protein kinase, was found. (Martin *et al.*, 1993). Subsequently in a two-hybrid screen, *Pto* was found to physically interact with a second ser/thr protein kinase Pti (for Pto-interacting). When *Pto* and *Pti* were overexpressed under a constitutive promoter, in transgenic tomato, and when *Pti* was overexpressed in tobacco, plants showed an enhanced hypersensitive response to *P. seringae* pv *tabaci* expressing *avrPto* (Martin *et al.*, 1993; Zhou *et al.*, 1995). Pti is phosphorylated by Pto, and is thought to act as a component downstream of Pto in a phosporylation cascade.

Transgenic rice expressing *Xa21*, which encodes a serine/threonine kinase receptor cloned by a map-based approach, displayed enhanced levels of resistance to *Xanthomonas oryzae* pv. *oryzae* race 6. Song *et al.* (1995) first isolated clones from a bacterial artificial chromosome and cosmid library with a probe that coseggregated with the *Xa21* locus in a *Oriza sativa* ssp. Indica x *O. longistaminata* cross. Overlapping subclones were transformed by particle bombardment into *O. sativa* ssp. Japonica var. Taipei. From 1500 transgenic plants screened, fifty containing a 9.6 kb *Kpn*I subclone showed reduction in lesion length, compared to controls, when inoculated with the *X. oryzae* pathogen. The associated subclone was further divided and overlapping subclones were used in additional transformations. This revealed a 3075 bp ORF (*Xa21*) encoding a leucine-rich repeat motif, a single pass transmembrane domain, and a protein kinase domain which are typical for transmembrane receptor kinases, suggesting a role in cell surface recognition of the pathogen ligand (Song *et al.*, 1995).

A few plant protein kinases have been shown to be stress induced (Ureo *et al.*, 1994; Anderberg and Walker-Simmons, 1992; Hwang and Goodman, 1995; and Shen and

Gulick, unpublished). Among those are a root specific kinase homolog from *A. thaliana* induced by dehydration, ABA, and NaCl (Hwang and Goodman, 1995), a wheat protein kinase, PKABA1, induced by ABA and NaCl (Anderberg and Walker-Simmons, 1992), and two protein kinases cloned from *A. thaliana*, that were shown to be calcium modulated, and are induced by high salt and drought (Ureo *et al.*, 1994). Lastly *Esi47*, cloned using differential screening and selective enrichment, from the salt-tolerant wheat grass *Lophopyrum elongatum*, encodes a putative serine-threonine protein kinase (Gulick and Dvorjak, 1990; Shen and Gulick, unpublished). The role of these stress-regulated protein kinases still remains unclear.

Genetic screening for ABA-response mutants thus far has been the most revealing approach to finding signalling elements related to protein phosphorylation that directly mediate a plant's response to dehydration stress. The locus for the ABA insensitive mutation, *Abi1*, was cloned independently by two labs, and by sequence comparison, appears to be a Ca^{2+} modulated phosphatase of type 2C (Leung *et al.*,1994; Meyer *et al.*, 1994). When this mutated phosphatase was introduced into transgenic *A. thaliana*, it blocked stomatal closure, the maintenance of seed dormancy, and the inhibition of plant growth. Its ability to arrest general responses to ABA suggests it is an early element of the signal transduction pathways of stress response.

The purpose of the present study was to examine the role of *Esi47*, which encodes a stress-induced serine/threonine protein kinase cloned from *Lophopyrum elongatum* (Shen and Gulick, unpublished). An attempt was made to characterize *Esi47* by overexpression in *Arabidopsis*, so that its effects on stress-induced responses could be

monitored. Expression vectors were also prepared for *Esi47* overexpression in *E. coli*, to be used to characterize its substrates, and for antibody production. In addition, promoter-reporter constructs with various forms of *Esi47's* 5'- region, originally cloned by Shen (1995, unpublished), were subcloned into the *Agrobacterium*-mediated transformation vector pBI121.

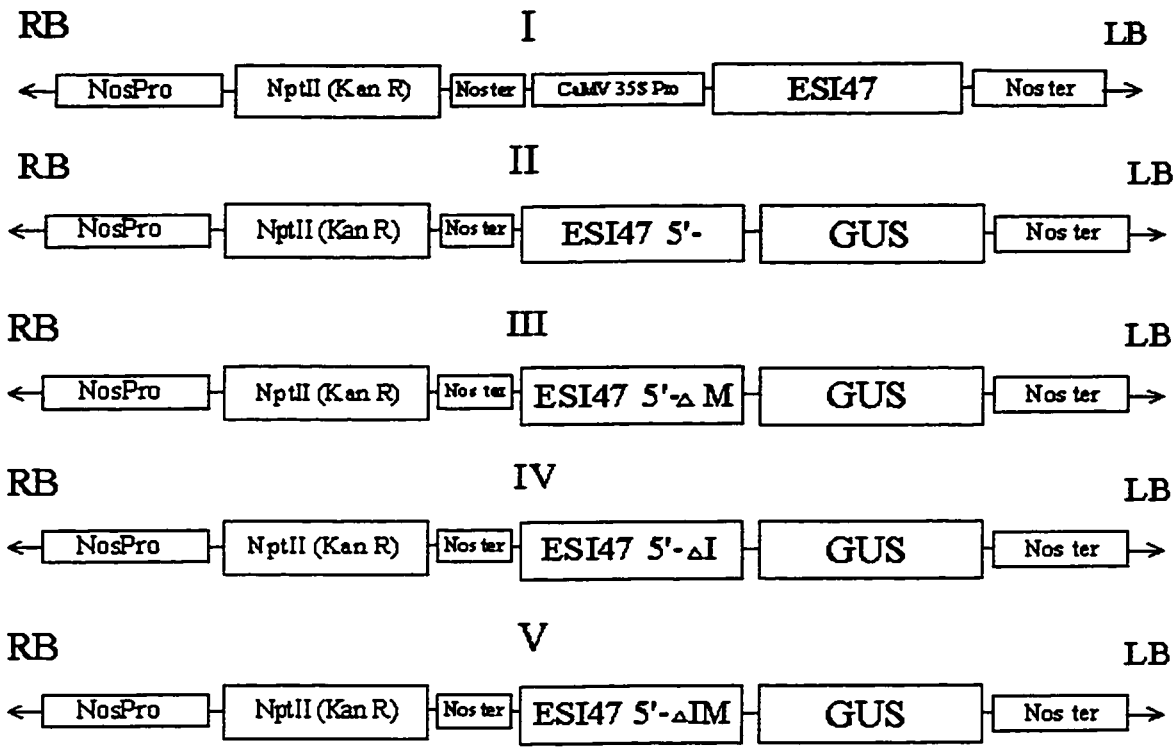
MATERIALS AND METHODS

1. Construction of plant expression vectors

Construct I (Figure 1) was prepared for the expression of *Esi47* under the control of a CaMV 35S promoter, which functions as a strong, constitutive gene promoter in plants. Essentially, the construct was made by replacing the β-glucuronidase (GUS) gene of the plant transformation vector pBI121 (Jefferon *et al.*, 1987) with the entire open reading frame (ORF) from of *Esi47*.

The vector plasmid pBI121 was linearized with *Sac*I, which was obtained from Bio/Can Scientific™ as were all of the enzymes used in this study. The linearized plasmid was ethanol-precipitated, and blunt-ended using T4 DNA polymerase. The polymerase was then heat inactivated, and then plasmid was precipitated again. Finally, it was subjected to *Xba*I digestion. Similarly, the *Esi47* 1.2 kbp ORF in pBluescript was linearized using *Apa*I, blunt ended with T4 DNA polymerase, precipitated, and *Xba*I digested. The final digestion of the *Esi47* clone was electrophoresed on a 1% agarose gel, the 1.2 kbp fragment (ORF) was isolated, and purified using Quiex™ gel extraction kit (Quiagen). The ligation was carried out in 20 µl with 90 ng of pBI121 (without GUS) and 110 ng of *Esi47* ORF. The mixture was allowed to ligate overnight at 16 °C. Since the vector was not purified from the gel, contaminants derived from incomplete *Xba*I digestion of the vector (in which the GUS was not removed) had to be eliminated. The ligation products were thus digested with *Sna*BI (a site unique to GUS), to linearize any vector with GUS still present. The plasmid was transformed into *E.coli* (XL1-Blue) using the heat-shock method with kanamycin selection (Ausubel *et al.*, 1988).

Figure 1. T-DNA regions of the plant transformation constructs prepared in pBI121. Construct I was used in this study to overexpress *Esi47* in transgenic *Arabidopsis*, while constructs II-V were prepared for further work, involving *Esi47* promoter analysis with transgenic *A. thaliana*. Construct II has the original 5'-upstream region *esi47*, III has the AT from the ATG of the first ORF removed, IV has the first intron removed, and V has both modifications. These modifications were constructed by Shen (1995, unpublished) in the vector pBluescriptSK$^-$.


RB
I
LB
NosPro
NptII (Kan R)
Nos ter
CaMV 35S Pro
ESI47
Nos ter
RB
II
LB
NosPro
NptII (Kan R)
Nos ter
ESI47 5'-
GUS
Nos ter
RB
III
LB
NosPro
NptII (Kan R)
Nos ter
ESI47 5'-Δ M
GUS
Nos ter
RB
IV
LB
NosPro
NptII (Kan R)
Nos ter
ESI47 5'-ΔI
GUS
Nos ter
RB
V
LB
NosPro
NptII (Kan R)
Nos ter
ESI47 5'-ΔIM
GUS
Nos ter

The plant transformation vector pBI121 (Jefferson *et al,* 1987) was also used as a base for construction of plant transformation vectors II-V (Figure 1). For these constructs, the CaMV 35S promoter and the GUS gene were removed and replaced by 4 forms of the 5' - upstream region of *Esi47* fused with GUS, previously cloned and modified by Wei Shen (unpublished). *Esi47* consists of a 5'- upstream promoter region followed by two ORFs. The first ORF is 51 bp long and is followed by a 312 bp intron and the serine/threonine protein kinase 1.2 kbp ORF. The variations of the upstream region consist of (1) the 3 kbp of intact genomic promoter region, (2) ΔM with the AT nucleotides removed from *Esi47's* first ORF start codon, (3) ΔI- with the first intron removed, and (4) ΔMI, with both modifications. These were termed plant transformation vectors II-V, respectively.

