The Interaction of the Stress Regulated Wheat (*Triticum aestivum*) Proteins, ESI2 and RAN1, and the Localization of Ipp-1 and Pr-1.

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Abstract

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and the Localization of Ipp-1 and Pr-1

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The Early Salt-Stress Induced 2 protein, ESI2, from wheat (*Triticum aestivum*) was identified as an interacting protein with the GTP-binding protein RAN1. Localization of both ESI2, and RAN1, has been performed using fluorescent protein fusions, transiently expressed in *Nicotiana belthamiana,* and observed using confocal fluorescent microscopy. The proteins fused to independent fluorescent partners localized to separate cellular compartments, with RAN1 primarily localized to the nucleus. However, the interaction of the two proteins detected by bimolecular fluorescent complementation, was found to be localized to the tonoplast. This interaction suggests a potential mechanism for the role of ESI2 in the sequestration of RAN1 and the regulation of cell division in response to stress. Two additional proteins, expressed in wheat during stress response and shown previously, by Y2H assays to bind with ESI2, where also localized. To further explore the effects of RAN1 on cell division in plants, a homozygous mutant for the *Arabidopsis Ran1* was identified and a change in root growth shortly after germination was noted.

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PART I. INTRODUCTION

In recent years, apprehension for global food shortages has increased due to increasing human populations. This concern is not without reason and as land devoted to agriculture is increased, there will be increased pressure to use marginal areas that are subject to environmental stresses. Compounding this concern, climate changes threaten previously thriving agricultural areas (Lane and Jarvis 2007). Currently, it has become essential to increase our understanding of tolerance to several environmental stress conditions with impacts on plant productivity, particularly those resulting in large losses, such as salinity and drought. Elucidating the complex mechanisms regulating tolerance to these stresses will provide insight into the basis of genetic mechanisms that can be improved or optimized. Ultimately, improved agricultural production has not only helped to feed people, but has positive repercussions, stabilizing volatile regions, and strengthening democratic governance.

1. Environmental Stress

The impact of environmental stress on plant growth and productivity can be measured by various criteria, but ultimately the most important global measure is reduction in yield. For most major crops, over 50% of their potential yield is lost due to abiotic stresses (Wang *et al*. 2003). This degree of loss does not include the impact of future climate change, which will shift suitable areas for some of the World's most important crops, with notable reductions in areas for major cereal crops (Lane and Jarvis 2007). Primary abiotic stresses include: drought, salinity, cold, heat, and chemical pollution. Gene expression profiles show similarities between these osmotic stresses, supporting the interconnectivity on the cellular level as each of these stresses has the potential to interfere with the water potential within a plant cell (Adie *et al*. 2007; Nakashima *et al.* 2005; Wang *et al*. 2003; Zhu 2001; Munns 2002). As such, emphasis placed on improving response mechanisms for any of these

stresses has the potential to diversely affect overall production yield.

As relatively stationary organisms, plants have evolved adaptations to tolerate many environmental stresses. A striking example of such adaptations is seen in *Mesembryanthemum crystallinum* as it changes carbon fixation pathways from C3 photosynthesis to Crassulacean acid metabolism (CAM) following salt stress (Cushman and Bohnert 1997), and even the use of specialized cells to serve as salinity storage organs (Agarie *et al.* 2007).

Although all cells are sensitive to stresses, actively dividing cells and photosynthesizing cells are more sensitive to ion toxicity produced by the increase in Cl^-, Na^+ and even K^+ in water stress related conditions. Even under what is considered non-stress conditions, salt accumulation is an ongoing problem for plants, as some plants can transpire more than 70 times the amount of water used for cell expansion and photosynthesis (Munns 2002). For tobacco, cytoplasmic ion concentrations above 100 mM, has been shown to inhibit essential enzymes (Munns 2002). This brings up the subtle, but essential, adaptations on the cellular level, with the transport and sequestration of ions to maintain homeostasis, preventing ion toxicity and reducing osmotic stress (Nakashima *et al.* 2005; Haseqawa *et al.* 2000; Morillon *et al.* 2001). To prevent toxic ion levels several options are available. One is the selective uptake by root cells and retention of ions to upper root systems and the vascular system (Gorham *et al.*1990). For most plants, intracellular ion compartmentalization is common. Cultured tobacco cells, grown in 430 mM NaCl, were measured to have a vacuole concentration of 780 mM Na+ and 625 mM Cl- while maintaining below 100mM cytoplasmic concentrations (Binzel *et al.* 1988).

Many of these examples of stress-tolerance responses result from developmental, and physiological mechanisms that involve many genes, but there are also more subtle, but still essential, biochemical adaptations on the cellular level. Some enzymes have been found to have reduced salt sensitivity (Ghosh et al, 2006). Characterization of the wild rice, *Porteresia coarctata*, gene PcINO1 product revealed that it is capable of retaining its enzymatic function even in presence of 500 mM

NaCl, as opposed to its salt-sensitive homolog (Ghosh et al, 2006).

These, and other tolerances, are recognized by the genetic diversity among species, as well as within species. Through plant breeding, this genetic diversity has been utilized to select desired traits, creating numerous cultivars adapted to a variety of climates leading to detectable genetic differences when comparing cultivars to each other, or to their crop wild relatives (Mardi *et al*. 2011; Hajjar and Hodgkin 2007).

In addition to the direct genetic makeup, acclimation through prior environmental conditions also affect stress tolerances. Many plants can be hardened by growth in non-freezing low temperatures, which markedly increases freezing stress tolerance (Tyler *et al.* 1980). Hardened plants physically respond quickly at the cellular level with increased stomatal closure and inhibition of cell growth and photoshynthesis (Shinozaki and Yamaguchi-Shinozaki 2006).

The genus *Triticum,* which includes cultivated wheats, has in excess of 20 species, each with numerous cultivars. *T. aestivum* (bread wheat) itself, has a diverse genetic background, with a large genome. After cold acclimation, cultivars of winter wheat can survive temperatures as low as -22ºC, where as spring cultivars, of the same species, can achieve freezing tolerance to only -8ºC. Spring and winter wheat cultivars differ by alleles of a single major gene, the vernalization locus Vrn1 (Fu *et al*. 2005), though genetic variation within the spring and winter types indicates that there are additional genes that contribute to freezing tolerance.

2. Gene Candidate Identification

The manifestation of genetic differences in tolerances to environmental stresses is seen clearly on the molecular level through comparative studies of differential gene expression between tolerant and sensitive genotypes (Galvez *et al*. 1993). This has been a major avenue to identify candidate genes that may contribute to the growth and survival of plants under extreme environmental conditions. The

increased production of the phytohormone abscisic acid (ABA) is noticeably increased under drought and salt stress conditions, and has been shown to cause molecular and cellular responses, such as stomatal closure, and the increased expression of stress-related genes (Aguirrezabal *et al.* (2006). However, there is evidence that shows both ABA dependent and ABA-independent regulatory systems for the expression of drought and salt-stress inducible genes (Yamaguchi-Shinozaki and Shinozaki 2005). This emphasizes the complexity of the stress response interaction network and the need for further investigation of the function of stress induced genes.

