FUNCTIONAL AND GENETIC ANALYSIS OF PLANT TRANSCRIPTION FACTORS INVOLVED IN THE PLANT GROWTH UNDER VARIOUS ENVIRONMENTAL CONDITIONS

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FUNCTIONAL AND GENETIC ANALYSIS OF PLANT TRANSCRIPTION FACTORS INVOLVED IN THE PLANT GROWTH UNDER VARIOUS ENVIRONMENTAL CONDITIONS

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Kun Yuan, daughter of Shiquan Yuan and Huafang Guo, was born on July 10, 1975, in Changsha, China. She attended Hunan Medical School, Changsha, China, for seven years, and graduated with a Medical Doctor Degree in July 2000. After graduation, she worked in People's Hospital of Guangdong Province, Guangzhou, China, from July, 2000 to July, 2001. She began graduate studies at Auburn University, Alabama, USA in Aug 2001, where she worked as a graduate research and teaching assistant. She married Guangbing Wu, son of Zhenxi Wu and Honglian Xue, at Auburn, Alabama, USA in 2003. She has two daughters, Linda Wu, two years old and Karissa Wu, one year old.

DISSERTATION ABSTRACT

FUNCTIONAL AND GENETIC ANALYSIS OF PLANT TRANSCRIPTION FACTORS INVOLVED IN THE PLANT GROWTH UNDER VARIOUS ENVIRONMENTAL CONDITIONS

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Arabidopsis serves as a model system for the plant molecular biology. Many biotic and abiotic factors affect plant growth from seed germination to mature plant growth. External factors studied in our research include nutrients and salinity. Phytohormone pathways interact with glucose and salt stress signaling pathways to affect plant growth. Abscisic acid (ABA) and gibberellic acid (GA) are two important phytohormones that impact the plant development. Exogenous glucose and salt stress delay seed germination in *Arabidopsis thaliana* not only in wild type (WT) but also in a number of mutants in hormone signaling pathways. Our study demonstrates that the *ABA*

Insensitive 3 (*ABI3*) gene in the ABA signaling pathway and the *RGA-like 2* (*RGL2*) genes in the GA signaling pathways playing important roles in the glucose-induced delay and salt inhibition of seed germination. Transcription of the *ABI3* and *RGL2* genes is up-regulated by glucose and salt. The study also demonstrates that several genes in ABA and GA signaling pathways have essential functions during early and later seedling development. This study suggests that different genes in ABA and GA signaling pathways are involved at different developmental stages under stress condition and glucose treatment. The possible crosstalk between different hormone signaling pathways exists under salt and osmotic stress conditions. Three genes in the GRAS family, *SCARECROW* (*SCR*), *SHORT ROOT* (*SHR*) and *SCARECROW-LIKE 3 (SCL3)* are involved in the salt-induced inhibition of seedling development. Transcription of the *SCR* and *SHR* genes is up-regulated by salt. There are differences in stress tolerance between genotypes or differences in stress tolerance at different developmental stages within a single genotype. This study also demonstrated the interaction among GRAS family genes (*SCR*, *SHR* and *SCL3*) and components (*SOS1*, *SOS2* and *SOS3*) in Salt overly sensitive (SOS) signaling pathway. The result indicated that *SCR* and *SHR* may play important roles in the salt-induced inhibition of seedling development via regulating transcription levels of *SOS1*, *2* and *3* in SOS signaling pathway.

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I. INTRODUCTION

Biotechnical significance of *Arabidopsis*

In recent years much progress has been made in the field of plant biology by using *Arabidopsis thaliana* as a model system. *Arabidopsis* has one of the smallest plant genomes, 120 Mb, that has been sequenced in its entirety. Its genome contains about 26,000 predicted genes, most of their functions are unknown. Conservation of basic mechanisms within the plant kingdom has been well documented (Peng *et al*., 1999). Isolation of mutants defective in various aspects of embryonic/postembryonic development, and various growth responses to plant hormones or to environmental abiotic "factors" has led to the identification of some key genes involved in these processes. Identification and understanding of the key pathways and interactions of all the molecular components involved will allow for generation and testing of crops to solve the world hunger problem.

The effect of sugars on plants

Complex regulatory networks have been used by different organisms to sense nutrient signals and regulate gene expression in order to adapt to environmental and metabolic cues (Wang *et al*., 2003). Plants are extremely sensitive and responsive to their surroundings because they are immobile and have few options for survival. Plant and microbial genes have similar responses to carbohydrate depletion but differ as well.

Sugar concentrations vary over a wide range in plant tissues. This range typically exceeds that found in more homeostatic systems (such as the mammalian blood stream) and provides plants a greater chance to adjust according to the environmental changes for survival. Microarray analysis suggested that expression levels of a very large number of genes respond to changes in glucose concentration (Price *et al*., 2004). Sugar regulates a broad range of gene types, ranging from stress responses and cellular metabolism to those involved in signaling/gene regulation. Sugar-regulated genes are important for plants to adjust to environmental changes. Genes for sucrose metabolism can be up-regulated in photosynthetic tissues following manipulations that cause sugars to accumulate. High sugar levels can strongly affect genes for sucrose metabolism. For example, those encoding ADPG-pyrophosphorylase (AGPase), a key step in starch biosynthesis, are markedly responsive to sugar in potato (Krapp *et al*., 1993). Not only genes encoding enzymes but also a large number of transcription factors (TFs) were found to be glucose-regulated. Many of them are up-regulated and others are down-regulated (Price *et al*., 2004).