Plant transformation plasmids II through V were prepared similarly. The vector was prepared by the digestion of pBI121 with *Hin*dIII. This vector was then blunt ended with the Klenow fragment of DNA polymerase. The reaction was extracted with phenol:chloroform (1:1) and ethanol-precipitation. Subsequently, the vector was digested with *Eco*RI. Similarly, the *Esi47*::*GUS* promoter fusions in pBluescript (prepared by Wei Shen), were cut with *Pst*I, blunt ended with Klenow, then cut with *EcoRI.* The DNA fragment containing these modified *ESI47*::*GUS* promoter fusions (5'-::*GUS*; 5'-ΔM::*GUS*; 5'-ΔI::*GUS*; and 5'-ΔIM::*GUS*) were gel purified as above and ligated overnight at 16°C, to the pBI121 (without *GUS*) vector. The ligation products were

PRIMER	SEQUENCE	USE
Primer I	5'-AGCTCGCGAACCGGAAGCACTA-3'	Sequencing plant transformation construct I
Primer II	5'-TCGCGAGCTCGGAGAAGGA-3' *Sac*I	PCR amplification, *trpE* fusion construct I and II
Primer III	5'-GAAGGTGAGGATCCGGAGGTC-3' *Bam*HI	PCR amplification, *trpE* fusion construct II
Primer IV	5'-GGAGATGGTCTAGAAGATTTG-3'	PCR amplification, *trpE* fusion construct III
Primer V	5'-CTCACAAGCTTGGGTGTC-3' *Hin*dIII	PCR amplification, *trpE* fusion construct I and III

Table 1.- Sequences and uses of primers. Restriction sites which were introduced into the gene by site directed mutagenesis are underlined.

digested with *Xba*I (which is unique to the MCS of pBI121 which would be left by an incomplete *Eco*RI digestion of pBI121) to linearize any recircularized vector. The treated ligation mixtures, for each of the four constructs were used for transformation. Colonies were isolated for minipreparations of plasmid by the alkaline lysis method (Ausubel *et al.*, 1989). Clones were verified by linearization with *Eco*RI. Clones for each construct were conserved for further analysis.

2. Genetic transformation of *A. thaliana*

Transformation of *Agrobacterium tumefaciens*

For plant transformation, construct I was introduced into competent *A. tumefaciens* cells by electroporation and the heat shock method. Five ml of LB with 50 μg/ml of streptomycin was inoculated with a fresh colony of *Agrobacterium tumefaciens* strain LBA4404, and incubated on a shaker at 30°C for 24 hours. Streptomycin was used to maintain the virulence plasmid (approximately 200 kbp) encoding the genes required for infection and transfer of the T-DNA from the binary plasmid. An overnight culture (5ml) was used to inoculate 100 ml of the same medium which was incubated for another 24 hours. Afterwards, the cells were chilled for fifteen minutes, centrifuged at 6800 xg for 15 minutes at 4°C, the supernatant was removed, the cells were washed in sterile water by vortexing, and centrifuged again at 6800 xg for 10 minutes. This step was repeated. Electroporation and heat shock (5 minutes on ice, 5 minutes at 37°C) were both attempted, followed by a 4-hour incubation period in 1 ml LB with 50 μg/ml streptomycin for phenotypic expression. A volume of 100 μl of this solution was plated

on LB medium containing streptomycin (50 μg/ml) and kanamycin (100 μg/ml). *A. tumfaciens* cells without the construct (streptomycin resistant), and *E.coli* transformed with construct I (kanamycin resistant) were streaked on the plates with both streptomycin and kanamycin as controls. The plates were then incubated at 30°C for 2 days.

Arabidopsis seed surface sterilization and plating

Several hundred seeds were placed in a microfuge tube and 200 μl of 70% ethanol was added. The tube was inverted several times, then microfuged at full speed for five seconds, and the ethanol was removed using a pipettman with a sterile tip. Five hundred μl of 50% commercial bleach was added and the tubes were shaken for 2 minutes. The seeds were spun down again and the bleach was removed. The seeds were then washed twice with sterile distilled water, each time seeds were vortexed, centrifuged and water removed. Finally, distilled water was added to the seeds, mixed, and a pipettman with a sterile tip was used to transfer the water with seeds to a growth plate (**GM**) containing 1 x MS (Murashige and Skoog, 1962) salts and vitamins, 10 g/l sucrose, and .8 % agar, or selection plate (**SP**) containing 1/2 MS, 1x B5 (Gamborg *et al.*, 1968), .8 % agar, and 50 μg/ml kanamycin. Seeds were vernalized two days at 4°C prior to incubation in the growth chamber.

Plant transformation using vacuum infiltration

The vacuum infiltration technique for transformation used was a modified version from Andrew Bent's protocol, posted on the internet in the *Arabidopsis* Research

Companion Gopher, Harvard/Massachusetts General Hospital Biological Gophers. It is derived from Betchold *et al.*, (1993).

A. thaliana strains RLD-1, LE, Col-2, Abi1, and Aba1 seeds (provided by the Arabidospsis Biological Resource Center, Ohio State University, Table2) were either surface sterilized and germinated *in vitro*, on GM medium or planted directly in soil. This was done to produce homogeneous plants for transformation. After approximately 10 days, seedlings were transplanted into 4 inch pots. The plants were then grown for another 10-14 days. Emerging flowering stems were clipped off to encourage growth of lateral bolts. Vacuum infiltration was carried out four days after pruning.

Agrobacterium carrying the plant transformation construct I was inoculated into 25 ml LB medium (50 μg/ml streptomycin and 100 μg/ml kanamycin) and cultured overnight. This culture was added to 500 ml of LB medium with the same antibiotics and incubated at 30°C for an additional 24 hours. *Agrobacterium* cells were harvested by centrifugation at 3000 x g for 10 minutes and resuspended in 600 ml of infiltration medium, (1/2 Murashige & Skoog (**MS**), 1 x B5 vitamins, 5.0% sucrose, 0.044 μM benzylamino purine). Two hundred millilitres were transferred to a small pyrex dish, the pots with seedlings were inverted gently so that the plants were immersed into the *Agrobacterium* culture, and the pyrex dish with the plants was placed in a bell jar. Vacuum was applied until bubbles formed, meanwhile the bell jar was rocked slowly to ensure proper mixing. The vacuum was then released rapidly. The infiltration solution was decanted into a beaker for reuse (once), and the plants were inverted back slowly holding the casserole over the pot so

A.- Wild type strains:

Strain Abbreviation	Name	Collector
RLD-1	-	M. Koorneef
LE	*Landsberg erecta*	E. Holub
Col-2	*Colombia*	M. Koorneef

B.- *Landsberg erecta* derived mutants:

Allele symbol	Gene name	Reference
Abi1-1	Abscisic acid insensitive	Koorneef *et al.*, 1984
Aba1	Abscisic acid deficient	Koorneef *et al.*, 1982

Table 2.- Wild type (**A**), and *Landsberg erecta* (**B**) mutant strains of *Arabidospsis thaliana* used for this study. Seeds were provided by the Arabidopsis Biological Ressource Center, Ohio State University.

that the soil would not fall out. The plants were kept under cellophane overnight, rinsed to remove excess infiltration medium, and placed in the greenhouse. Plants generally flowered within days and set seed within 3-4 weeks.

Seeds were harvested throughout plant growth, when the siliques on the plants were dry. They were surface sterilized and plated on the kanamycin selection plates (**SP**) described above. The Petri plates were then placed in a growth chamber under a sixteen hour light/eight hour dark photoperiod at 25 °C.

Transformation of *Arabidopsis thaliana* with tissue culture

The protocol used for *Arabidopsis thaliana* transformation by the tissue-culture method is derived from Valvekens *et al.* (1988), except that whole seedlings were used in contrast to using only the roots. *A. thaliana* RLD-1 and Col-2 seeds were surface sterilized, and inoculated in a 250-ml Erlenmeyer flask with 50 ml of liquid GM (MS salts and vitamins with 10 g/l sucrose). This was placed for 2 weeks on a shaker at approximately 100 rpm at room temperature. The seedlings were then placed for three days on **c**allus **i**nducing **m**edium (**CIM**: B5 salts and vitamins, 30 g/l sucrose, 0.05 mg/l kinetin, and 0.5 mg/l 2,4-D) in a growth chamber (16 hours light/8 hours dark). The seedlings were then sliced into 0.5 cm pieces and placed in a test tube containing 1.0 ml of *A. tumefaciens* overnight culture with 9 ml liquid CIM. After two minutes of shaking, the solution was decanted, and the seedling explants were plated on CIM agar for 2 days (co-cultivation with *A. tumefaciens*) and placed back in the growth chamber. Subsequently, the explant material was rinsed in liquid CIM containing 1 g/l cefotaxime,

blotted on sterile filter paper, and plated on shoot inducing medium (SIM: B5 salts and vitamins, 30 g/l sucrose, 0.4 mg/l benzylamino purine (BAP), 0.2 mg/l naphthalene acetic acid (NAA), 1 g/l cefotaxime, and 100 μg/ml kanamycin). After one week, regenerated shoots were transferred to GM agar with 1 g/l cefotaxime and 100 μg/l kanamycin (in 50 ml cylindrical plastic tubes), and newly formed callus was subcultured onto fresh SIM with the same antibiotics. As a control, untransformed RLD-1 seeds were surface-sterilized and incubated on the same GM with cefotaxime and kanamycin. After two weeks of growth on the selection GM, regenerated shoots were transferred to moistened sterile soil under a clear plastic cover, and placed in the greenhouse, while callus was subcultured every 10 days onto fresh SIM. After two subcultures, regeneration from callus was attempted by subculturing on SIM (1000 mg/l cefotaxime and 100 μg/ml kanamycin) with the following phytohormone combinations:

- 0.5 mg/l NAA, 1 mg/l BAP
- 0.5 mg/l NAA, 5 mg/l BAP
- 0.5 mg/l NAA, 10 mg/l BAP.