2.1. Microarray Data

With the large production of genomic, cDNA and EST sequences provided by sequencing projects, microarray analysis of genome-scale gene expression became a standard to sift through all potential genes involved in plant stress-response (Schena *et al.* 1995; Eisen and Brown 1999). A multitude of microarray expression comparisons have since been completed for a number of stresses, and plants such as Arabidopsis, (Seki *et al.*, 2001; Seki *et al.,* 2002; Krebs *et al.,* 2002), rice (Rabbani *et al.,* 2003), and wheat (Gulick *et al.,* 2005; Monroy *et al.,* 2007). Cold stress response is measured after growth at 4°C for one, and 24 hours. Drought is measured by the removal form the medium and desiccation for a similar amount of time. High salinity stress is provided by hydroponically grown plants in a solution of 100 mM NaCl for Arabidopsis plants in the array done by Kreps *et al* (2002), and 250mM NaCl in Arabidopsis arrays run by Seki *et al* (2002), as well as those done for rice. The cold-stress microarray data for wheat, observed time intervals over a course of 36 days, with temperatures ranging from 6ºC to 2ºC for day and night respectively. These arrays do narrow down the genes of interest that could be related to pertinent stress responses, however, a large number of candidate genes remain. With many candidate genes, group organization is almost mandatory. Shinozaki's work on drought stress response and tolerance classifies the drought regulated proteins in two groups. The first

as 'Functional Proteins', such as chaperones, late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases. The second major group is that of regulatory proteins, which include genes encoding transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism and calmodulin-binding proteins (Shinozaki and Yamaguchi-Shinozaki 2006).

2.2. Protein-Protein Interaction and Hub-Proteins

Large scale yeast two hybrid (Y2H) screens have been carried out in order to develop insight into regulatory networks for stress response. Proteins with numerous interaction partners appear as hub-proteins in protein-protein interaction networks and are thought to play a role as central regulators. Hub-proteins can differ from non hub-proteins in their primary protein structure, often containing multiple and repeated domains, (Ekman *et al*. 2006), but are only readily found through protein-protein interaction screens. In wheat, genes that were initially identified as being differentially expressed during cold acclimation in spring and winter cultivars (Gulick *et al.* 2005), were used in a yeast two hybrid screening for protein-protein interaction partners (Tardiff *et al*. 2007). A low temperature induced G-RAN-like protein used as a bait was found to interact with a protein encoded by the previously identified Early Salt Stress Induced *Esi2* (Tardiff *et al*. 2007, Galvez *et al.* 1993). *Esi2* was also found to interact with a cold-induced pathogen-related protein (PR-1), and an inorganic pyrophosphatase.

3. The ESI2 Protein

ESI2 is a novel protein characterized by a repeated amino acid motif, rich in methionine, proline and serine that, so far, appears to have no highly similar gene outside of the Triticeae tribe, and is

unlike any previously reported stress-regulated protein. In response to cold treatment, the levels of gene induction increased respectively to the tolerance of freezing stresses, with the highest expression in the tolerant winter wheat cultivars of Norstar, LT_{50} -19ºC, and Musketeer, LT_{50} -21ºC, and reduced levels in the more cold sensitive, spring wheat cultivars of Glenlea LT_{50} -8ºC, and Concorde LT_{50} -8ºC (Unpublished, Gulick**)**. ESI2 is somewhat reminiscent of Late Embryogenesis Abundant (LEA) genes in that it is strongly up regulated by salt stress, is hydrophilic in nature, apparently non enzymatic, and has repeated amino acid motifs. However, LEA proteins have been categorized into seven distinct categories (Battaglia *et. al*. 2008), none of which resembles the *Esi*2 sequence.

4. Lea Proteins

Genes encoding LEA, RAB and dehydrin-like hydrophilic proteins were among the first genes shown to be strongly up-regulated by drought, low temperature and salt-stress, as well as by treatment with ABA (Hong, *et al*. 1988; Curry, *et al.* 1991). The proteins encoded by these genes are characterized by repeated amino acid sequence motifs with no apparent domain for catalytic enzymatic activity and are archetypal examples of stress regulated genes, affected by several environmental stresses. Although first discovered in cotton and wheat twenty years ago (Dure 1993), and have since been well categorized, the underlying function of both LEA and other dehydrin proteins has still yet to be fully elucidated as they do not possess strict enzymatic properties (Battaglia *et. al*. 2008). Even so, LEA proteins have been shown to confer tolerance to water-deficit conditions, (Xu *et al.* 1996; Zhang *et al*. 2000). Reyes *et al*. (2005) used *in vitro* assays of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) to show the presence of LEA proteins could protect these enzymes against the reduction of their activity induced by dehydration conditions over a wide range of water potentials.

Unpublished results from Dr. Gulick's lab indicate that *Esi*2 is up regulated during cold acclimation and that the level of induction parallels the degree of low temperature tolerance found

among different wheat genotypes and related species. This evidence and the fact that ESI2 appears to interact with a number of proteins, suggests *Esi*2 to be integrated, and perhaps integral in some of the extreme stress tolerance of wheat. In order to study this further, three genes that are possible interactors with the protein coded by *Esi*2 were selected for localization to confirm interactions. These genes include the Ran-related GTP-binding protein, an inorganic pyrophosphatase like protein, and a pathogen-related protein, first reported in Tardiff *et al.* (2007).

5. *Arabidopsis thaliana* and *Triticum aestivum*

Arabidopsis thaliana is an important model organism for plant biology and genetics. It has a colourful research history, first being discounted for potential research due to its small size and the difficulty in distinguishing different chromosome pairs (Meyerowitz 2001). Even so, *A. thaliana*'s relatively small genome of 157 Mbp, 28-day life cycle, seed production numbering in the thousands per plant and self-fertilizing reproduction habit have lead it to be the most popular plant model organism (Muller 2010). The genome sequence was completed in 2000, (Theologis *et al.* 2000), and a gene knockout library has been compiled based on random T-DNA insertional mutants, made in a collection of over 300,000 independent transgenic lines. Genetic transformations are also very simple with the *Agrobacterium*-mediated transformation method (Clough *et al.* 1998). In spite of the advantages of the *A. thaliana* experimental system, there is no clear ESI2 homolog in Arabidopsis, however, the interacting Ran-related GTP-binding protein found in wheat is nearly identical (94% identity) to the *A. thaliana* Ran3 (AT5G55190) protein, and therefore it should be possible to study the interaction of ESI2 with the RAN in Arabidopsis.