The levels of glucose and other sugars have effect on seed germination and early seedling development. Different concentrations of sugars may exert varying effects. Both low and high concentrations of glucose delay seed germination but even 0.33M glucose cannot totally inhibit seed germination (Ullah *et al*., 2002; Price *et al*., 2003; Dekkers *et al*., 2004). A glucose analog, 3-O-methylglucose, which is a very poor substrate for hexokinase was also shown to delay seed germination at similar concentrations as glucose (Dekkers *et al*., 2004). It indicated that the delay of seed germination by

relatively low concentrations of exogenous glucose and 3-O-methylglucose may occur via a hexokinase-independent mechanism. In contrast, a better substrate for hexokinase, mannose, was found to completely inhibit seed germination at concentrations as low as 5–10 mM. Neither glucose nor 3-O-methylglucose has a significant effect on seed germination at such low concentrations (Cortes *et al*., 2003). Mannose inhibition of seed germination may be via a hexokinase-dependent pathway whereas glucose induced germination delay appears to be independent of hexokinase (Pego *et al*., 1999).

Besides mediating early developmental events, soluble sugars also affect the formation of more adult structures, such as leaves, nodules, pollen, tubers and roots. The formation of extra organs, such as extra tubers and adventitious roots can be induced by sugar application (Xu *et al*., 1998). Under- and over expression of hexokinases 1 and 2 fail to alter tuber yield, suggesting that hexokinases do not play a major role in the regulation of tuber formation (Veramendi *et al*., 1999). Glucose and fructose increase the formation of adventitious roots but mannose and sorbitol do not. It indicates that the effect is not due to alterations in the osmotic potential of the media and that only metabolizable sugars are effective in indication of adventitious root formation. The effects of sugars on the formation of adventitious roots are dependent on sugar concentrations (Druege *et al*., 2004).

The effect of salt stress on plants

Soil salinity is one of the most significant abiotic stresses limiting plant growth. High salinity causes both hyperionic and hyper osmotic stress and can lead to plant death. Salt stress essentially results in a water deficit condition in the plant and takes the form of

a physiological drought. Excess salts adversely affect all major metabolic activities in plants including cell wall damage, accumulation of electron-dense proteinaceous particles, plasmolysis, cytoplasmic lysis and damage to ER, and overall decline in germination and seedling growth (Garcia *et al*., 1997; Khan *et al*., 1997; Sivakumar *et al*., 1998). The major ions involved in salt stress signaling, include Na^+, K^+, H^+ and Ca^{2+}. It is the interplay of these ions, which brings homeostasis in the cell. Although Na^+ is a plant micronutrient, excess Na+ levels are toxic for plant growth. Influx of Na^+ dissipates the membrane potential and facilitates the uptake of Cl^- down the chemical gradient. Na^+ is toxic to cell metabolism and has deleterious effect on the functioning of some of the enzymes (Niu *et al*., 1995). High concentrations of Na^+ causes osmotic imbalance, membrane disorganization, reduction in growth, inhibition of cell division and expansion. High Na^+ levels also lead to reduction in photosynthesis and production of reactive oxygen species (Yeo, 1998). Ca^{2+} has role in providing salt tolerance to plant. Externally supplied Ca^{2+} reduces the toxic effects of NaCl, presumably by facilitating higher K^+/Na^+ selectivity (Liu and Zhu, 1998). High salinity results in increased cytosolic Ca^{2+} that is transported from the apoplast as well as the intracellular compartments. This transient increase in cytosolic Ca^{2+} initiates the stress signal transduction leading to salt adaptation.

The adaptation of plant cells to stress conditions involves triggering a network of signaling events. Little was known about the salt stress signaling pathways until the SOS (salt overly sensitive) genes in SOS pathway in *Arabidopsis* were identified. SOS genes were identified through positional cloning. *Sos1* (Salt Overly Sensitive 1), 2 and 3 mutants were identified by using a root-bending assay on NaCl containing agar plates

(Wu *et al*., 1996; Liu *et al*., 1997; Zhu *et al*., 1998). *SOS1* gene encodes the plasma membrane Na^+/H^+ exchanger (antiporter) (Shi *et al*., 2000). *SOS2* gene encodes a serine/threonine type protein kinase which activity is required for salt tolerance (Liu *et al*., 2000). The N-terminal kinase catalytic domain of *SOS2* is similar to that of the yeast Suc nonfermenting1 (SNF1) and AMP-activated (AMPK) kinases (Hardie, 1999). The N-terminal catalytic domain and the C-terminal regulatory domain of *SOS2* are essential for *SOS2* normal function. *SOS3* encodes a protein that acts as a Ca^{2+} sensor in the plant. The sequence of *SOS3* has a significant similarity with that of the calcineurin B subunit from yeast and neuronal calcium sensors from animals (Liu and Zhu, 1998). SOS3 and SOS2 interaction has been shown in the yeast two-hybrid assay and the analysis of double mutant *sos3/sos2* suggested that *SOS2* and *SOS3* work in the same pathway (Halfter *et al*., 2000). *SOS2* and *SOS3* genes not only interact together but also regulate *SOS1* gene expression. *SOS1* gene expression level is low in wild-type (WT) in the condition without salt but is up regulated by salt stress. *SOS2* and *SOS3* partially control *SOS1* gene expression level during salt stress (Shi *et al*., 2000). SOS2 and SOS3 are also required for the activation of SOS1 Na^+/H^+ exchanger (Shi *et al*., 2000). Quintero et al (2002) reconstituted the SOS system in yeast cells and found that the SOS2/SOS3 kinase complex promoted the phosphorylation of SOS1 and activated the expression of SOS1 in yeast. The coexpression of SOS1, 2 and 3 enhanced the salt tolerance of a yeast mutant without Na^+ transporters. SOS2 protein activity partially phosphorylated SOS1 independently of SOS3, but single SOS2 protein was not as efficient as the SOS2/SOS3 kinase complex for activation of SOS1 *in vivo* (Quintero, 2002). In addition, the

increased salt tolerance in plants may be induced by the co-over expression of *SOS1, 2* and *3* (Guo et al., 2004). These results suggested that SOSs function in the same signaling pathway. *SOS1* and SOS2 expression can be detected under the normal conditions in the root. SOS1 and SOS2 expression in the root is up-regulated by salt stress (Liu *et al*., 2000; Shi *et al*., 2000).