Isolation of plant genomic DNA and PCR

Plant DNA used in this study was isolated from leaf tissue of *A. thaliana* and *L. elongatum* using a modified miniprep technique from Dellaporta *et al.* (1983). Several pieces of leaf tissue were placed directly in a microfuge tube and liquid nitrogen was added, as the nitrogen evaporated the tissue was ground with a small glass pestle. A few

drops of extraction buffer were used to wash off the glass rod, and then another 750 µl were added to the microfuge tube. The extraction buffer was made up of:

- 50 mM Tris-Cl pH 8.0
- 10 mM EDTA pH 8.0
- 100 mM NaCl
- 1 % SDS
- 10 mM β-mercaptoethanol

The tube was incubated for 10 minutes at 65°C. Three hundred and thirty three µl of 3 M potassium acetate was added and the tube was inverted 6 to 8 times and incubated on ice for 20 minutes. The microfuge tube was then centrifuged for ten minutes and the supernatant was decanted into a clean tube. This tube was filled with isopropyl alcohol and centrifuged at full speed in a microfuge. The DNA pellet was washed with 70% ethanol, dried in a Speedvac™, and redissolved in 50 µl of water.

The polymerase chain reaction (PCR) was used to examine the presence of *Esi47* in plant genomic DNA. A dilution series of DNA from *L. elongatum* was used to establish optimal PCR conditions. Ten µl of diluted plant genomic DNA (1/1000 dilution factor of a minipreparation) was found to be optimal. Primers II and III (Table 1) were used with the following reaction conditions and temperature cycling program:

1 µl Taq polymerase [8 u/µl]
10 µl 10 x Taq buffer (Bio/Can Scientific)
10 µl DNA
2 µl dNTPs [10 mM]
10 µl primer II, 10 µM
10 µl primer III, 10 µM
57 µl sterile distilled water.

The program used for the thermal cycling was :

- Initial denaturation
  4 min at 95°C, denaturation

- 30 cycles:
  1 min at 95°C, denaturation
  1 min at 50°C, annealing
  2 min at 72°C, elongation

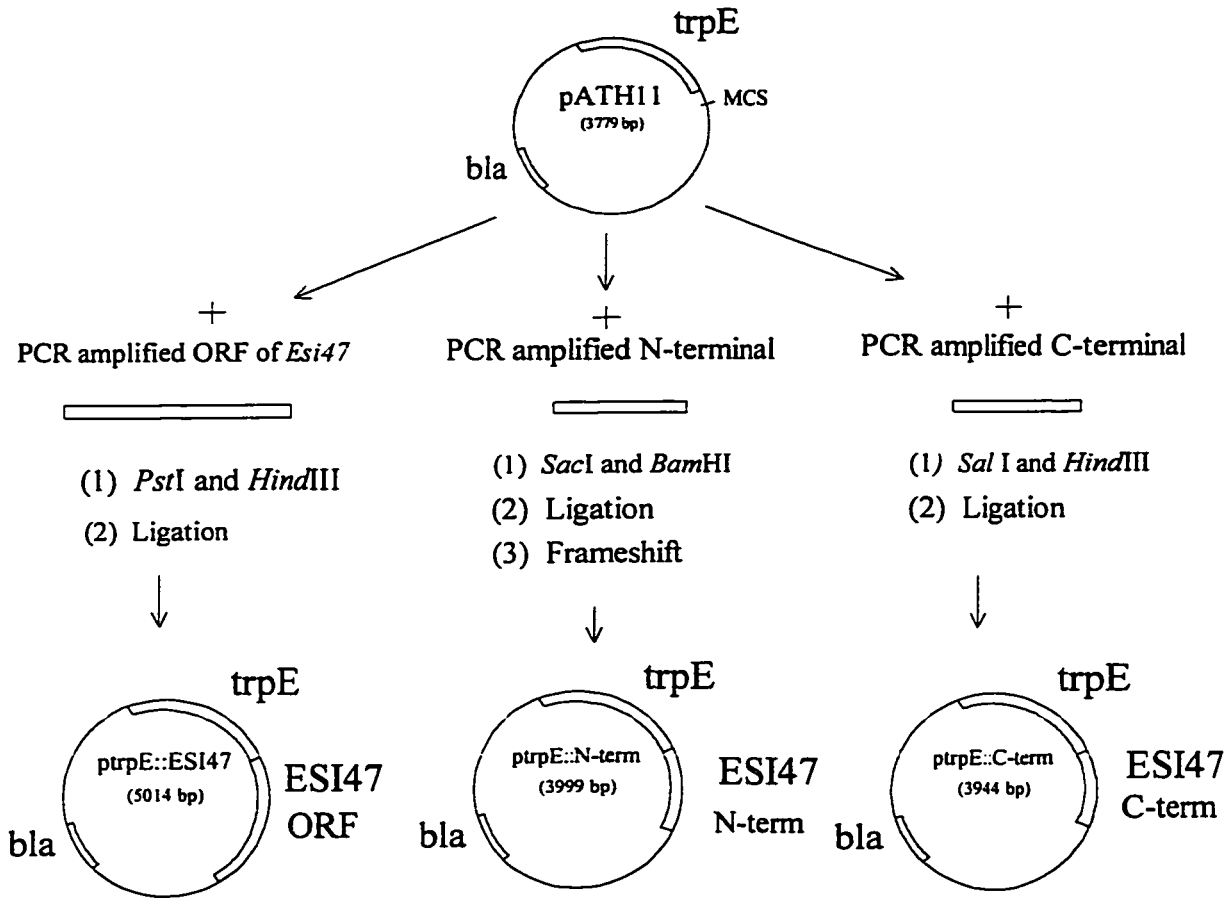
- Final elongation:
  5 minutes at 72°C.

3. Protein production in *E. coli*

TrpE fusion constructs

Three expression vectors were prepared using the pATH vector (Koerner *et al.*, 1991) for overexpression of a trpE::Esi47 fusion protein in *E. coli*. The pATH vectors consist of a *trpE* fusion under the control of the tryptophan operon. The three constructs are shown Figure 2. The *trpE::Esi47* fusions prepared were *trpE* fused to (I) the 1.2 kbp ORF of *Esi47*, (2) the first 198 nucleotides of the ORF, and (3) the last 180 nucleotides of the *Esi47* ORF (Figure 2). PCR was used to amplify each of the three fragments and cloning sites were introduced into the PCR products using mutagenic oligonucleotides. For construct I, the vector was prepared by digestion with *Pst*I and *Hin*dIII, and the insert (1.2 kbp ORF of E*si47*) was amplified using the PCR primers II and III (Table 1). The reaction conditions and thermal cycling program are described above with the following modifications: 1 µl of *E. coli* plasmid DNA (2.5 ng), and 67 µl of sterile distilled water were used.

Figure 2. pATH11 *trpE* fusion expression vectors used to overexpress *Esi47* in *E.coli.* PCR was used to amplify and introduce endonuclease restriction sites for subcloning (I) the full ORF of the *Esi47*, (II) it's N-terminus, and (III) it's C-terminus. These were fused to the N-terminus of *trpE* which is under the control of the Trp operon. A correctional frameshift was perfomed on construct II. *bla* is β-lactamase, the gene required for ampicillin resistance.


trpE
pATH11
(3779 bp)
MCS
bla
+
PCR amplified ORF of *Esi47*
(1) *Pst*I and *Hind*III
(2) Ligation
trpE
ptrpE::ESI47
(5014 bp)
ESI47
ORF
bla
+
PCR amplified N-terminal
(1) *Sac*I and *Bam*HI
(2) Ligation
(3) Frameshift
trpE
ptrpE::N-term
(3999 bp)
ESI47
N-term
bla
+
PCR amplified C-terminal
(1) *Sal* I and *Hind*III
(2) Ligation
trpE
ptrpE::C-term
(3944 bp)
ESI47
C-term
bla

The PCR reaction product was phenol:chloroform extracted and ethanol precipitated. It was digested with both *Pst*I and *Hin*dIII. Both the vector and the insert were electrophoresed on a 1% agarose gel, and purified as mentioned above. The vector and insert were ligated, and the ligation solution was then mixed with frozen heat shock competent *E. coli* cells (strain JF1754, *hsdS,* λ-, *lac, metB, leuB, hisB436)* and transformed. DNA was prepared from twenty four colonies and all contained the insert.

For construct II (Figure 2) pATH11 was digested *Sac*I and *Bam*HI. *Esi47's* N-terminus was PCR amplified (with primers IV and V, Table 1), as described above. The N-terminal fragment was digested subsequently with *Sac*I and *Bam*HI. The insert was electrophoresed, gel purified, then ligated to the vector overnight at 16°C in 15 µl volume. The ligation was ethanol precipitated and heat inactivated for 15 minutes at 65°C. The ligation mixture was digested with *Sma*I to remove any contaminating recircularized vector which remained, and then used for heat shock transformation of *E. coli* strain JF1754. Twelve colonies were screened, and four were shown by restricion digestion to contain the expected insert. One clone was selected to realign the reading frame of the N-terminus, since the pATH11 *trpE* was two bp out of frame with *Esi47.* The plasmid was digested at the *trpE::Esi47* junction with *Sac*I, then blunt ended with T4 DNA polymerase, the polymerase was heat inactivated, then T4 DNA ligase and *Sac*I were added to religate, and cut any contaminating plasmids which were not blunt ended. The ligation mixture was used to transform *E. coli* as above. Plasmid was prepared from

22 colonies, and the construct checked by *Sac*I digestion. Twenty one of 22 transformants had lost the *Sac*I site.