As an allohexaploid, direct genetic studies on wheat using transformation and mutagenesis continue to remain a difficult experimental task. Most genes identified in wheat are expected to be present in three homeologous copies located on three homeologuous chromosome pairs belonging to

the A, B and D genomes (Ridha Farajalla and Gulick 2007).

6. The Ran Protein

6.1 The Wheat Ran

The GTP binding protein, Ran, a candidate interactor with ESI2, is a highly conserved member of the Ras superfamily. A precise understanding to the functions of Ran in plant systems has yet to be fully elucidated; however, an overexpression of the tobacco Ran protein suppresses the phenotype of the temperature dependent cell regulatory mutant *pim1-46* in fission yeast (Merkle *et al.* 1994). Ran genes, in yeast and humans, have been extensively studied, and play an essential role in a number of cell functions (Belhumeur *et al.* 1993).

The wheat Ran protein shares 76% identity and 85% similarity across 98% coverage to that of the *Saccharomyces cerevisiae* GTP binding proteins GSP1p and GSP2p. Although GSP1p and GSP2p are nearly identical to each other, only GSP1p is an essential gene (Belhumeur *et al.* 1993). This is most likely due to the expression of GSP2p being ten fold lower and supported by the recovery of the GSP1p mutant through over-expression of GSP2p (Belhumeur *et al.* 1993). The RAN ortholog in humans shares 77% identity, 84% similarity with a 93% coverage to that of the wheat RAN, with the *Arabidopsis* RAN ortholog sharing 94% identity, 95% similarity over 100% of the gene (Benson *et al.* 2005).

6.2 Ran in Nucleocytoplasmic Transport

Like other GTPases, Ran exists in two forms, GTP or GDP bound states, RanGTP and RanGDP, respectively. This GTPase switch changes the interaction of Ran with effectors (Vetter *et al.* 1999). RanGTP is formed in the nucleus through the interaction with a guanine nucleotide exchange factor, GEF, known as RCC1, Regulator of Chromosome Condensation 1 (Bischoff *et al.* 1991). There is a

putative chromosome condensation factor in *T. aestivum*, with 43% sequence similarity that also shares the same cl08266 conserved domains for the RCC1 repeat (Renault *et al.* 1998: Marchler-Bauer *et al.* 2011). The GTPase activity of Ran is greatly enhanced by the cytoplasmic GTPase activating protein, GAP, stimulating the hydrolysis of GTP to GDP (Bischoff *et al.* 1994). Since the GEF is localized to the nucleus, and the GAP is localized to the cytoplasm, an asymmetric distribution of RanGTP/GDP occurs, with RanGTP found in high concentrations in the nucleoplasm, and RanGDP found in high concentrations in the cytoplasm (Izaurralde *et al.* 1997; Gorlich and Kutay 1999). Although Ran is mainly soluble and mobile, it does not diffuse through the nuclear pore complexes (NPC).

In the cytoplasm, an import complex is formed between a karyopherin and a nuclear localized protein allowing transport through the NPC. Once in the nucleus, the complex is uncoupled through the hydrolysis of RanGTP to RanGDP (Ribbeck *et al.* 1998; Vetter *et al.* 1999). Exporting cargo from the nucleus, both mRNA, and tRNA, involves a karyopherin with cargo bound to RanGTP, which can then diffuse through the NPC, releasing the cargo upon the hydrolysis and disassociation of RanGDP in the cytoplasm. RanGDP, in the cytoplasm, is then actively imported back to the nucleus, by means of the nuclear transport factor-2, NTF2 (Ribbeck *et al.* 1998).

6.3 Ran in Spindle Assembly

The generation of RanGTP by RCC1 is required for the activation of the major mitosis-promoting factor, cyclin-dependent kinase-1-cyclin B protein kinase (Clark *et al.* 1995). To separate the role of Ran during the cell cycle, from Ran's nucleocytoplasmic transport, a cell-free model system, using *Xenopus laevis* egg extracts, was used. Although the full mechanism is yet to be elucidated, this model system also showed a disruption in the balance of RanGTP/GDP disrupted the formation of microtubule asters and spindles (Zhang *et al.* 1999). In somatic cells, RAN localizes to the centrosomes, however, when the levels of RanGTP are reduced, by increasing the quantity of the

binding protein RanBP1, the centrosomal cohesion is lost (Zhang *et al.* 1999). RanGTP has been shown to regulate the microtubule-crosslinking motor protein Eg5, required for the formation of a bipolar spindle (Wilde *et al.* 2001). Also, the addition of excess RanGTP to the *Xenopus* system, in the absence of centrioles or chromatin, still induces small, spindle-like structures (Halpin *et al.* 2011).

6.4 Ran in Mitotic Progression

More evidence using the *Xenopus* egg extracts reveals RAN to be crucial to the spindle assembly checkpoint. When RanGTP levels are increased, the spindle checkpoint is inactivated, by removal of the regulators from the kinetochores allowing attachment to the spindle microtubules (Musacchio and Hardwick 2002). Addition of RanGDP restores the spindle checkpoint, supporting the hypothesis that it is not only the presence of RanGTP, but also the ratio, and distribution of RanGTP/GDP that regulates the spindle assembly check point.

6.5 Ran in Post-Mitotic Nuclear Envelope Assembly

When beads coated with Ran were added to the *Xenopus* system, there is a rapid accumulation of membrane vesicles that fuse to form a continuous lipid layer. The membrane also incorporates nucleoporins and the formation of NPCs, all in the absence of chromatin (Zhang, *et al.* 2000). Ran has also been shown to, not only, accelerate the nuclear envelope assembly, but is also essential to it's proper formation (Hughes *et al.* 1998). RanGTP hydrolysis is also necessary for this function, as when RanGAP is inhibited, therefore, reducing the RanGTP to RanGDP transformation, the vesicle binding for the nuclear envelope is reduced.

7. Fluorescent proteins

7.1 GFP and Derivatives

The notoriety and usefulness of the green fluorescent protein (GFP), first isolated from *Victoria aquaporins*, has continued to increase in recent years. Its significance as an important research tool was acknowledged with the Nobel prize in Chemistry in 2008, awarded jointly to Samu Shimomura, Martin Chalfie and Roger Y. Tsien, for its discovery and development. GFP is widely used as a fusion protein and expressed transgenically to determine the localization of a protein of interest. The wild type GFP, wtGFP, is composed of 238 amino acid residues and forms a typical beta barrel structure. This form of GFP is not commonly used for molecular localization studies as it is sensitive to both chloride and pH, and does not fold well at 37ºC (Tsien 1998). The enhanced GFP, EGFP (F64L/S65T), however, is not as sensitive to these conditions, and has a single excitation peak at 488 nm, as opposed to the double excitation peak of 488 nm and 395 nm for that of the wtGFP (Tsien 1998). Most current experiments, and those described in this thesis utilizing GFP, employ the EGFP derivative. Like the EGFP protein, the enhanced yellow fluorescent protein, previously known as YFP 10C, EYFP, is also a derivative of the wtGFP with the substitutions S65G/V68L/S72A/T203Y (Shi *et al.* 2007).