Phytohormones and external factors, sugars and salt stress, effects on seed germination

The effects of sugars on seed germination and early seedling development are complicated and may involve several phytohormone-response pathways. ABA has a crucial role in seed dormancy and GA is required for seed germination (Debeaujon I *et al*., 2000). ABA plays essential roles in many physiological processes, such as embryogenesis, seed dormancy, leaf transpiration, and stress tolerance (Koornneef *et al*., 1998; Leung and Giraudat 1998). Many lines of evidence indicate that there are multiple ABA perception and signaling mechanisms. Seed germination-based genetic screens have identified mutants affected in ABA biosynthesis or sensitivity. The latter include ABA-insensitive mutants (*abi*) and ABA-hypersensitive mutants. ABI1 is a negative regulator of ABA signaling, as indicated by the enhanced sensitivity of the recessive mutants to ABA in seeds and in vegetative tissues (Gosti *et al*., 1999). *ABI1* and *ABI2* genes have overlapping functions during seed development, seed dormancy, and leaf transpiration (Leung and Giraudat 1998). *ABI3*, *ABI4*, and *ABI5*, all encode transcription factors (Giraudat *et al*., 1992; Finkelstein and Lynch 2000). *ABI3*, *ABI4*, and *ABI5* act in a combinatorial network, rather than a regulatory hierarchy to control seed development

and ABA response (Soderman *et al*., 2000**)**. *ABI4* may down-regulate *ABI3* gene or act in a parallel pathway at later stage (Soderman *et al*., 2000). Mutations in *ABI3, ABI4*, and *ABI5* have their greatest impact on gene expression during seed maturation because they are only expressed to a limited degree in vegetative tissues (Finkelstein *et al*., 1998; Finkelstein and Lynch, 2000). In vegetative tissues, ABA and various abiotic stresses activate the expression of a large number of genes, which may play important roles in stress adaptation (Zhu *et al*., 1998). During germination, ABI5 is a sensor for osmotic status and a part of a growth repression mechanism during germination when the seeds imbibe under unfavorable conditions (Carles *et al*., 2002). *ABI5* gene expression increases during germination in the presence of ABA and/or salt (Lopez-Molina *et al*., 2001).

Plant hormone, ABA, seems to play a role in sugar signal transduction pathways that affect seed germination and other aspects of plant growth processes (Finkelstein and Lynch, 2000; Gazzarrini and McCourt, 2001; Gibson *et al*., 2001; Pourtau *et al*., 2004). The possible mechanism of glucose-induced delay of seed germination may be related to the slower decline of endogenous ABA in the presence of exogenous sugar (Price *et al*., 2003). Many of the Glc-insensitive and Suc-insensitive mutants turned out to involve genes related to ABA biosynthesis or ABA signaling pathways (Arenas-Huertero *et al*., 2000). However, the tests of exogenous glucose supply effect on the seed germination rates of different ABA-metabolic and ABA-response mutants have suggested that glucose delay of seed germination does not involve any of the expected ABA-response pathway components such as ABI1, ABI2, ABI4 or ABI5 (Brocard-Gifford *et al*., 2003; Dekkers

et al., 2004). At least, *abi8* and *abi4* are resistant to both ABA inhibition of seed germination and glucose inhibition of early seedling development (Brocard-Gifford *et al*., 2004). This indicates that some of the genes in ABA signaling pathway are involved in the glucose effect on plant growth/development but it appears that different ABA signaling pathways may interact with sugar signaling in a stage and tissue dependent manner.

ABA plays a crucial role in the response of plants to abiotic and biotic stresses. Stress-responsive genes are regulated by both ABA-dependent and ABA-independent signaling pathways (Zhu, 2002). ABA biosynthesis is up-regulated by the osmotic stress. The genetic studies on the ABA-deficient mutants *los5/aba3* and *los6/aba1* of *Arabidopsis* indicated that ABA plays a crucial role in osmotic stress-regulated gene expression (Xiong *et al*., 2001; Xiong *et al*., 2002).

GA is another hormone that plays essential role in plant growth and seed germination. Most of the genes encoding enzymes for GA biosynthesis and catabolism have been identified (Olszewski *et al*., 2002). Genes encoding several GA-signaling components have also been identified (Yang *et al*., 2004). Recent work in *Arabidopsis* has identified several putative transcription factors belonging to GRAS family to be involved in the GA signal transduction pathway. GRAS (GAI/RGA/SCR) family of plant specific putative transcription factors, which includes 33 *Arabidopsis* sequences with some of them corresponding to genes with known functions (Pysh *et al*., 1999; Bolle, 2004). These include five closely related sequences containing DELLA motif, *GAI* (GA insensitive), *RGA* (repressor of *ga1-3*) and *RGL1-3* (RGA-like) (Bolle, 2004). Four of

these genes encode negative regulators of GA response, the function of the fifth one, *RGL3*, is unknown (Wen and Chang 2002; Tyler *et al*., 2004). Other GRAS proteins regulate several different aspects of plant growth and development including root development, lateral branch development, and phytochrome A signal transduction (Bolle C, 2004). The GRAS-like sequences of unknown function have been referred to as *SCLs* (*SCR-LIKE* sequences; Pysh *et al*., 1999). The predicted protein sequences of all the family members share well-conserved C-termini (Pysh *et al*., 1999). Based on the homology over the entire protein, including quite variable N-termini, the family can be further subdivided into several subfamilies each consisting of two to five closely related members (Bolle, 2004).