Construct III added the C-terminus of the ORF (180 bp) into the pATH11. Vector pATH11 was digested with *Sac*I and *Hin*dIII. PCR was used to amplify the C-terminus of *Esi47* (with primers IV and V, Table 1), as described above. The PCR fragment was digested with *Sal*I and *Hin*dIII. Each sample was run on a 0.6 % agarose gel, then purified as above. The insert was ligated to the vector and *E. coli* strain JF1754 was transformed by heat shock with the ligation mixture (Ausubel *et al.*, 1989). Ten colonies were used for plasmid preperation which was screened by digestion. The positive clones were linearized with *Pst*I and not affected by *Bam*HI digestion. Samples were digested separately with either *Pst*I or *Bam*HI and were electrophoresed on a 1% agarose gel. Most clones had the C-terminus properly inserted.

Overexpression of *Esi47::trpE* fusions in *E. coli*

E. coli transformed with the *Esi47::trpE* fusion were grown in 100 ml of modified M9 (Koerner *et al.*, 1991) with 1x M9 salts (Ausubel *et al.*, 1989), 5 g casamino acids/100 ml, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2% glucose, 10 μg/ml thiamin B_1, 20 μg/ml tryptophan, and 50 μg/ml ampicillin. The culture was incubated overnight at 37°C on a shaker. Cells were then centrifuged at 3000 x g for 5 minutes, the M9 medium poured off, and 100 ml of M9 without tryptophan was added. The cells were resuspended by vortexing, transferred to a sterile 500-ml Erlenmeyer flask, and incubated on a shaker at 37°C for 1.5 hours. After this, 0.5 ml of IAA (2 mg/ml in 95% ethanol) was added to

relieve repression and attenuation (Koerner *et al.*, 1991), and the cells were left to grow for 8 to 16 hours.

Protein extraction

Protein was isolated from *E. coli* according to Koerner *et al.* (1991). Cells were harvested by centrifugation at 3300 x g, washed in 10 mM Tris-HCl pH 7.5, and centrifuged again. The cell pellet was then lysed by resuspending in 20 ml of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1.4 ml 5 M NaCl, and 1.5 ml 10 % Nonidet P-40. The solution was mixed by inversion and sonicated on ice for 3 bursts of approximately 10 seconds. The cell lysate was centrifuged for 10 minutes at 9000 x g. The supernatant was discarded, and the insoluble fraction was washed once in 20 ml of 10 mM Tris-HCl, pH 7.5, 1 M NaCl, and once in 10 mM Tris-HCl, pH 7.5. The final pellet was resuspended in 1 ml of 10 mM Tris-HCl, pH 7.5 and transferred to a microfuge tube and homogenized with a small glass pestle. The protein was then stored at -20°C.

For analysis, a 10-µl aliquot of the homogenized pellet was mixed with 2 x protein loading buffer (Ausubel *et al.*, 1989) boiled for 10 minutes, and separated by 12 % SDS-PAGE. A preparative isolation of the fusion protein was carried out with 500 µl of protein preparation, electrophoresed on a 12 % SDS-polyacrylamide gel, and stained with Coomassie blue. The appropriate band was excised from the gel and stored at -20°C.

RESULTS

1. Transformation of *A. thaliana* by vacuum infiltration

Five series of plant transformations were attempted using the vacuum infiltration technique. Each series included, 64 plants per cultivar for each of the five cultivars (RLD-1, LE, Col-2, Abi1, and Aba1) for a total of 320 plants. Flowering began approximately one week after infiltration and continued for two to three weeks thereafter. Silique maturation required several more weeks. Most plants survived the infiltration process to produce considerable seed yield in all but one series, where all the plants died except for a few RLD-1, LE, and Col-2 specimens. These wilting plants died a few days after the vacuum treatment. Seed yield varied from a half to a full microfuge tube per cultivar in each series.

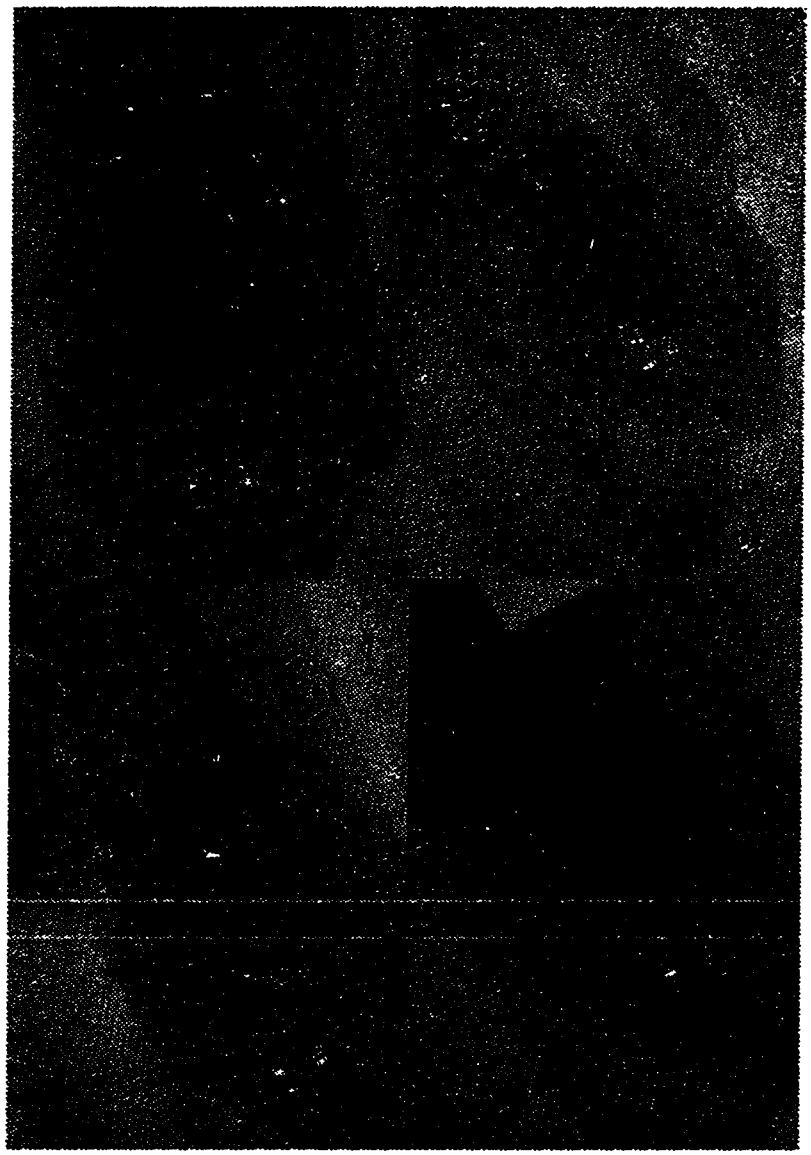
Seed germination on the kanamycin (50 μg/ml) selection plates was above 95 %, and most seeds germinated within one week, to produce one cm seedlings. After this, most seedlings scenesed, turned yellow and died within a few days. No growth was observed, nor did any of the seedlings produce secondary leaves or undergo root development. Approximately 15 seedlings (RLD-1 and LE) maintained vigor on the selection plates after 10 days, although no signs of growth were observed. They were transferred to soil, and upon transfer, growth resumed then developed into mature fertile plants. A leaf from each plant was cut off, genomic DNA was extracted and used for a PCR assay to detect the presence of *Esi47* sequence (with primers II and III, Table 1). No PCR product was observed from the *Arabidopis* specimens, while positive controls from

Lophopyrum genomic DNA and *E.coli* with the plant transformation construct I (Figure 1) amplified the *Esi47* N-terminal 180 nt fragments successfully (data not shown).

2. Transformation of *A. thaliana* by tissue culture method

Three series of *Agrobacterium* transformations with the ORF of *Esi47* (plant transformation construct I, Figure 1) using plant tissue-culture were undertaken. The first series was the only one that induced cell growth on the kanamycin (100 μg/ml + 1000 μg/ml cefotaxime) SIM plates. In this series, RLD-1 produced 10 regenerated shoots after one week on SIM (Figure 3). Both multiple and single shoots were observed (Figure 3A and 3C respectively). In addition, two explants produced shoots with multiple buds but remained stunted (Figure 3B). All regenerated plants developed secondary leaves within this first week of growth, and shoots grew to a length of approximately 1.5 cm. The shoots were transferred to 50-ml plastic cylindrical tubes with GM containing 100 μg/ml kanamycin + cefotaxime 1 g/l and growth progressed with further leaf and root formation. Shoots lengthened to approximately 3 inches within 2 weeks. Regenerated shoots were transferred to moistened sterile soil under a clear plastic cover and placed in the greenhouse. Transplanted plants wilted within the first half hour, and withered completely within 24 hours. Culture was continued to allow possible growth arising from the roots, however this did not occur. The RLD-1 explants from this series did not produce any significant callus. As a control,

Figure 3.- Reproductions of 7 day old *A. thaliana* RLD-1 regenerated seedlings on GM (1 g/L cefotaxime + 100 μg/ml kanamycin) medium. Seedlings A and C showed normal growth, while seedling B had a highly developed axial buds but remains stunted. D shows surface sterilized RLD-1 seeds two weeks after inoculation on the same medium.



surface sterilized RLD-1 seedlings were placed on the same medium as the regenerated shoots (Figure 3D). The seeds germinated but were unable to grow, and died soon thereafter.