Although wtGFP has many derivatives, not all fluorescent proteins used are a derivative of wtGFP. The fluorescent red protein, DsRed, originally isolated from the genus *Discosoma*, has provided many powerful red derivatives. DsRed, however, forms a dimer in its natural state, therefore, among the more used proteins is the monomeric mCherry, which has five amino acid substitutions relative to DsRed (Q66M, T147S, M163Q, M182K, T195Q) (Shaner *et al*. 2004).

Multiple fluorescent molecules can be used simultaneously; however, the excitation and emission spectras for each must be distinguishable. In conjunction with either the EGFP or EYFP, mCherry has a distinct excitation maximum of 587 nm, and emission maximum of 610 nm, allowing different expressions to be identifiable within the same field of view.

7.2 Bimolecular Fluorescent Complementation

Bimolecular Fluorescence Complementation, BiFC, takes advantage of the stability of the fluorescent proteins to validate other protein-protein interactions within living cells. The beta barrel shape found in wtGFP and its derivatives enable the protein to fold into its native conformation from a single polypeptide chain, or from two fragments of the chain. The fluorescent EYFP can be successfully formed from two fragments of the full length EYFP, one truncated at residue 154, YN154, (the N-terminal fragment contains amino acid residues 1-154) and the second fragment extending from residue 155, YC155, to the C terminus (the C-terminal fragment contains residues 155-238) (Hu *et al*. 2002). Two proteins are separately fused to complementary fragments of the EYFP and co-expressed in the same cell. If the two proteins interact, they bring the two fragments of YFP into proximity and the native beta barrel forms. This allows for fluorescence to occur, demonstrating the protein-protein interaction within the cell and providing sub-cellular localization of the protein-protein interaction using fluorescent microscopy. The BiFC method is very sensitive, especially with rapid or transient interactions, as the formation of the beta barrel is very stable, even if the fused protein interaction is not. The BiFC method has been developed to test protein-protein interaction in plant cells using a system of *Agrobacterium tumafaciens* to mediate transient transformation of the epidermal cells of leaves of the wild tobacco species *Nicotiana benthamiana* (Sparkes *et al.* 2006).

8. Summary

In this thesis, the role of the stress inducible *Esi*2 gene of *T. aestivum* is investigated. Homeologous copies of the gene family for *Esi*2 and its candidate interaction proteins were identified in the GenBank EST databases. *Agrobacterium* mediated transformation of *N. benthamiana* epidermal cells was used to express the full length fluorescent fusion proteins of ESI2 and its interaction candidates, RAN1, PR-1, IPP, to determine cellular localization. The BiFC assay for protein-protein interaction was used to characterize the interaction between ESI2 and the GTPase, RAN1.

In addition, *Arabidopsis* mutant lines, with T-DNA insertions in the promoter region for the *RAN*1 gene were identified, and a comparison in root length was compared with wild type plants, one and two days after germination.

PART II. MATERIALS AND METHODS

1. Full Length Sequence for *ESI2*, *Ran1 IPP* and *PR-1* in *Triticum aestivum*

The *Esi*2-2 cDNA clone LMB04-6N02 was sequenced in its entirety and submitted to GenBank (GI:62861390). In order to identify other members of the gene family, this sequence was used to search GenBank for corresponding EST sequences with at least 90% sequence identity. The EST sequences were compiled using CAP3 to determine the relative orientation of the sequences and Clustal X was used to align and identify the SNPs in the sequences in order to separate sequences derived from different homeologs. Three distinct contigs were then created with CAP3 using sequences sharing at least 100bp of consecutive sequence identity.

Genbank's EST sequence database was searched for sequences with at least 95% sequence-identity to the coding regions for the Ran-related GTP binding protein *Ran*1-1 from wheat (GI:19919693). The EST sequences were compiled using CAP3 to determine directionality, and Clustal X to separate the sequences derived from different homeologous copies. Overlapping sequences that shared at least 100bp of 100% consecutive sequence identity were grouped and assembled into contigs with CAP3. Two contigs were not previously reported in Genbank and were assembled and submitted as *Ran*1-2 and *Ran*1-3 (JQ673329, JQ673330). The sequence for *Ran*1-2 had the greatest number of EST for the Ran gene family from among the FGAS EST sequences. The full length FGAS clone L6B027-N_11 (GI:39624144) was sequenced and use for subsequent experiments.

The cDNA clone J882 which encodes an inorganic pyrophosphatase-like gene was sequenced and submitted to GenBank (JQ673331). The two corresponding homeologs were identified using the methods described above for *Ran1*. The two additional homeologous sequences were submitted to Genbank as, *Ipp*1-2 JQ673332, and *Ipp*1-3 JQ673333.

The Pathogen-related protein clone PR-1 was sequenced in its entirety and found to match a

gene previously submitted to GenBank (GI:334903111). The two corresponding homeologs were identified using the methodology described above for *Ran1*, and have also been submitted to GenBank, as PR-1-2 (GI:334903113) and PR-1-3 (GI:334903115).

2. Vector Constructs

2.1 Clone Selection:

Plasmid constructs for expression of fluorescent fusion protein in plant cells were created using the two step Gateway cloning technology (Hartley et al. 2000). Each gene was PCR amplified from a full length cDNA clone with the stop codons omitted. The following clone IDs were used: *ESI2*: GI:323903580, *Ran1:* GI:39624144, *IPP:* GI:39571794, and *PR-1:* GI:26355792. See appendix for primers.

The Gateway entry clone *ESI2-ent*, for creating the plant fluorescent constructs was made with *ESI2-attB* PCR product gateway cloned into pDonR207. The entry clones *Ran1-ent, PR-1-ent*, and *IPP1-ent* were made with *Ran1-attB*, *IPP1-attB-* and *PR-1-attB* PCR products, gateway cloned into pDonR201.

2.2 Plant Expression Clones:

The green fluorescent protein fusions for each of the genes *ESI2::eGFP*, *IPP1::eGFP*, and *PR-1::eGFP* were made using the vector pK7WG2.1. The *Ran::D2Red* red fluorescent fusion was made using the vector pH7RWG2.

The *ESI2:C-YFP* BIFC construct was formed using the pBatL-L-CYFP destination vector. The *Ran1:N-YFP* BIFC construct was formed using the pBatL-B-SYFP-N vector.

The inducible ESI2:Glox over-expression clone was created by cloning *ESI2-attB-s* product (with the stop codon included) into pDonR201. The Entry clone was then gateway cloned into the

destination vector pJCGLOX.