Unlike other members of the GRAS family, the five proteins in the RGA subfamily have a well-conserved N-terminal domain called DELLA after an amino acid motif contained therein (Peng *et al*., 1997; Silverstone. *et al*., 1998). This DELLA domain has been implicated in GA signaling (Wen and Chang, 2002). In *Arabidopsis*, there are five DELLA proteins, namely GAI, RGA and RGL1, RGL2 and RGL3.Two members, RGA and GAI interact synergistically to repress a set of GA-induced growth processes. Phenotypes affected by *rga* and *gai* null mutations include leaf expansion, stem elongation, juvenile-to-adult phase change in leaf development, vegetative-to-reproductive transition, and apical dominance. GAI and RGA, are partially redundant negative regulators in GA signaling pathway (King *et al.*, 2001)**.** The presence of the amino acid sequence in the DELLA motif has an essential role for GA inducible RGA

protein disappearance (Dill *et al*., 2001). GA can induce the degradation of four DELLA proteins in *Arabidopsis,* namely GAI, RGL1, RGL2 and RGL3 (Hussain *et al*., 2007).

The DELLA subfamily of GRAS family not only contains RGA and GAI but also RGL1, RGL2 and RGL3 (Dill and Sun 2001). In addition to overall homology between DELLA proteins they all contain two highly conserved N-terminal regions, I and II, which are critical for GA signaling (Peng *et al*., 1999; Richards *et al*., 201; Dill and Sun 2001). The high similarity of N-termina regions (I and II) among RGA, GAI, RGL1, RGL2 and RGL3 suggest that RGLs (RGL1, RGL2 and RGL3) may also act as negative regulators of GA signaling pathways. Based on the analysis of RGL1 overexpressors it was proposed that RGL1 acts as a negative regulator of GA signaling for seed germination (Wen and Chang, 2002). Other findings based on the *rgl1* mutant analysis, however, do not support this conclusion (Lee *et al*., 2002).

Analysis of loss-of-function mutations in *GAI*, *RGA*, *RGL2* and *RGL1* revealed that *rgl2* alleles conferred strong resistance to PAC inhibitory effect on germination, whereas *rgl1-1*, *gai-t6* and *rga-t2* did not (Lee *et al*., 2002). Lee *et al.* also showed that *RGL2* transcription levels raise rapidly following seed imbibitions and then decline rapidly as germination proceeds, implying that the expression of wild type *RGL2* is induced by imbibitions and it is dynamic. *RGL2* loss-of-function mutations did not suppress the dwarf phenotype of *ga1-3* (GA-deficient mutant). These observations indicate that RGL2 is a negative regulator of GA-responses that control seed germination specifically rather than stem elongation. The transition of a seed from dormancy to germination is controlled by both external environmental cues (including light quality,

moisture, and transient exposure to cold) and by the internal growth hormone regulators positively by GA and negatively by ABA.

The *Arabidopsis* gene *SPY* (SPINDLY) encodes an *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase. SPY is a repressor of GA responses that regulates various developmental processes, including seed germination, shoot elongation, flower initiation and flower development (Izhaki *et al*., 2001). Previous study showed that SPY, which does not belong to GRAS family, plays an important role in regulating GA signaling pathway (Jacobsen *et al*., 1996). SPY gene product affects both GA and cytokinin responses indicating that SPY is important for crosstalk between phytohormone pathways (Greenboim-Wainberg *et al*., 2005). *SPY* gene is expressed throughout the plant organs (Swain *et al*., 2002). Mutations at the *SPY* locus partially restore the phenotypes of mutant *ga1*, including failure to germinate, short stem growth, delayed flowering and male sterility (Jacobsen *et al*., 1996). All the *spy* alleles tested partially suppress the phenotypes of *ga1-1*. In addition, *spy* alleles suppress the nongerminating phenotype conferred by *ga1-2* (an allele of *ga1*) as tested by that *spy/ga1-2* double-mutant seeds that germinate in the absence of exogenous GA (Jacobsen and Olszewski, 1993). It indicates that SPY is an important regulator of the GA-mediated control of both stem elongation and seed germination. The function of SPY as a negative regulator has been suggested to be related to activation or stabilization of DELLA genes, or by leading them to localize to the site where they exert their regulatory action (Shimada *et al*., 2006).

The SPY gene has been cloned from *Arabidopsis* (Jacobsen *et al*., 1996) and barley (*HvSPY*, Robertson *et al*., 1998) and found to have significant similarity to animal

tetratricopeptide repeat (TPR)-containing serine and threonine-*O*-linked *N*-acetylglucoseamine (*O*-GlcNAc) transferase (OGT). An actively repressive complex was created by the interaction between TPRs of SPY and other proteins (Tseng *et al*., 2001). The hypothesis was set up that SPY plays a role in plant development not only through its role in GA signaling but also through other ways. Except its role in GA response,

Alteration of the cellular distribution of bioactive GAs caused by various environmental factors such as light and temperature control seed germination (Yamauchi *et al*., 2004). Both hormone and sugar levels modulate α-amylase production in barley embryos (Perata *et al*., 1997), suggesting potential interaction between sugar and GA on plant developmental processes. Whether GA biosynthesis or signaling pathway plays a role in sugar induced signal transduction is unknown. Further study is needed to clarify the relationship between GA and sugar signaling pathways since both of them play pivotal roles in plant growth and seed germination. Also, there is not much known about potential relationship between GA and salt signaling pathways.

SCR and SHR in *Arabidopsis*

Several key components essential for hormone regulated growth processes (Richards *et al*., 2001) and for radial patterning of plant organs (Helariutta *et al*., 2000; Wysocka-Diller *et al*., 2000) have been identified through mutational analysis. Some of these key developmental genes have been shown to function in the development of different organs throughout both embryonic and postembryonic development. Two such genes, *SCARECROW* (*SCR*) and *SHORTROOT* (*SHR*), are involved in both the root and shoot development patterning by controlling some key cell divisions (Fukaki *et al*., 1998;

Wysocka-Diller *et al.*, 2000). In the stems they are also essential for correct specification of endodermis, the site of gravity perception in *Arabidopsis* shoots (Fukaki *et al*., 1998).