Col-2 produced no shoots but callus was readily induced on SIM selection medium containing 100 μg/ml kanamycin and 1 mg/ml cefotaxime with 0.4 mg/l BAP and 0.2 mg/l NAA (Figure 4A). Calli arose primarily from the roots, and appeared non-photosynthetic, since they lacked pigmentation. The callus exhibited rapid growth, and doubled in size in the first week. The tissue was thus subcultured onto fresh SIM (kanamycin 100 μg/ml + 1 mg/ml cefotaxime). The callus line was subcultured two more times on a weekly basis, and then growth slowly subsided.

In the third series, two somatic embryos differentiated directly from meristematic tissue on Col-2 explants, seven days after transformation (Figure 4B). They were subcultured onto GM (100 μg/ml kanamycin + 1000 μg/ml cefotaxime), but failed to grow further. As well, three week old callus lines (Figure 5) were transferred to three different media (modified SIM) with 0.5 mg/l NAA, and different treatments of BAP (1 mg/l, 5 mg/l, and 10 mg/l). Such treatments are known to shock the callus into shoot formation and in some cases revive the tissue (Street, 1973). However, this proved unsuccessful in this case.

Figure 4.- Reproductions of (A) *A. thaliana* 10 day old Col-2 calllus tissue induced directly after transformation with plant transformation construct I on SIM (1 g/l cefotaxime + 100 μg/ml kanamycin) media. (B) Photograph of 7 day old somatic embryoes (to the right of the X) induced after transformation with the same construct, on SIM (cefo.+ kan.) medium.

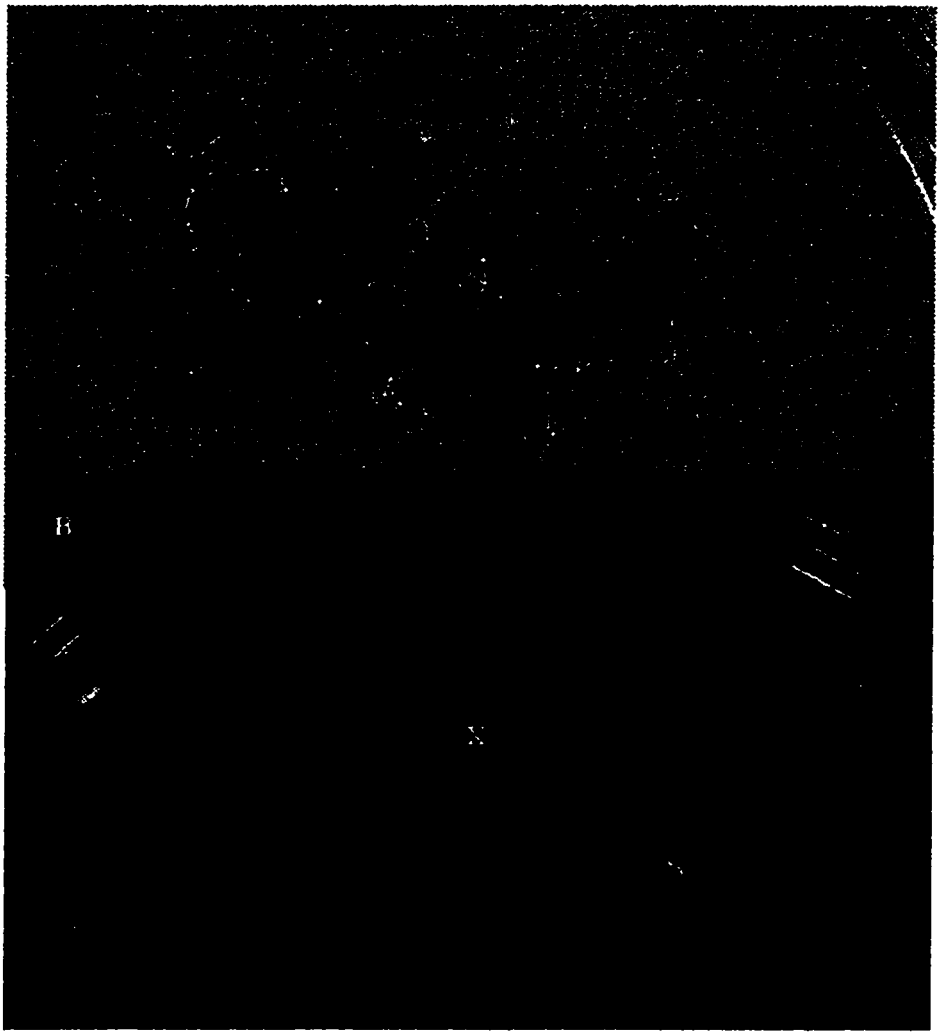

B

Figure 5.- Reproduction of 3 week old *A. thaliana* callus tissue, subcultured weekly on modified SIM with 0.5 mg/L NAA and 5 mg/BAP (with 1000 mg/l cefotaxime+100 mg/l kanamycin). Callus arose primarily from the roots, as can be seen by the extensions out of the core of the explant.

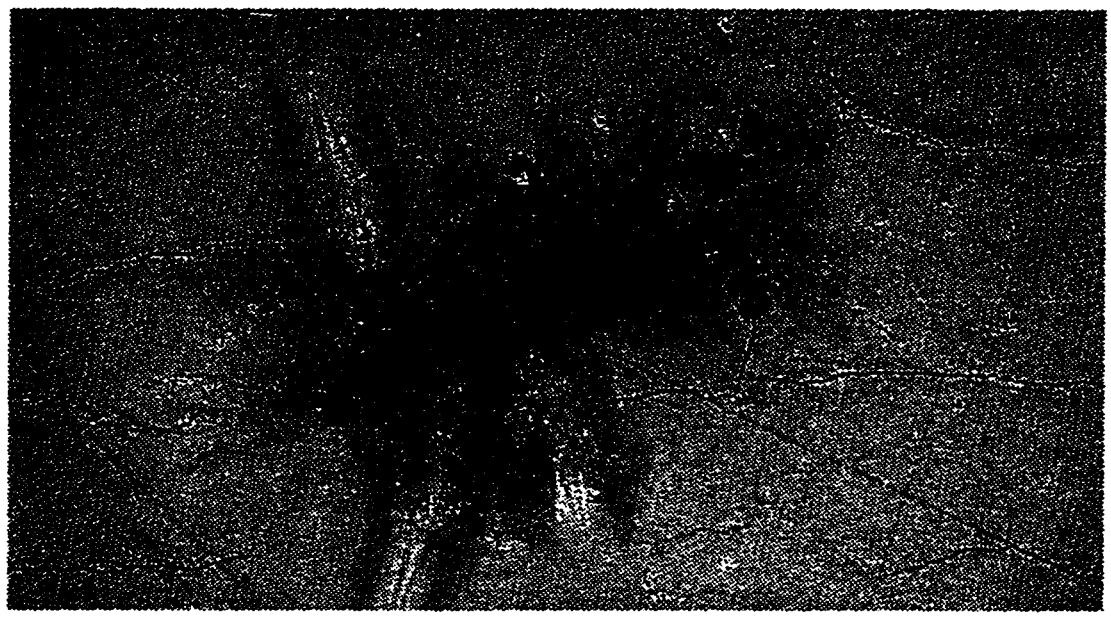
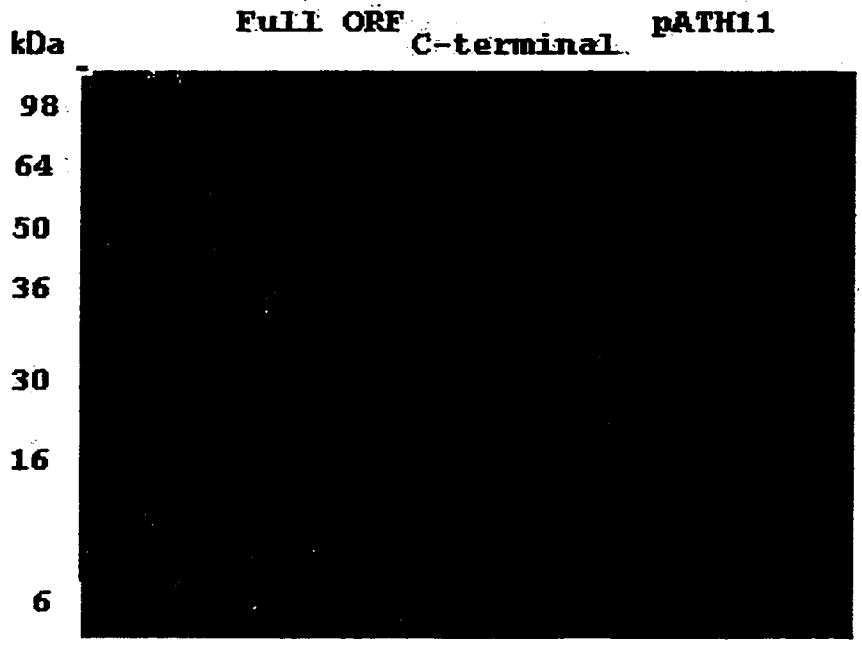


Figure 6.- 12% SDS-polyacrylamide gel stained with Coomassie blue of *trpE::esi47* fusions overexpressed in *E. coli*, for antigen production and in vitro kinase characterization. The full ORF of *Esi47* was fused to *treE* from the pATH11 expression vector (lane 1) and the expressed protein runs smeared from 75 to 40 kDa. The C-terminus of *Esi47* fused to *trpE*, when overexpressed and runs at 45 kDa (lane 2). pATH11 expression vector without an insert in its MCS is in lane 3.