3. Agrobacterium Mediated Plant Transformation

Each clone was separately transformed into the *Agrobacterium tumerfaciens* strain AGL1 using electoporation. The strains were then separately grown as a liquid culture to log phase, and collected by centrifugation at 3000g for 10 min at 21ºC. The cells were then re-suspended in an activating solution, containing 1.5 mM acetosyringone and 0.1 M $MgCl_2$ to an OD_{600} of 0.4 (English et al., 1996). The solutions of *Agrobacterium* were then incubated at 25ºC for six hours. For each transformation, 800µl of each of the desired constructs was mixed with an additional 800µl of the gene-silencing inhibitor, P19. The suspensions were taken in syringe, without a needle, and injected directly into the underside of a tobacco leaf.

The wild tobacco (*Nicotiana benthamiana*) plants used were four to five weeks old, and only mature leaves were selected for transformation. The plants were grown in a soil mixture of 4:3:2:1 of Black earth, vermiculite, perlite and peat moss respectively. The plants were grown at 25ºC, under 16 h day conditions and covered to increase humidity and ease of transformation. Transformation was done in the evening to allow time for the plants to recover without the need to shelter them from direct light.

4. *In vivo* Protein Expression using Confocal Microscopy

Glass microscope slides were prepared with a single layer of parafilm, heated until it adheres to the surface of the slide.. A ~0.8 cm square segment was removed from the centre of the parafilm after attaching it to the glass slide. Four days after transformation, 0.5 cm square segments were cut from the *Agrobacterim* infiltrated *N. benthamiana* leaves. The segments were wet mounted with the bottom side of the leaf facing up, on the prepared microscope slides with a drop of 0.1M $MgCl_2$ solution and covered with a No.1 cover slip.

Immediately after preparation, the samples were observed using the Leica TCS SP2 microscope system from the Center for Structural and Functional Genomics. A 488 nm and 543 nm laser scanner was used to excite the eGFP/eYFP and mCherry/DsRed proteins respectively. Acquisition of the emission spectra used Leica's spectrophotometric system.

5. Arabidopsis thaliana Ran1 mutant plants

Arabidopsis thaliana T-DNA insertion mutant lines SALK 066148, SALK 066157 and SALK 067649 which had insertions in, or upstream, of the *RAN1* gene, AT5G20010 were obtained from the Salk Institute Genomic Analysis Laboratory. Seeds were germinated on a soil mixture of 4:3:2:1 of Black earth, vermiculite, perlite and peat moss respectively which was previously oven baked at 150ºC for 4 hours. The seeds were cold treated at 4ºC for 24 hours prior to sowing, and were grown under 16 hour days at 22ºC. Identification of lines with T-DNA insertions was done using PCR with gene specific primers LP and RP, and the T-DNA specific primer, LBb1.3 (see appendix for the sequences). The primers were designed with the aid of the Salk institutes primer design tool, SiGnAL.

Identified mutants were grown on MS media, ½ salt MS media, and ½ salt MS media with 1% added D-Glucose plates, mounted at a 75º angle. Each plate was observed for germination. One, and two, days after germination of the seed. Root measurements were taken from the base of the seed to the root tip. For each collection of measurements the average, standard deviation, and a Student *t*-Test was performed using Excel.

PART III. RESULTS

1. Full Length Sequences for *Esi*2-2 in *Triticum aestivum*

A cDNA clone for *Esi*2-2 from *T. aestivum* corresponding to the EST sequence from the Functional Genomics of Abiotic Stress (FGAS) database LMB04-6 N02, was sequenced in its entirety and the sequence was submitted to Genbank (GI:323903580). The cDNA sequence is 1226 nt with a coding region of 966 nt, which encodes a protein of 321 amino acids. This clone shares 95% nucleotide identity and 95% and 97% amino acid identity and similarity respectively, with the *T. aestivum* cDNA clones *Esi2-1* (GI:62861390) *Esi2-3* (GI:241990857). The ESI2-2 protein contains a sequence, between the 33rd and 118th amino acids, with high similarity to the conserved plastocyanin-like domain pfam02298. It also has a repeating amino acid sequence motif, 17 amino acids long, repeated 6 times starting at the 141st amino acid. *Esi*2-2 also shares high sequence similarity with *Esi*2 genes in *Lophopyrum elongatum* (tall wheat grass) (*Esi*2-1 GI:62861392, *Esi*2-3 GI:62861388) and with a *Hordeum vulgare* (barley) cDNA. The overall nucleotide sequence identity is 87% and 93% respectively in matching regions but the proteins differ by the number of repeated motifs, with one fewer in *L. elongatum* (5), and one more in *H. vulgare* (7). Although repeated amino acid sequence motifs are found in other proteins whose genes are highly induced by stress and dehydration, such as Late Embryogenesis Abundant (LEA) proteins (Battaglia *et al,* 2008) and dehydrins (Garay-Arroyo, 2000), ESI2 like proteins share no sequence similarities with LEA and dehydrin proteins.

2. Identification of Potential Homeologs of *Triticum aestivum G*enes *ESI*2, *Ran1*, *Pr-1*, and *Ipp*.

Three *Esi*2 contigs were formed using GenBank EST sequences from *T. aestivum*. One contig is identical to *Esi*2-1 (GI:62861390). The second matches *Esi*2-3 (GI:241990857), while the third contig was novel, and the sequence was deposited at as *Esi*2-2 (GI:323903580). *Esi*2-1 and *Esi*2-3

both share 97% sequence identity in a pattern consistent to that of homeologous copies of genes in hexaploid *T. aestivum*, i.e. orthologous genes derived from the three ancestral A, B and D genomes. The third sequence, *Esi*2-2 shares a 95% sequence identity to both *Esi*2-1 and *Esi*2-3 a degree of similarity consistent with another homeologous gene copy.

The *T. aestivum, Ran*1-1 gene, encoding a small Ran-related GTP-binding protein (GI:19919693) has been previously reported, (Wang *et al,* 2006). Potential protein-protein interactions were observed using the yeast two hybrid system, with a homeologous gene, *Ran*1 clone J925 (GI:16903081). To identify a possible third homeolouge for Ran1, all ESTs with high sequence similarity to *Ran*1-1 in the Genbank database were compiled and compared to each other. The EST set was assembled into three homeologous gene sequences. The one contig was identical to *Ran*1-1 (GI:19919693). Two additional homeologous genes, named *Ran*1-2 and *Ran*1-3 were assembled and the sequences were submitted to GenBank (JQ673329, JQ673330). The GenBank accession that was previously deposited in GenBank by Gao *et al*. in 2001 (GI:16903081) was not identified among the EST sequences.

An inorganic pyrophosphatase-like gene was also identified. The assembly of all similar EST sequences from GenBank, comprised three contigs with sequence similarity consistent with homeolougus sequences. The sequences were submitted to Genbank as *Ipp*1-1, *Ipp*1-2, *Ipp*1-3 (JQ673331, JQ673332, JQ673333, respectively).

The sequences encoded proteins with high similarity to inorganic phosphatases and have an Inorganic pyrophosphatase domain, cd00412, between the 49th and 202 amino acid.