SCR has been cloned and sequence suggested a role as a transcriptional regulator (Di Laurenzio *et al*., 1996; Pysh *et al*., 1999). The SCR gene product is one of the founding members of the GRAS (GAI/RGA/SCR) family of plant specific putative transcription factors (Pysh *et al*., 1999; Bolle, 2004). *SCR* was the first GRAS gene (functionally) identified and used as the defining sequence for the family (Di Laurenzio *et al*., 1996; Pysh *et al*., 1999). Mutations in either SCR or SHR (alsp a member of GRAS family) lead to cell layer deletions resulting from an absence of some key cell divisions. The *scr* roots have only a single cell layer in place of the two present in the WT (Scheres *et al*., 1995; Di Laurenzio *et al*., 1996). This single remaining cell layer in *scr* mutant roots expresses differentiated characteristics of both the cortex and the endodermis (Di Laurenzio *et al*., 1996, Wysocka-Diller *et al*., 2000). The endodermis may not function normally in *scr* mutant. In *shr* roots the single ground tissue layer differentiates into cortex resulting in the complete deletion of root endodermis (Benfey *et al*., 1993; Helariutta *et al*., 2000). One of *SCARECROW-LIKE (SCL)* genes in GRAS family, *SCL3*, showed a tissue-specific expression pattern in the root similar to *SCR* (Pysh *et al*., 1999). It suggested that SCL3 may play a role in endodermal specification by regulating *SCR* expression or by being regulated by *SCR*. In addition to the morphological phenotypes both *scr* and *shr* are shoot agravitropic (Fukaki *et al*., 1998) and have reduced salt tolerance (J. W-D unpublished data). SHR and SCR play similar essential roles in a number of developmental processes. However, their functions, unlike the GAI/RGA

situation, don't seem to be redundant. The cloning of SHR revealed that these two genes, SCR and SHR, not only function in the same processes but also are molecularly related. Thus, both SCR and SHR are the members of the GRAS family. However, neither of these two genes falls into any of the subfamilies. Nor are they more closely related to each other than to any other sequence in the family. This may partially explain the lack of functional redundancy.

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II. RESEARCH OBJECTIVES

My dissertation was focused on the following objectives:

1. Sugar effect on seed germination and early seedling development
    a. Identify phytohormone signaling pathway components involved in sugar-mediated delay of germination.
    b. Whether any of these genes acting in the same pathway or not.
    c. Sugar effect on the early seedling development and phytohormone signaling pathway components involved in this process.
2. Salt and osmotic stress effect on seed germination and early seedling development
    a. Identify phytohormone signaling pathway components involved in salt and osmotic stress effect on seed germination and early seedling development.
    b. The interaction and crosstalk among these genes and pathway involved in this process.
3. Identify the function of GRAS family genes under the stress condition
    a. Investigate the phenotypes of several gene mutants in GRAS family, *scl3*, *scr* and *shr* under salt stress.
    b. The interaction and crosstalk between several signaling pathways involved in plant response to salt stress.
    c. Identify the genes in phytohormone pathways to response to salt stress during the later seedling development.

III. PHYTOHORMONE SIGNALING PATHWAYS INTERACT WITH SUGARS DURING SEED GERMINATION AND SEEDLING DEVELOPMENT

Abstract

Exogenous glucose delays seed germination in *Arabidopsis thaliana* not only in wild type (WT) but also in a number of mutants in hormone signaling pathways. Our study demonstrates that the *ABA Insensitive 3* (*ABI3*) gene in the ABA signaling pathway and the *RGA-like 2* (*RGL2*) and *SPINDLY* (*SPY*) genes in the GA signaling pathways all play important roles in the glucose-induced delay of seed germination. Transcription of the *ABI3* and *RGL2* genes is up regulated by glucose. This study also supports the idea that different sugars such as the hexose stereoisomers, glucose and mannose, delay or inhibit seed germination via different branches of hormone signaling pathways. Analysis of post-germination seedling development of wild type plants indicates that exogenous glucose supplied after germination may have a concentration dependent stimulatory effect on root and shoot growth. Comparison of WT and *spy* seedling growth on different glucose concentrations suggests that the stimulatory effect of glucose is partially exerted via the GA or cytokinin signaling pathways. The effects of glucose on plant growth and development may be stimulatory or inhibitory depending on the developmental stage. The inhibitory effect on seed germination seems to be accomplished via activation of ABA signaling pathway, through ABI3, and inactivation of the GA signaling pathway

through RGL2 and SPY. On the other hand, the stimulatory effect of glucose on seedling growth may involve GA and/or cytokinin signaling pathways.

Key words: Abscisic acid, *Arabidopsis*, Germination, Gibberellic acid, Glucose signaling

Introduction

Seed germination is a critical step in the plant life cycle, and is regulated by many biotic and abiotic factors. In *Arabidopsis thaliana* seeds, the transition from dormancy to germination is controlled by external environmental factors such as light quality, moisture, and transient exposure to cold as well as several internal growth regulators (reviewed by Koornneef et al., 2002). Among the photohormones that play a role in *Arabidopsis* seed germination, gibberellin (GA) and abscisic acid (ABA) have the most pronounced effects (Koornneef et al., 2002). ABA establishes and maintains dormancy of seeds, whereas GA has the opposite effect, breaking dormancy and inducing seed germination (Steber et al., 1998). It appears that there are multiple ABA detection and signaling mechanisms (Himmelbach et al., 2003). Seed germination-based genetic screens have identified mutants affected in ABA biosynthesis or sensitivity. The latter include ABA-insensitive mutants and ABA-hypersensitive mutants (reviewed by Finkelstein et al., 2002). Many genes encoding enzymes involved in GA biosynthesis and catabolism have also been identified (Olszewski et al., 2002). Several genes encoding GA-signaling components involved in seed germination are also known (Swain et al., 2002; Tyler et al., 2004). RGL2 negatively regulates GA responses that primarily control seed germination (Lee et al., 2002). The *Arabidopsis* gene *SPY* is also a negative regulator of GA signaling. However, unlike RGL2 it also regulates all other developmental processes involving GA (Izhaki et al., 2001). In addition, SPY is an activator of cytokinin signaling pathway (Greenboim-Wainberg et al., 2005).