Full ORF
C-terminal
pATH11
kDa
98
64
50
36
30
16
6

3. Protein production in *E. coli*

E. coli strain JF 1754 with the pATH11 vector successfully overexpressed the *trpE::esi47* full ORF, and the *trpE::esi47* carboxy teminal fusions (*trpE* fusion constructs I and III, Figure 2), when induced in the absence of tryptophan in the medium. When the soluble fraction was run on a 12% SDS-polyacrylamide gel, it showed no evidence of fusion products. When the insoluble fraction was solubilized in protein loading buffer (Ausubel *et al.*, 1989) and run on a 12 % SDS-polyacrylamide gel (Figure 6), the full ORF fusion (lane 1) ran as a smear between 75 kDa and 40 kDa. Apparent smearing was likely due to large quantity of protein in the sample, as well as partial degradation of product in *E. coli*. The two lower bands at 33 kDa and 34 kDa represent a doublet of the genomic copy of trpE while the faint band that can be seen at approximately 30 kDa is probably β-lactamase, the gene required for ampicillin resistance (Koerner *et al.*, 1991). The trpE::esi47 carboxy-terminal fusion product (lane 2) was evident as an approximetly 45 kDa product (Figure 6). β-lactamase at approximately 30 kDa (lane 2) appears to be strongly induced in this protein extract. The vector, pATH11, without an insert in the MCS, was induced as well as a control (lane 3). The trpE protein from this extract ran as a doublet at 33 kDa and 34 kDa. The *trpE::esi47* amino-terminal fusion (*trpE* fusion construct II in pATH11, Figure 2) did not produce a readily visible product (data not shown).

DISCUSSION

To investigate the role of *Esi47* from *L. elongatum* in the regulation of stress response, it was introduced into transgenic *Arabidopsis* under the control of a constitutive promoter. Upon transfer, it would be possible to monitor its effects on stress-inducible responses. *Arabidospsis* is an excellent model organism since many stress-induced genes and inducible promoters have been cloned and characterized in this species (Wang *et al.*, 1995; Yamagushi-Shinozaki and Shinozaki, 1994; Ureo *et al.*, 1994; Welin *et al.*, 1994; Kiyosue *et al.*, 1994a; Kiyosue *et al.*, 1994b; Yamagushi-Shinozaki and Shinozaki, 1993; Horvath *et al.*, 1993; Nordin *et al.*, 1993; Nordin *et al.*, 1991), and both ABA biosynthetic (Koorneef *et al.*, 1982) and ABA-insenitive response mutants are available (Koorneef *et al.*, 1984). The ABA mutants have permitted the classification of these genes to the signal transduction pathways by which they are mediated, either the ABA -dependent or -independent. As well, both physiological and morphological responses to cellular dehydration have been well characterized (Koorneef *et al.*, 1982; Koorneef *et al.*, 1984; Vartanian *et al.*, 1994). Finally, *Agrobacterium*-mediated transformation of *A. thaliana* with tissue culture (Valvekens *et al.*, 1988) and *in vivo* vacuum infiltration have been reported (Bent *et al.*, 1994; Betchold *et al.*, 1993).

Plant genetic transformation has been successful in the characterization of the protein kinases *Pto*, *Pti*, and *Xa21*, involved in plant-pathogen response, and the protein phosphatase *ABI1*, which mediates ABA responses including those that are dehydration related. The binary vector pBI121 used in this study is appropriate for both transformation protocols (Valvekens *et al.*, 1988; Bent *et al.*, 1994; Betchold *et al.*,

1993). The β-glucuronidase reporter gene was removed from pBI121 and replaced by the 2nd open reading frame of *Esi47* (Jefferson *et al.*, 1987) which encodes a serine/threonine protein kinase. This places *Esi47* under the constitutive control of the CaMV 35S promoter. Martin *et al.* (1993) and Zhou *et al.* (1995) placed the pathogen-response protein kinases *Pto* and *Pti* under the control of the CaMV 35S promoter in pBI121 and used *Agrobacterium*-mediated transformation of both tomato and tobacco in a similar manner.

The first approach used in this work was the vacuum infiltration technique (Bent *et al.*, 1994). This technique is based on the *Agrobacterium*-mediated transformation process (reviewed by Zupan and Zambryski, 1995). It involves the inoculation of *A. thalinana* prior to flowering with *Agrobacterium* carrying the engineered plasmid. The plants are placed in a vacuum, which is released rapidly enabling the *Agrobacterium* to infiltrate intracellular spaces. Seed is then collected and plated on selection plates that contain antibiotics, in this case kanamycin. This approach was unsuccessful in this study. While a few seedlings maintained vigor on the selection plates, those expressing the kanamycin selectable marker should have undergone secondary growth (Bent *et al.*, 1994). Growth was not observed until seedlings were transferred to soil. Possibly seed yields or the number of seeds screened may have been insufficient to compensate for the low transformation efficiencies that are related to this approach (F. Belzile, personal communication). Rinsing the plant after the inoculation may have lowered the *Agrobacterium* densities. Aside from the experimental procedure, it is possible that the transgenic seeds may have been altered such that they were unable to survive the surface

sterilization process that preceds plating. It is plausible that *Esi47* alters the ABA-dependant signaling required for proper seed maturation. To test this hypothesis, *Agrobacterium* carrying a control transformation plasmid such as pBI121 could be used to monitor relative transformation efficiencies.

The tissue culture approach was partially successful. Developed by Valvekens *et al.* (1988), it has been widely used for the transformation of *Arabidopsis*, including studies of transgenics expressing *ABI1*, the ABA response mediating phosphatase (Meyer *et al.*, 1994; Leung *et al.*, 1994). Transformation of *Arabidopsis* cv. RLD-1 produced 10 regenerated shoots after one week on shoot-inducing kanamycin selection medium. These shoots grew rapidly and were transferred to hormone free growth medium, again with kanamycin. Rooting occurred and rapid growth persisted. The seedlings exhibited single and multiple shooting, which are commonly observed in micropropagation. Additionally, two seedlings remained stunted but underwent healthy bud development, presumably as a result of the stress of *in vitro* culture, which may have induced somaclonal variation (Bhojwani and Razdan, 1983). This rapid growth is a strong indication of the expression of *nptII* found within the transferred DNA (T-DNA) incorporated within the plant genome. Three weeks later, the seedlings were planted in the greenhouse under a clear plastic wrap to maintain humidity but wilted rapidly upon transfer.

Although it is common for plants to be weakened during acclimatization, constitutive expression of *Esi47* may be deleterious. Such effects were observed by Leung *et al.* (1994) in the transfer of *ABI1* from the ABA -insensitive mutants to wild

type *Arabidopsis* cv. C24. In their transformation experiment, 17 of the 20 primary transformants exhibited ABA-resistant root growth. While the progeny (T1) from these transgenics had increased tendency to wilt during propagation in the greenhouse, seven of these lines were more severe in this phenotype than the original *abi1-1* mutant (Leung *et al.* 1994). The T2 seed progeny homozygous for the transgene also showed reduced seed dormancy (Leung *et al.* 1994), demonstrating that seed developmental processes can be altered by the transfer of genes involved in ABA signaling.

It must be determined if *Esi47* is blocking the pathways required to overcome the aclimatization process that plants undergo when transferred from test tubes to the greenhouse. This phenotype, and the possible alteration in the seed maturation processes, add technical complications, rendering their study difficult. Since the kanamycin resistant plants regenerated from tissue culture were likely transgenic, it may be possible to grow such plants to maturity *in vitro*, then the stress response phenotype could be tested on the progeny. The genotype of the plants should also be confirmed by Southern analysis. If antibodies are available, Western analysis should be performed to confirm expression of *Esi47*. A comparative analysis with untransformed plants would be informative in terms of the effects of *Esi47* on *A. thaliana*'s physiology and gene expression in response to dehydration stress. If normal seed developmental processes are affected, preventing *in vitro* plants from producing seed, axenic cultures could be used to propagate the transformed genotype, used later for studies in stress response. These could include Northern analysis on a variety of stress induced genes, Na^+/K^+ ratios in tissue, guard cell responses, wilting, and biomass accumulation upon various levels of

dehydration. Alternatively, *Esi47* could be placed under the control of an inducible promoter. An inducible/repressible system developed by Gatz (1995), which can control gene expression in eukaryotic organisms by tetraccycline treatment, would probably be the most useful in regulating an *Esi47* transgene. It uses a *tetR* operator::CaMV 35S promoter which can be sterically blocked by a coexpressed tetR bacterial repressor. Using this system, the expression of *Esi47* could be regulated, insuring that its respective phenotype would not be affecting the transformation efficiencies.

Arabidopsis cv. RLD-1 used for transformation regenerated into shoots, however was poor at producing callus. In contrast Col-2 readily produced callus which could be maintained for three passages on shoot inducing medium with kanamycin, after which growth subsided. The ability to regenerate *in vitro,* from *de novo* meristem, depends on the genotype used and can vary between species (Bhojwani and Razdan, 1983). Shoot inducing medium, which consists of a high cytokine/auxin ratio will affect plants in a genotype specific manner, and must be adjusted for each strain used. As for the growth of Col-2 callus on SIM, while it appeared healthy early on, it should have been possible to maintain it indefinitely. Adjustments to the medium, including balancing the cytokine/auxin ratio, type of phytohormone either synthetic or natural, supplements such as yeasts extracts, and basic salt and vitamin combinations, all of which may affect growth, and consequently regeneration, should be examined (Street, 1973). Cefotaxime, which was used to prevent *Agrobacterium* growth, may have also hindered regeneration from Col-2 explants. Alternatives, such as vancomycin (Valvekens *et al.*, 1988) should be investigated. Embryogenesis observed in Col-2 demonstrated one of the possible

avenues for regeneration for this genotype (Narayanaswamy, 1977; Bhojwani and Razdan, 1983).

The pATH11 expression system (Koerner *et al.*, 1991) expressed *trpE::esi47* fusions, with the exception of the N-terminal fusions. The full open reading frame and the C-terminal fusions expressed 75 kDa and 45 kDa proteins, respectively, when induced by media lacking tryptophan. These correspond well with the predicted size of the fusions (Koerner *et al.*, 1991), and in comparison with the vector without insert which ran at 34 kDa. Mutations are known to occur in the *trpE* promoter region. This may have been the reason for low levels of N-terminal fusion product (Koerner *et al.*, 1991).