The pathogen-related protein *Pr*-1, contains the SCP_PR-1_like domain cd05381, between the 28th and the 164th amio acid (HQ541963.1). All similar EST sequenced were obtained from GenBank. A single contig was formed, which matched *Pr*-1 (GI:334903111). Two other full length gene sequences are present in GenBank, , *Pr-1-2* (GI:334903113) and *Pr-1-3* (GI:334903115).

3. Sub-Cellular Protein Localization for ESI2-2, RAN1-2, IPP1 and PR-1

ESI2-2, was expressed as a green fluorescent fusion protein, ESI2::eGFP, in *Nicotiana benthamiana* leaf epidermal cells, and viewed using a confocal microscope. The fusion protein was localized to the tonoplast (i.e. vacuolar membrane) while none was localized to the nucleus or other organelles (Fig. 1a). Tonoplast localization is seen as fluorescence localized along the complete outline of the cell with varying degrees of intensity. When viewed at the right plain of focus a fluorescence outline around the nucleus is also observed, and strands of fluorescence seen crossing the interior of the cell in places where adjacent vacuoles press against each other (Fig. 1a). Proteins localized to the plasma membrane (PM), also appear as an outline of the cell, however, the expression is more uniform and only localized to the periphery of the cell (Fig. 3c). As conformation, the tonoplast marker, gammaTIP::mCherry (Nelson and Cox 2007), was expressed (Fig. 1b, and Fig. 2b) simultaneously with the ESI2::eGFP (Fig. 1c and Fig 2c); it a nearly complete overlap between the two.

RAN1-2, expressed as a red fluorescent fusion protein, RAN1::DsRed, was localized to the nucleus in *N. benthamiana* epidermal cells (Fig. 3a). The nucleus is easily distinguishable in these cells as a large, mostly circular, organelle pressed between the tonoplast and the PM. Images were also taken using the colour 5 m look up table provided by Leica`s microscope software, which facilitates viewing extreme difference in expression levels by using multiple colours. The lowest measured intensity is set to red, greater levels of expression are shown in green, and then the highest levels are shown in blue. Using this setting, fluorescence outlining the periphery of the entire cell was visible, however, in much lower concentrations than that seen within the nucleus. To confirm nuclear expression, the RAN1-2:DsRed fusion protein was colocalized with the nuclear expression of the B-Zip transcription factor expressed as a Bi-Molecular Fluorescent Complementation (BiFC) pair, since the protein forms a dimer in the nucleus. The expression of binary B-Zip::eYFP-C and B-Zip::eYFP-N proteins localized with Ran1-2::DsRed in the nucleus (Fig. 4d).

IPP1, expressed as an eGFP fusion protein, IPP::eGFP, was localized to the ER network in leaf epidermal cells in the wild tobacco, *N. benthamiana,* (Fig. 5a). The ER localization is seen in the upper focal planes of the cell as a network and in the central focal plane of the cell it is seen as punctate localizations on the periphery of the cell, typical of a cross section of a network (Fig. 5b). When viewed as a three dimensional construct, the ER is seen as a webbed network between the tonoplast and the PM. IPP::eGFP was colocalized with the ER network marker AtWAK2::mCherry (Nelson 2007) (Figure 5c).

PR-1, expressed as an eGFP fusion protein, PR-1::eGFP, was localized to the plasma membrane in leaf epidermal cells in *N. benthamiana* (Fig. 5a). PM localization is seen as a clear outline of the periphery of the entire cell. It differs from tonoplast localization in the clarity and evenness of expression, however, more importantly, there is no localization across the interior of the cell as is seen with tonoplast localized proteins (Fig. 5b). PR-1::eGFP was colocalized with the plasma membrane marker AtPIP2A::mCherry (Nelsom 2007). (Fig. 5c).

Figures 1a-1c

Figures 2a-2c

Landscape pages

Figures 3a-3c

Figures 4a-4d

Landscape pages

Figures 5a-5c

Figures 6a-6c

Landscape pages

Figures 7a-7c

Landscape pages

4. ESI2 and RAN1 *in vivo* protein-protein interaction and sub-cellular localization.

Using Bimolecular Fluorescent Complementation, BiFC, ESI2-2 and RAN1-2 were seen to interact along the tonoplast, in *N. benthamiana* epidermal cells. ESI2-2 was fused with the carboxy portion of the eYFP while RAN1-2 was fused with the amino portion of the eYFP. When expressed together in the same cell, ESI2-1 was seen to interact with RAN1-2 by yellow fluorescence localized to the tonoplast. The signal was seen outlining the periphery of the cell, with strands of fluorescence crossing through the interior of the cell, marking the borders between adjacent vacuoles (Fig. 7a). The ESI2::eYFP-C and RAN1::eYFP-N were simultaneously expressed with the gammaTIP::mCherry tonoplast marker (Nelson 2007), which showed complete overlap with the ESI2-RAN1 interaction with the tonoplast marker. There was no observable localization of the interaction in nucleus (Fig. 6b and c).

***5. Arabidopsis thaliana* RAN1 mutant plants**

Three seed lines with potential T-DNA insertional mutations of the *Arabidopsis thaliana RAN*1 gene, AT5G20010 were selected and screened. Line SALK 066148 and SALK 066157 have been identified at the SIGNaL T-DNA Express public data base (http://signal.salk.edu/cgi-bin/tdnaexpress) as having T-DNA insertion within the coding portion of the *RAN*1 gene, however, over 50 plants from these lines were PCR screened and no individuals with insertions could be identified. Line SALK 067649 has been identified in the T-DNA Express database as a line with a T-DNA insertion within the promoter of the *RAN*1 gene. Two mutant plants, one homozygous for the T-DNA insertion event, and one heterozygous for the T-DNA insertion event, were identified. PCR amplification using LBb1.3 and RB primers yielded a 700bp product in plants carrying the T-DNA insertion and amplification using gene specific LB-RB primers yielded a 1,000bp product in heterozygous plants and plants lacking the insertion.

Root length was measured for wild type Col-0 and homozygous SALK067649 seeds, germinated on MS media plates, half-salt MS media plates, and half-salt MS media plates with 1% D-Glucose grown at a 75º angle. The root length was measured one and two days after the first signs of germination.

Root growth for *Ran*1 mutant plants SALK067649 root growth grown agar based media. Eleven wild type and 99 homozygous plants germinated on MS media plates. Average root length two days after germination was 6.8 mm and 8.2 mm for wild-type and mutant plants respectively, A Student *t*-Test indicated that the differences were significant with a P value of 0.02 (Table 1).