Sugars also act as regulatory molecules and play a pivotal role in the plant life cycle. Exogenous glucose application has been shown to delay seed germination and inhibit seedling development (reviewed by Gibson, 2005). Different levels of sugar supply have different effects on various stages of plant development, which may be related to endogenous ABA levels (Gibson, 2005). A delay in *Arabidopsis* seed germination was observed at glucose concentration as low as 0.5% with the delay increasing with increasing glucose concentrations (Price et al., 2003; Dekkers et al., 2004). While intermediate glucose concentrations (1.5-3%) dramatically delay WT seed germination, similar concentrations of sorbitol or mannitol have very little effect, indicating that the effect of glucose is not simply osmotic (Price et al., 2003; Dekkers et al., 2004). Because ABA and GA are key internal regulators of *Arabidopsis* seed germination, it seems likely that glucose and other sugars exert their inhibitory effects via the biosynthesis, degradation or signaling pathways for these hormones. Based on experiments with exogenous application of hormones, hormone precursors and hormone synthesis inhibitors, Dekkers et al. (2004) concluded that glucose is not acting via biosynthesis of ABA, GA or ethylene. Price et al. (2003) suggested that glucose at intermediate and high concentrations may delay germination by slowing the decline of endogenous ABA concentration. Although these results suggest that glucose at higher concentrations may at least partially act via ABA, the components of the ABA response pathway involved in seed germination delay have not been identified (Price et al., 2003). It has been suggested that the glucose-induced delay of seed germination involves neither increasing ABA biosynthesis nor activation of ABA signal transduction via ABI1, ABI2,

ABI4 or ABI5 gene products (Dekkers et al., 2004). Dekkers et al. (2004) also reported that *spy-5* is sensitive to glucose for the timing of germination and suggested that glucose does not act via GA signaling.

High concentrations of glucose increased the accumulation of ABA in seedlings (Arenas-Huertero et al., 2000) and many of the sugar insensitive mutants for post-germination seedling developmental abnormalities have been found to be allelic to genes involved in ABA biosynthesis or ABA signaling pathways (Gibson, 2005). For example, the *Arabidopsis* sucrose uncoupled-6 gene was found to be identical to one of the ABA-insensitive genes, *ABI4* (Huijser et al., 2000). In addition, mannose induced inhibition of germination has been demonstrated to also involve *ABI4* gene which has no function in glucose pathway in seeds (Pego et al., 1999; Dekkers et al., 2004).

In this study we address the possible mechanism of glucose-induced delay of seed germination via the stimulation of ABA signal transduction and/or inhibition of the GA signaling pathway. We analyzed seed germination and seedling development of several mutants of ABA and GA signaling pathways during glucose treatment. Our results indicate that ABA signal transduction is involved in glucose-induced delay of seed germination via ABI3 gene. The GA signaling pathway is also involved in glucose effects on seed germination via RGL2 and SPY genes. Our data also suggest that there may be crosstalk between the ABA, GA, and glucose-signaling pathways that involves ABI3 and RGL2 during seed germination.

Results

Components of both ABA and GA signaling pathways are involved in seed germination delay caused by exogenous glucose

We investigated the seed germination kinetics of several mutants involved in the ABA and GA signaling on plates containing intermediate concentrations of glucose. High glucose concentrations were not used here because under those conditions the delay of germination is partially caused by osmotic effects (Price et al., 2003). We tested mutants in ABA and GA signaling pathways, *abi3-1* and *rgl2-1* that are known to be involved in germination but have not been included in previous studies. We also retested and used as a control *spy5* that was previously found to be glucose sensitive, or even hypersensitive for the germination delay (Dekkers et al., 2004).

Germination of Ler (wild type control), *abi3, rgl2 and spy* seeds in the sugar-free control plates approached 90-100% after 42 hours. Moderate concentrations (1.5% and 2.5%) of glucose delayed seed germination at 42 hours of all the lines tested as shown in Figure 1. However, the mutants were affected less severely than the wild type (WT). The mutant seeds of *rgl2*, *spy* and *abi3*, all have a significantly higher germination rate than WT for both glucose concentrations used (Fig. 1). The dramatic increase of germination frequency of *abi3* begins at 36 hours and approaches 100% at 72 hours on 1.5% glucose plates. The germination frequency of WT on 1.5% glucose begins to increase at 48 hours and only approaches 40% at 72 hours. The germination frequency of *abi3* begins to increase dramatically at 42 hours and approaches 100% at 96 hours on 2.5% glucose. The

germination of WT increases very slowly on 2.5% glucose and reaches only 31% at 120 hours.

The germination kinetics of *rgl2* mutant seeds is similar to *abi3* seeds (Fig. 1). Dekkers et al. (2004) also reported that the *spy* mutant was as sensitive as or even more sensitive than WT to glucose. However, our results indicate that *spy* is partially resistant to glucose during germination. The germination of *spy* seeds was above 90% at 120 hours on 1.5% glucose plates and about 80% at 120 hours with 2.5% glucose treatment (Fig.1B and C). Our data suggest that both the ABA signaling pathway via ABI3 and GA signaling pathway via RGL2 and SPY are involved in glucose delay of seed germination.