The plant transformation vectors II-V were prepared for promoter analysis by removing the CaMV 35S promoter from pBI121 and replacing it with modified 5'-regions of *Esi47*. If the promoter is inducible in *A. thaliana*, the constructs will give insight into the tissue specific expression of *Esi47*. The factors that should be examined are the inducibility by salt-stress or ABA, or any possible developmental or tissue specificity. Furthermore, by comparing the expression of the GUS reporter gene, the modifications will provide further insight into the role of the first 51 bp ORF in the regulation of *Esi47*.

This and proposed work will develop a greater understanding of the salt-stress induced Esi47 protein kinase from *Lophopyrum elongatum* and its role in the control of stress-responses by phosphorylation. Plant protein kinases, as mediating factors that may alter the activity of other enzymes, proteins, or transcriptional activators through phosphorylation, are of great interest (Stone and Walker, 1995). Their study will

undoubtedly lead to a better understanding of the responses they control. Control transformations to assess the efficiency would be highly beneficial, especially to ascertain the possible toxicity or crippling effect of *Esi47*. If *Esi47* does control the responses to dehydration related stresses in transgenic *A. thaliana*, it will be difficult for the plants to withstand the transformation itself, which is inevitably stressful. If circumvented by maintaining the plants *in vitro*, as "bubble" plants of sorts, or placing *Esi47* under the control of a repressible-inducible promoter, in conjunction with a comparative analysis with non-transgenic controls, knowledge will be obtained into the role of *Esi47* and of phosphorylation in dehydration stress-response in plants.

REFERENCES

ANDERBERG, R.J. AND M.K. WALKER-SIMMONS. 1992. Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases. *Proc. Natl. Acad. Sci. USA* **89**:10183-10187

AUSUBEL, F.M., BRENT, R., KINGSTON, R.E. MOORE, D.D, SEIDMAN, J.G., SMITH, J.A., AND K. STRUHL. 1989. Short Protocols in Molecular Biology. John Wiley & Sons, New York.

BETCHOLD, N., ELLIS, J., AND G. PELLETIER. 1993. In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C.R. Acad. Sci. Paris, Life Sciences* **316**:1194-1199

BENT, A.F., KUNKEL, B.N. DAHLBECK, D., BROWN, K.L., SCHMIDT, R., GIRAUDAT, J., LEUNG, J. AND B.J. STASKAWICZ. 1994. RPS2 of *Arabidopsis thaliana*: A leucine rich repeat class of plant disease resistance genes. *Science* **256:** 1856-1860

BHOJWANI, S.S, AND M.K. RAZDAN. 1983. Plant Tissue Culture, Theory and Practice. Elsevier. New York.

BIDWELL, R.G.S. 1979. Plant physiology. 2d ed. New York: Macmillan.

BOHNERT, H.J., NELSON D.E., AND R.G. JENSON. 1995. Adaptations to environmental stresses. *Plant Cell.* **7**:1099-1111

BOSTOCK, R.M., AND R.S. QUATRANO. 1992 Regulation of *Em* gene expression and abscisic acid. *Plant Physiol.* **98**:1356-1363

BOWLER, C., AND N.-H. CHUA. 1994. Emerging themes of plant signal transduction. *Plant Cell* **6**:1529-1541

BRAY, E.A. 1993. Molecular responses to water deficit. *Plant Physiol.* **103**:1035-1040

CHEN, H.H., LI, P.H., AND M.L. BRENNER. 1983. Involvement of abscisic acid in potato cold acclimation. *Plant Physiol.* **71**:362-365

COHEN, A., AND E.A. BRAY. 1990. Characterization of three mRNAs that accumulate in wilted tomato leaves in response to elevated levels of endogenous abscisic acid. *Planta* **182**:27-33

CSONKA, L. 1989. Physiology and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **53**:121-147

DELLAPORTA, S.L., WOOD, J., AND J.B. HICKS. 1983. A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* **1**:19-21

DELAUNEY, A.J., AND D.P.S. VERMA. 1993. Proline biosynthesis and osmoregulation in plants. *Plant J.* **4**:215-223

DING, J.P., and B.G. PICKARD. 1993. Mechanosensory calcium-selective cation channels in epidermal cells. *Plant J.* **3**:83-110

DVORAK, J., EDGE, M., AND K. ROSS. 1988. On the evolution of the adaptation of *Lophopyrum elongatum* to growth in saline environments. *Proc. Natl. Acad. Sci. USA.* **85**:3805-3809

DVORAK, J., EPSTEIN, E., GALVEZ, A.F., GULICK, P.J., AND J.A. OMIELAN. 1991. Genetics of salt tolerance in highly salt tolerant *Lophopyrum elongatum*, salt sensitive wheat, and their amphiploid. (R. Choukr-Allah, ed) *in* Plant Salinity Research, Proceedings of the International Conference on Agricultural Managment of Salt-affected areas held in AGADIR MOROCCO. **April 26 - May 3 1991**

DVORAK, J., INSKI, E. 1993. Compatible solutes of halophystic eubacteria-Molecular principles, water-solute interaction, stress protection. *Experentia* **49**:487-496

DVORAK, J., NOAMAN, M.M., GOYAL, S., AND J. GORHAM. 1994. Enhancement of the salt tolereance of *Triticum tugidum* L. by *Knal* locus transferred from the *Triticum aestivum* L. chromosome 4D by homologous recombination. *Theor. Appl. Genet.* **87**:872-877

GALVEZ, A.F., GULICK, P.J., AND J. DVORAK. 1993. Characterization of early stages of genetic salt-stress response in salt-tolerant *Lophopyrum elongatum*, a salt-sensitive wheat, and their amphiploid. *Plant Physiol.* **103**: 257-265

GAMBORG, O.L., MILLER, R.A., AND K. OJIMA. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res* **50**:151-158

GATZ, C. 1995. Novel inducible/repressible gene expression systems, *in* Methods in Cell Biology (Galbraith, D.W., Bourque, D.P. and H.J. Bohnert, eds). Volume 50, Academic Press, New York.

GIRAUDAT, J., PARCY, F., BERTAUCHE, N., GOSTI, F., LEUNG, J., MORRIS, P.C., BOUVIER-DURAND, M., AND N. VARTANIAN. 1994. Current advances in abscisic acid action and signalling. *Plant Mol. Biol.* **26**:1557-1577

GIULANO, G., PICHERSKI, E., MALIK, V.S., TIMKO, M.P., SCOLNIK, P.A., AND A.R. CASHMORE. 1988. An evolutionary conserved protein binding sequence upstream of a plant light-regulated gene. *Proc. Natl. Acad. Sci. (USA)* **85**:7089-7093

GUILTINAN, M.J., MARCOTTE, W.R., AND R.S. QUATRANO. 1990. A plant leucine zipper protein that recognizes an abscisic acid element. *Science*. **250**:267-271

GULICK, P.J., AND J. DVORAK. 1987. Gene induction and repression by salt treatment in roots of the salinity-sensitive Chinese Spring wheat and the salinity-tolerant Chinese Spring x *Elytrigia elongata* amphiploid. *Proc. Natl. Acad. Sci. USA.* **84**:99-103

GULICK, P.J., AND J. DVORAK. 1990. Selective enrichment of cDNAs from Salt-stress induced genes in wheatgrass, *Lophopyrum elongatum* by formamide-phenol emulsion reassociation technique. *Gene* **95**:173-177

GULICK, P.J., AND J. DVORAK. 1992. Coordinate gene response to salt stress in *Lophopyrum elongatum. Plant Physiol.* **100**:1384-1388

HARPER, J.F., SUSSMAN, M.R., SCHALLER, G.E., PUTNAM-EVANS, C., CHARBONNEAU, H., AND A.C. HARMON. 1991. A calcium dependent protein kinase with a domain similar to calmodulin. *Science* **252**:951-954

HARPER, J.F. BIMDER, B.M.., AND M.R. SUSSMAN. 1993. Calcium and lipid regulation of an *Arabidopsis* protein kinase expressed in *Escherichia coli. Biochemisrty* **32**:3282-3290

HORVATH, D.P., MCLARNEY, B.K., AND M.F. THOMASHOW. 1993. Regulation of *Arabidopsis thalinana* L. (Heyn) *cor78* in response to low temperature. *Plant Physiol.* **103**:1047-1053

HWANG, I. AND H.M. GOODMAN. 1995. An *Arabidopsis thaliana* root-specific kinase homolog is induced by dehydration, ABA, and NaCl. *Plant J.* **8**:37-43

IZAWA, T., FOSTER, R., AND N.-H. CHUA. 1993. Plant bZip protein DNA binding specificity. *J. Mol. Biol.* **230**:1131-1144

JEFFERSON, R.A., KAVANAGH, T.A., AND M.W. BEVAN. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**:3901-3907

KAWASKI, T., HAYASHIDA, N., BABA, T., SHINOZAKI, K., AND H. SHIMADA. 1993 A novel calcium-dependent protein kinase near the gene encoding starch branching enzyme I (*sbe* 1) is specifically expressed in developing rice seeds. *Gene* **129**:183-189

KEIBER, J.J., ROTHENBERG, M., ROMANG., FELDMANN, K.A., AND J.R. ECKER. 1993. CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. *Cell* **72**:427-441

KIYOSUE, T., YAMAGUSHI-SHINOZAKI, K., AND K. SHINOZAKI. 1994a. Characterization of two cDNAs (ERD10 and ERD14) corresponding to genes that respond rapidely to dehydration stress in *Arabidopsis thaliana. Plant Cell Physiol.* **35**: 225-231