Table 1								
	Day one				Day two			
	ms no D-Glucose				ms no D-Glucose			
	Wild type	Homozygous mutant			Wild type	Homozygous mutant		
	1.0	NA	0.6	2.4	6.4	NA	9.6	6.4
	1.8	2.0	0.2	0.6	6.4	6.0	8.0	7.0
	NA	2.8	1.0	1.2	NA	11.2	3.2	10.2
	1.2	4.0	1.8	1.8	6.0	13.2	4.8	7.2
	2.0	3.8	1.8	2.2	9.0	10.4	10.0	7.2
	2.0	1.8	1.8	1.8	7.0	8.4	8.0	8.0
	1.6	1.4	1.8	0.8	4.0	9.2	6.4	5.0
	2.4	2.2	1.6	0.8	8.8	6.8	9.4	9.2
	0.2	1.8	2.0	2.4	5.0	7.0	4.8	8.0
	3.0	1.8	1.6	1.0	7.4	8.4	8.4	8.2
	1.2	1.8	3.0	2.8	5.0	8.4	8.0	5.0
	3.4	4.6	1.8	1.2	9.4	13.0	11.2	7.4
		2.4	2.2	3.0		9.2	8.2	5.4
		1.8	2.2	2.0		7.6	9.8	9.0
		1.0	2.4	2.0		8.4	11.0	9.2
		2.8	3.0	1.8		9.2	9.0	9.6
		2.2	2.2	3.0		9.8	10.4	7.4
		2.4	NA	2.8		9.4	9.0	10.4
		2.2	2.4	1.8		8.0	8.4	7.6
		2.8	3.0	1.2		7.4	8.2	11.6
		1.0	2.8	4.0		9.6	5.0	9.6
		3.0	3.6	3.8		10.2	7.6	8.0
		NA	2.4	2.0		NA	8.2	6.0
		2.2	1.8	0.4		9.6	8.6	10.2
		1.4	3.2	1.4		8.8	7.2	9.2
		1.0	3.2	1.4		5.6	12.2	9.6
		1.8	1.4	2.0		9.6	10.2	8.6
		2.4	0.2	0.6		9.4	5.8	7.8
		2.2	0.8	1.4		10.6	4.0	9.0
		2.0	2.4	1.2		6.0	6.0	5.8
		2.2	0.2	1.4		8.2	8.0	4.6
		2.0	2.0	2.0		5.0	7.6	6.6
		2.0	2.2	2.4		9.6	8.4	7.0
		2.0	1.8	1.8		9.0	8.4	7.4
N=	11			99	11			100
AVERAGE=	1.8			2.0	6.8			8.2
STDEV=	0.9			0.9	1.8			1.9
TTEST=				0.511				0.022

Table 1: Root growth comparison for *Ran1* mutant plants, one and two days after germination on MS media plates showing a statistically significant difference in growth, P=0.02.

When plants were grown on half strength MS media, the average root length two days after germination was 2.9 mm and 3.8 mm for wild-type and mutant plants respectively, and the A Student *t*-Test gave a value of 0.145, indicated that the differences were not significant. (Table 2)

Table 2				
	Day one		Day two	
	½ ms no D-Glucose		½ ms no D-Glucose	
	Wild type	Homozygous mutant	Wild type	Homozygous mutant
	1.4	2.4	2.4	5.0
	1.4	NA	4.0	NA
	1.2	2.4	2.4	5.6
	2.0	0.2	5.6	1.0
	1.8	1.8	4.4	4.8
	2.0	NA	3.0	NA
	1.8	0.8	2.0	2.6
	0.8	0.8	1.8	2.0
	NA	0.8	NA	2.8
	1.4	0.2	1.8	0.4
	1.4	1.8	2.8	4.6
	0.6	0.2	1.4	0.8
		2.2		5.8
		3.0		4.0
		2.2		5.0
		2.4		5.6
		3.0		5.4
		2.4		4.0
		2.2		4.4
N=	11	17	11	17
AVERAGE=	1.4	1.7	2.9	3.8
STDEV=	0.5	1.0	1.3	1.8
TTEST=		0.356		0.145

*T*able 2: Root growth comparison for *Ran1* mutant plants, one and two days after germination on 1/2 MS media plates.

In a third experiment, plants were grown on half strength MS media with 1% glucose and the average root length two days after was 8.2 mm and 10.1 mm for wild-type and mutant plants respectively, A Student *t*-Test gave a P value of 0.08, indicating that the differences were significant.

Table 3				
	Day one		Day two	
	½ ms 1% D-Glucose		½ ms 1% D-Glucose	
	Wild type	Homozygous mutant	Wild type	Homozygous mutant
	3.8	1.8	6.2	9.0
	2.2	2.8	6.0	9.2
	3.2	2.2	9.4	9.6
	3.4	3.4	12.2	12.8
	3.2	3.4	9.6	12.2
	2.2	3.2	11.0	10.4
	0.2	3.2	5.2	11.0
	2.2	3.0	4.2	13.0
	2.4	2.2	9.8	9.2
		3.4		9.6
		1.4		4.8
		2.2		9.6
		2.6		10.0
		2.6		10.2
		2.2		9.2
		2.8		12.2
		2.0		9.4
		4.2		11.4
		2.4		8.0
		2.8		8.6
		2.0		10.8
		3.8		10.4
		3.6		12.8
N=	9	23	9	23
AVERAGE=	2.5	2.7	8.2	10.1
STDEV=	1.1	0.7	2.8	1.8
TTEST=		0.587		0.079

Table 3: Root growth comparison for *Ran1* mutant plants, one and two days after germination on 1/2 MS media plates with 1% added D-Glucose showing a statistically significant difference in growth, P=0.08

PART IV. DISCUSSION

This study reports on the sub cellular localization for the interaction between the wheat proteins ESI2 and RAN1. Also, I characterized a change in root growth shortly after germination for homozygous mutants of the *Arabidopsis* RAN homolouge. In addition two proteins, previously shown by Y2H assays to bind with ESI2, were localized by transient expression in plant cells.

1. ESI2 and Ran interaction along the Tonoplast

The overexpression of RAN in both rice and *Arabidopsis* causes a reduced tolerance to salinity and osmotic stress (Zang *et al.*2010). Since the molecular responses to salt-stress and drought overlap with acclimation to low temperatures, it is not surprising that exposure to low temperature causes a reduction in Ran expression in wheat (Gulick *et al.* 2005). The discovery that ESI2 and RAN1 can interact directly suggested that ESI2 may be acting in the signaling pathways stimulated by RAN (Tardiff *et al.* 2007). Whereas *Ran1* showed a reduction in expression in response to stress, *Esi2* showed a rapid increase in expression in winter wheat, in response to both cold (Gulick *et al.* 2005), and salt-stress (Gulick and Dvorak 1990; Galvez *et al*.1993), suggesting ESI2 may be a negative regulator of RAN1. The results reported here show the interaction between ESI2 and RAN1 to be along the tonoplast, where in the absence of ESI2, RAN1 is localized predominantly to the nucleus, suggests ESI2 may sequester RAN1 to the cytoplasm. The binding of ESI2 to RAN1 may interfere with essential mechanisms in which RAN1 has been demonstrated to play an important role, namely cell division and nuclear transport. For RanGDP to be activated, it must enter the nucleus. The RAN1 guanine nucleotide exchange factor, RanGEF, is required for activation (Kleb *et al.* 1995), and is localized solely to the nucleus (Cushman *et al.* 2004). The replacement of the GTP bound form with GDP form of RAN directly blocks DNA replication, and prevents the cell from entering into mitosis

(Kornbluth *et al.* 1994). Wang *et al.* (2006) postulates that a change in *Ran1* expression disrupts cell division indirectly by interfering with the movement of proteins imperative for the G2 to mitosis cell cycle transition, such as the cytosolic B1-type cyclin, which requires interaction with RanGTP to enter the nucleus and bind to chromosomes for the cell to enter mitosis (Inze, 2005). Additionally, an increase of RanGDP causes activation of the spindle checkpoint (Musacchio and Hardwick 2002). The disruption of these processes are related directly to cellular growth.