Mannose affects germination via ABI3 but not via RGL2 or SPY

Arabidopsis seed germination is inhibited by mannose in a concentration-dependent manner starting at concentrations much lower than other sugars (Pego *et al*., 1999). We tested whether mutants in GA and ABA signaling pathways, *abi3-1*, *rgl2-1* and *spy* that were shown here to be resistant to glucose are also resistant to mannose inhibition of seed germination. The effect of mannose on *spy* and *rgl2* germination has not been reported and the reported results for *abi3-1* have been conflicting. Laby et al. (2000) found that *abi3-1* is as sensitive as WT to 1.7 mM mannose while Huijser et al. (2000) reported that *abi3-1* shows partial resistance to 5 mM mannose. We tested WT and mutant seeds on three concentrations of mannose. Germination of mutant seeds was assayed at the 8^{th} and 10^{th} days after transfer to the growth chamber. A comparison of germination on mannose-free plates and three concentrations (5 mM, 7.5 mM and 10 mM)

of mannose is shown in Fig. 2. The germination frequency of *abi3* seeds on 5 mM mannose plates approached 40% while those of WT, *rgl2* and *spy* were all below 10% on day 10 (Fig. 2). The germination frequency of *abi3* seeds on higher concentrations of mannose, 7.5mM and 10mM, was only about 10% but no seeds of WT, *rgl2* and *spy* germinated under these conditions (Fig. 2). These results indicate that mannose repression of *Arabidopsis* seed germination does not involve GA signaling pathways through RGL2 or SPY gene products but that ABA signaling pathway is involved in this process via ABI3 in addition to the previously reported effect of ABI4 (Pego et al., 1999).

Effects of ABA and GA on rgl2, abi3 and spy seed germination

To determine if there might be an interaction between the ABA and GA signaling pathways during glucose-induced delay of germination we tested glucose insensitive alleles used in our study for resistance to PAC, an inhibitor of GA biosynthesis, and to ABA during seed germination. *Spy-3* has been shown to be partially ABA insensitive and *abi3-1* is known to germinate in the presence of GA biosynthesis inhibitors (Nambara et al., 1991; Steber et al., 1998). We found that *spy-5* shows WT sensitivity to ABA. While the germination of *spy-5* and WT seeds is inhibited by ABA application (Fig. 3A and 3B) the germination of *rgl2-1* seeds is greater than that of WT with both 1μM and 3μM ABA treatment (Fig. 3A and 3B). These data demonstrate that the *rgl2-1* mutant is at least partially resistant to ABA in a concentration dependent manner. As shown in Figure 3c and 3d, *abi3-1* seed germination reached a high percentage (>90%) on day 6 on both 10^{-5} M and 10^{-4} M PAC plates. The germination of *abi3* seeds is similar to that of *rgl2* and *spy*

but the germination of *abi3* seeds is slightly delayed on the higher concentration of PAC (Fig. 3C and D). All three mutants, *abi3*, *rgl2* and *spy* were highly resistant to PAC inhibition of seed germination. These results suggest that ABI3 may inhibit seed germination via negative regulation of GA signaling pathway. ABI3 is a transcription factor, and could therefore exert its influence on the GA signaling pathway via transcriptional activation of negative regulators such as RGL2 or SPY.

Transcription of genes involved in glucose-induced delay of seed germination

We analyzed the RGL2 transcription levels in *abi3* and WT seeds imbibed in H_2O at 4^oC in dark for 7 days. The relative RGL2 mRNA levels in *abi3* mutant seeds decreased three fold relative to WT seeds (Table 1). This result suggests that ABI3 positively controls the *RGL2* gene expression. Since the *rgl2* mutant is slightly resistant to ABA inhibition of seed germination and RGL2 belongs to the GRAS family of putative transcriptional regulators (Pysh et al., 1999), we also compared the ABI3 transcription levels in *rgl2* and WT seeds. ABI3 mRNA levels in *rgl2* mutant seeds decreased by seven fold compared to those in WT seeds (Table 1). This suggests that RGL2 may be involved in activation of *ABI3* expression. There was no significant difference in *SPY* expression in either *rgl2* or *abi3* backgrounds (Table 1). Our data suggest that both ABI3 and RGL2 may be involved in the crosstalk between the ABA and GA signaling pathways and that it may be accomplished by reciprocal transcriptional activation.

Because the *ABI3* and *ABI4* genes both encode transcription factors and seem to be involved in the mannose-induced inhibition of seed germination we wanted to determine whether these genes act in the same pathway by controlling each others expression. We compared the ABI3 transcript levels in *abi4* and WT (Col) as well as the ABI4 transcript accumulation in *abi3* and WT (Ler) seeds. The expression of these genes is the same in WT and mutant backgrounds (data not shown) suggesting that ABI3 and ABI4 do not control each other's transcription.

To further investigate the effect of ABI3 and RGL2 on seed germination delay by glucose, we compared ABI3 and RGL2 mRNA levels in WT seeds imbibed in 2.5% glucose to seeds imbibed in H_2O alone. ABI3 mRNA level in seeds imbibed in glucose is more than 2500 times higher than that of H_2O treated seeds (Table 1). This indicates that glucose dramatically induces ABI3 gene expression on a transcriptional level. The RGL2 mRNA level in seeds treated with glucose is more than 42 times higher than that of H_2O treated seeds (Table 1). These data suggest that glucose delays seed germination via transcriptional induction of both RGL2 and ABI3 genes.

Glucose has a stimulatory effect on seedling growth in a concentration dependent manner.

High glucose concentrations, 6% and above, have been shown to have an inhibitory effect on seedling growth and development (Gibson, 2005). This inhibitory effect is restricted to a very narrow time window of about 48 hours from the start of seed imbibition (Gibson et al., 2001). All the mutants used in this study show WT sensitivity

to this inhibitory effect of glucose (not shown). We investigated the effects of different concentrations of glucose on later stages of seedling development. To avoid the effects of glucose on the timing of germination and on early seedling development, all seeds were first germinated on sugar-free plates and then similar-sized seedlings were transferred on day four to plates containing different concentrations of glucose (1.5%, 2.5%, 5% and 7% glucose). WT seedlings on all glucose concentrations proceeded to develop true leaves (Fig. 4A). The effects of different concentrations of exogenous glucose application on seedling growth are evident several days after transfer from glucose-free media (not shown). Figure 4A shows representative WT shoots, on day 18, from glucose-free plate and four different glucose concentrations. Seedling growth on sugar-free plates is stunted (Fig. 4). Primary roots are very short, and true leaves are not visible on day 10 (not shown). Seedlings from plates containing increasing concentrations of sorbitol alone are indistinguishable from seedlings from sugar-free plates on day 18 (Fig. 4A-C). In contrast, seedlings from plates containing glucose have much longer roots and larger shoots (Fig. 4A-C). Seedlings from intermediate glucose concentrations, 1.5% and 2.5%, have the longest roots and the largest leaves (Fig. 4A-C). Root length and shoot weight increased with intermediate levels of glucose and decreased at high glucose concentrations. Rosette development of WT on 7% glucose plates is better than that on 1.5% glucose plus 5.5% sorbitol plates (Fig. 4A). The above results indicate that glucose has a stimulatory effect on WT seedling development and that the inhibitory effect of very high levels of glucose is osmotic. Application of 1.5-5% glucose resulted in a significant growth stimulation. WT roots were on average at least nine times longer (Fig.