KIYOSUE, T., YAMAGUSHI-SHINOZAKI, K., AND K. SHINOZAKI. 1994b. Cloning of cDNAs for genes that are early-responseve to dehydration stress (ERDs) in *Arabidopsis thaliana* L.: Identification of three ERDs as HSP cognate genes. *Plant Mol. Biol.* **25**:791-798

KNIGHT, C.D., SEHGAL, A., ATWAL, K. WALLACE, J.C., COVE, D.J., COATES, D., QUATRANO, R.S., BAHADUR, S., STOCKLEY, P.G. AND A.C. CUMMING. 1995. Molecular response to abscisic acid and stress are conserved between moss and cereal. *Plant Cell.* 7:499-506

KOERNER, T.J., HILL, J.E., MYERS, A.M., AND A. TZAGOLOFF. 1991. High expression vectors with mutliple cloning sites for construction of *trpE* fusion genes: pATH vectors. *Methods in Enzymology* **194**: 477-490

KOORNEEF, M., JORNA, M.L., BRINKHORST-VAN DER SWAN, D.L.C, AND C.M. KARSEN. 1982. The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive line of *Arabidopsis thaliana* (L.) Heynh. *Theor. Appl. Genet.* **61**:385-393

KOORNEEF, M., REULING, G., AND C.M. KARSSEN. 1984. The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana. Physiol. Plant.* **61**:377-383

LEUNG, J., BOUVIER-DURAND, M., MORRIS, P.C. GUERRIER, D., CHEFDOR, F., AND J. GIRAUDAT. 1994. *Arabidopsis* ABA-response gene *ABI1*: features of a calcium-modulated protein phosphatase. *Science* **264**:1448-1452

MARCOTTE, M.R., RUSSEL, S.H., AND QUATRANO. 1989. Abscisic acid-responsive sequences from the *em* gene of wheat. *Plant Cell* **1**:969-976

MARTIN, G.B., BROMMONSCHENKEL, S., CHUNWONGSE, J., FRARY, A., GANAL, M.W., SPIVEY, R., WU, T., EARLE, E.D., AND S.D. TANKSLEY. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262**:1432-1436

MEYER, K., LEUBE, M.P., AND E. GRILL. 1994. A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana. Science* **264**:1452-1455

MOONS, A.A, BAUW, G., PRINSEN, E., MONTAGU, M.V., AND D. VAN DER STRAETEN. 1995. Molecular and physiological response to abscisic acid and salts in roots of salt-sensitive and salt-tolerant indica rice varieties. *Plant physiol.* **107**: 177-186

MUNDY, J., YAMAGUSHI-SHINOZAKI, K., AND N.H. CHUA. 1990. Nuclear proteins bind conserved elements in the abscisic-acid responive promoter of a rice *rab* gene. *Proc. Natl. Acad. Sci. USA.* **87**:1406-1410

MURASHIGE, T. AND F. SKOOG. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**:473-497

NARAYANASWAMY, S. 1977. Regeneration of plants from tissue cultures, *in* Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture (Reinert, J. and Y.P.S. Bajaj, eds). Springer-Verlag, New York.

NORDIN, K. HEINO, P. AND E.T. PALVA. 1991. Separate signal pathways regulate the expression of a low temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.*. **16**:1061-1071

NORDIN, K., VAHALA, T., AND E.T. PALVA. 1993. Differential expression of two related low temperature induced genes in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* **21**:641-653

OEDA, K., SALINAS, J. AND N.H. CHUA. 1991. A tobacco bZIP transcriptional activator (TAF-1) binds to a G-box-like motif conserved in plant genes. *EMBO J.* **10**:1793-1802

POTTS, M. 1994. Desiccation tolerance in prokaryotes. *Microbiol. Rev.* **58**:755-805

PUTNAM-EVANS, C., HARMON, A.C., AMD M.J. CORMIER. 1990. Purification and characterization of a novel calcium-dependant protein kinase from soybean. *Biochemistry* **29**:2488-2495

SCHAEFFER, H.J., FORSTHOEFEL, N.R., AND J.C. CUSHMAN. 1995. Identification of enhancer and silencer regions invovled in salt-responsive expression of Crassulacian acid metabolism (CAM) genes in the facultative halophyte *Mesembryathemum crystallinum*. *Plant Mol. Biol.* **28**:205-218

SCHROEDER, J.I. 1995. Anion channels as central mechanism for signal transduction in guard cells and putative functions in roots for plant-soil interactions. *Plant Mol. Biol.* **28**: 353-361

SCHROEDER J.I., SCHMIDT,C., SHEAFFE, J., 1993. Identification of high-affinity slow channel blockers and evidence for stomatal regulation by slow anion channels in guard cells. *Plant Cell* **5**: 1831-1841

SKERRET, M. AND D. TYERMAN. 1994. A channel that allows inwardly-directed fluxes of anions in protoplasts derived from wheat roots. *Planta* **192**: 295-305

SKIVER, K., AND J. MUNDY. 1990. Gene expression in response to abscisic acid and osmotic stress. *Plant Cell.* **2**:503-512

SONG, W.Y., WANG, G-L., CHEN, L.L., KIM, H.S., PI, L-Y, HOLSTEN, T., GARDNER, J., WANG, B., ZHAI, W.X., ZHU, L.-H., FAUQUET, C., AND P. ROLAND. 1995. Receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science* **270**:1804-1806

STEWART, C.R., AND G. COETBERG. 1985. Relationship between stress induced ABA and proline accumulation. *Plant Physiol.* **63**:24-27

STONE, J.M. AND J.C. WALKER. 1995. Plant protein kinase families and signal transduction. *Plant Physiol.* **108**:451-457

STREET, H.E. 1973. Plant tissue and cell culture. University of California Press, Berkeley and Los Angeles.

SUEN, K.L., AND J.H. CHOI. 1991. Isolation and sequence analysis of a cDNA clone for carrot calcium-dependent protein kinase: homology to calcium calmodulin-dependent protein kinases and to calmodulin. *Plant Mol. Biol.* **17**:581-590

UREO, T., KATAGIRI, T., MIZOHUSHI, T., YAMAGUSHI-SHINOZAKI, K., HAYASHIDA, N., AND K. SHINOSAKI. 1994. Two genes that encode Ca^{2+}-dependant protein kinases are induced by drought and high salt stresses in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **244**:331-340

UREO, T., YAMAGUSHI-SHINOZAKI, K., URAO, S., AND K. SHINOSAKI. 1993. An *Arabidopsis myb* homologue is induced by water stress and its gene product binds to the conserved MYB-recognition sequence. *Plant Cell* **5**:1529-1539

VALVEKENS, D., MONTAGU, M.V., AND M.V. LUSEBETTENS. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. (USA)* **85**:5536-5540

VARTANIAN, N.M., MARCOTTE, l., AND GIRAUDAT. 1994. Drought Rhizogenesis in *Arabidopsis thaliana*. *Plant Physiol.* **104**:761-767

VILARDEL, J., GODAY, A., FREIRE, M.A., TORRENT, M., MARTINEZ, C., TORNE, J.M., AND M. PAGES. 1990. Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. *Plant Physiol. 14:423-432*

WANG, H., DALTA, R., GEORGES, F., LOEWEN, M., AND A.J. CUTLER. 1995. Promoters from *kin1* and *cor6.6*, two homologous *Arabidopsis thaliana* genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum and dehydration. *Plant Mol. Biol.* **28**:605-617

WELIN, B.J., OLSON, A., NYLANDER, A., AND E. PALVA. 1994. Characterization and differential expression of *dhn/lea/rab*-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*. *Plant Mol. Biol.* **26**:131-144

YAMAGUSHI-SHINOZAKI, K., AND K. SHINOZAKI. 1993. Characterization of the expression of a desication-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. *Mol. Gen. Genet.* **236**:331-340

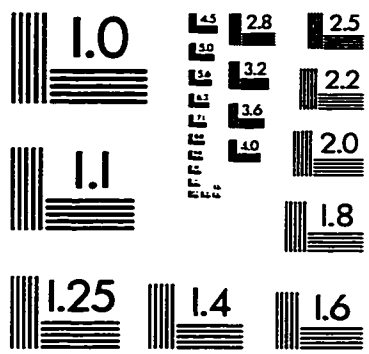
YAMAGUSHI-SHINOZAKI, K., AND K. SHINOZAKI. 1994. A novel *cis*-acting element in *Arabidopsis* gene is involved in the responsivness to drought, low-temperature, or high-salt stress. *Plant Cell* **6**:251-264

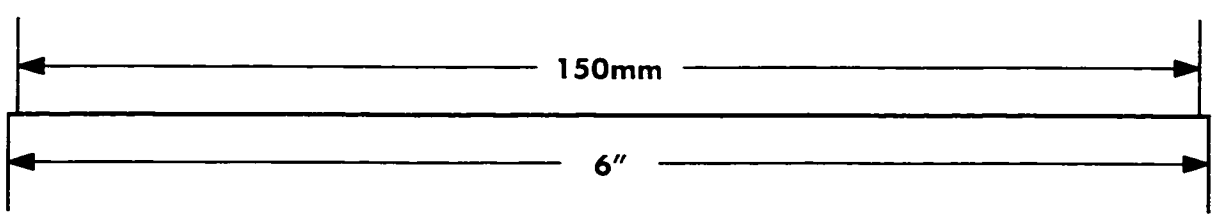
YEOMAN, M.M. 1973. Tissue (callus) cultures-techniques, *in* Plant Tissue and Cell culture (Street, H.E. ed). University of California Press. Berkeley and Los Angeles.

ZHOU, J., LOH, Y.-T., BRESSAN, R.A., G.B. MARTIN. 1995. The tomato gene *Pti1* that encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell* **83**:925-935

ZUPAN, J.R., AND P. ZAMBRYSKI. 1995. Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiol.* **107**:1041-1047

TEST TARGET (QA-3)





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