A reduction in plant growth occurs rapidly after the onset of stress (Fricke *et al.* 2006; Aguirrezabal *et al.* 2006; Bartels and Sunkar 2007), and is independent of both photosynthetic rates, and plant carbon status (Skiycz *et al.* 2010). Fricke *et al.* reported that the addition of NaCl, to a final concentration of 100 mM to the root medium of barley plants, (also in the Triticeae tribe) caused the rapid reduction in the elongation of leaves. This rapid arrest in shoot and leaf growth is a common response to the tolerance of abiotic stresses. However, root growth and structure is different, and roots will continue to elongate under mild stress conditions (Pardo 2010), suggesting a multifarious aspect to stress-response mediated cellular growth arrest. Investigation of the mechanisms that regulate cell division and growth in plants in response to stress is a field of growing interest, and the role of ESI2 as a potential regulator of cell division is a significant advancement in our understanding of this phenomenon.

2. Arabidopsis thalianta Ran1 Mutant Plants

Homozygous mutant plants with a T-DNA insertion in the promoter region of *RAN1*, when grown on MS media plates were found to have a larger increase in root length two days after germination compared to the wild relative. This implicates the involvement of RAN in the architecture of root formation. However these results are preliminary and a fuller understanding of the role of RAN and its interaction with ESI2 in root development in response to stress require further investigation.

This mutation provides a promising avenue to characterize the role of the ESI2-RAN interaction and further study the effect of this interaction on root growth and structure. Though there is no clear homologue of ESI2 in the genome of *Arabidopsis*, the high conservation of RAN between wheat and *Arabidopsis* does provide a method to study the regulation of the *Arabidopsis* RAN by interaction with ESI2. In addition there are *Arabidopsis* genes with very weak sequence similarity to ESI2 that may play a similar role in Arabidopsis to that of ESI2 in wheat .

3. Inorganic Pyrophosphatase

Inorganic Pyrophosphatase, Ipp, was localized to the ER network. The closest *Arabidopsis* homologe of Ipp1, PPa1 (GI:15223288), shares 88% sequence similarity and has been localized in the cytoplasm in clusters along the ER network (Bruneel *et al.* 2005; Lodish *et al.* 2008). In this experiment, the upper and lower planes of view, common for clear ER network expression, was poor. However, in the central planes, where ER localization fluorescence appear punctate, there was a clear overlap between Ipp-GFP and the ER marker, suggesting Ipp is also clustered around the ER network.

Like the Ran protein, Ipp has been shown to be essential for yeast viability (Lahti 1990), also, like Ran, the expression of Ipp homologs are increased in several forms of cancer cells (Chen *et al.* 2002). The enzymatic properties of Ipp, the cleavage of pyrophosphates (PP_i) provides a thermodynamic driving force for a range of anabolic processes (de Jardin *et al.* 1995), notably amino acid activation, the elongation of DNA and RNA, and nucleotide synthesis. Both the *de novo* and salvage pathways for nucleotide synthesis are reversible reactions in the absence of Ipp and the removal of PPi during these reactions drives irreversibility (Bruice 1998). The interaction of ESI2 with Ipp has the potential to interfere with the driving force of these reactions, and even causing them to be reversible. Removing the driving force for nucleotide synthesis would accompany a decrease in cell division, which we hypothesized to be a role for the ESI2-RAN interaction. This effect may also cause

the accumulation of precursors of nucleotide synthesis that would be available for resumption of cell division once environmental stress conditions end.

4. Pathogenesis-Related Protein 1

The Pathogenesis-Related Protein 1, Pr-1-1 is the first in group one of the pathogen-related proteins found in wheat (Lu *et al.* 2011). As well as abiotic stress conditions, Pr-1 gene expression is induced by a pathogen challenge. This expression was seen to be triggered in response to *Xanthomonas campestris*, (Buell and Somerville 1997) the cause of black chaff in wheat. The closest *Arabidopsis* homologe (GI:1228950), with 68% similarity has been localized to the cell wall and the extracellular region, (Boudart *et al.* 2005). Results reported here indicate the localization for the wheat Pr-1 protein is along the plasma membrane, indicating the protein might function differently than its closest homologe in *Arabidopsis*. As the molecular mechanisms of Pr-1 have yet to be determined, it is too soon to suggest possible effects of its interaction with ESI2.

5. Future Work

ESI2 appears to be limited to the Triticeae tribe and is a member of a small gene family, and is present in a number of closely related variety of species (Gulick and Dvorak 1992). The striking difference between the number of repeats of the motif present in ESI2 may have a functional role. It is important for future work to determine which of the domains of ESI2 interact with Ran and if the same domain is involved in the interaction with other proteins. Exogenous expression of wheat *Ran1* has already shown to affect cell division in yeast (Wang *et al*. 2006), and provides a platform for directly examining the effects of ESI2-RAN binding on cell division, cycle phases, and tolerance to stresses in an inducible manner.

Arguably, the foundation of healthy plants is in the architecture of the roots. The influence of

RAN1 on root growth and structure and the affect on adaptation to stress conditions warrants further analysis, especially through the investigation of the ESI2-RAN interaction. Root growth, is heavily reliant on auxins, a phytohormone sensitive to stress response. Auxin has been shown to cause a rapid increase in *Ran1* transcription (Wang *et al.* 2006), and an increase of auxin drastically changes root structure, inhibiting elongation and steering growth towards the formation of adventitious roots (Kim *et al.* 2001; Wang *et al.* 2006). The effect of ESI2-RAN binding on the sensitivity to auxin would be an interesting avenue of research.

The use of *Arabidopsis PPa1* mutant lines (*Arabidopsis Ipp* homolog) and overexpression of the Wheat *Ipp* in *Arabidopsis* could also revival more information on any effects of Ipp on the cell cycle, or stress survivability. If the interaction between ESI2 and Ipp can be confirmed using BiFC or other reliable method, it would be fruitful to investigate the parallel pathways that ESI2 may be acting in and to investigate its role as an instrumental hub protein in the stress response system for Wheat.

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