4B) and rosettes attained at least five times larger mass (Fig. 4C). There were no significant differences between root and shoot growth of the *rgl2* mutant and WT at day 18 (Fig. 4D-F). In contrast, *spy* grows considerably better than WT without glucose and similarly to WT seedlings with glucose (Fig. 4D-F). The roots of the *spy* mutant are much longer, at least 5 times, than those of WT on glucose-free plates (Fig. 4E). The rosettes of *spy*, on glucose-free plates, appear at least twice the size of WT on day 18 (Fig. 4D) and attain three times higher mass (Fig. 4F). These results indicate that glucose has a stimulatory effect on both root and shoot growth in WT seedlings and that *spy* is less dependent on glucose supply for seedling growth.

Discussion

Glucose levels as low as 0.5% considerably delay *Arabidopsis* seed germination (Price et al., 2003). This delay is not due to osmotic stress because concentrations below 6% of sorbitol have no effect on the timing of seed germination. Glucose at intermediate and high concentrations may affect germination by slowing the decline of endogenous ABA concentration (Price et al., 2003). However, the components of the ABA signaling pathway such as ABI1, ABI2, ABI4 and ABI5 are not involved in this process (Price et al., 2003; Dekkers et al., 2004).

There have been at least three different glucose-signaling pathways proposed, two of which appear to be hexokinase-dependent (Moore et al., 2003). Because the glucose analog, 3-O-methylglucose has very similar effect on the timing of germination as glucose and because it is not a good substrate for hexokinase (HXK) it was suggested that

glucose delays seed germination via an HXK-independent pathway (Dekkers et al., 2004). Because mannose, a stereoisomer of glucose, is a good substrate for HXK and it inhibits *Arabidopsis* seed germination at very low concentrations, concentrations at which other sugars have no effect, it was suggested that it acts via HXK-dependent pathway (Pego et al., 1999). The idea that mannose and glucose inhibit germination via different pathways is further supported by the findings that ABI4 is involved in mannose but not glucose effects on germination (Pego et al., 1999; Dekkers et al., 2004). The data presented here show that *rgl2* and *spy* mutants in GA signaling pathway are resistant to glucose-induced delay but not mannose inhibition of germination. These data are consistent with the idea that mannose and glucose inhibit germination through different pathways, and further demonstrate that mannose inhibition of seed germination is not via the GA signaling pathway through RGL2 or SPY.

One of the ABA-insensitive genes may be involved in both glucose-induced delay of germination and mannose inhibition of germination. The *abi3-1* mutant is resistant to glucose and partially resistant to mannose inhibition of seed germination, which suggests that ABI3 may be a common factor in both mannose and glucose pathways to inhibit seed germination. Therefore, mannose inhibits seed germination not only via ABI4 in ABA signaling pathway but also via ABI3. It remains to be determined whether ABI3 and ABI4 exert their effects through the same branch of ABA signaling pathway or possibly act in parallel.

It is clear from our results that glucose delays germination by activating the ABA signaling pathway via ABI3 and repressing the GA signaling pathway via SPY and

RGL2. We have shown that glucose application has a pronounced effect on ABI3 and RGL2, up regulating both at the transcriptional level. Our data also show that the *rgl2* mutant is partially resistant to ABA application and confirm that the *abi3* mutant is resistant to PAC indicating that RGL2 may be involved in the ABA signaling pathway and that ABI3 is involved in GA signaling pathway. Our data on SPY, RGL2 and ABI3 transcription in *rgl2* and *abi3* mutant backgrounds indicate that RGL2 up regulates ABI3 transcription and that ABI3 up regulates RGL2 transcription. These data suggest that there is crosstalk between the ABA and GA signaling pathways and that RGL2 and ABI3 gene products are involved in this process.

Our data also show that both *ABI3* and *RGL2* genes are induced by glucose, although whether glucose induces both genes "directly" remains to be determined. Figure 5 shows our model of possible mechanism of glucose-induced delay of seed germination. Glucose leads to a dramatic increase in *ABI3* gene expression at the transcriptional level, which in turn activates the ABA signaling pathway resulting in inhibition of seed germination. The ABI3 gene product may also activate RGL2 transcription and thus lead to inactivation of GA signaling pathway at the same time. Glucose also up regulates RGL2 expression either directly or via ABI3 thus leading to inhibition of seed germination by turning off the GA signaling pathway. Our data also suggest that RGL2 may activate the ABA signaling pathway by up regulating ABI3 transcription.

Glucose not only plays a role in germination but also in other aspects of the plant life cycle, such as seedling development. *Arabidopsis* seedlings germinated on 5%

glucose plates fail to develop expanded cotyledons or true leaves (Laby et al., 2000). Our data demonstrate that when young seedlings are transferred to glucose containing media, following germination without glucose, that glucose has a stimulatory effect on both root and shoot growth and development. Moderate glucose levels (e.g. 1.5% to 5%) strongly stimulate root and true leaf development. Very high glucose levels also lead to better growth than the complete absence of glucose. Poorer growth on very high concentration as compared to moderate concentrations of glucose may simply be associated with osmotic stress.

The interaction between sugar and plant hormone response pathways was indicated by studies that characterized sugar-hypersensitive and resistant mutants for seedling development. The mutants in the ABA signaling pathways such as *abi1*, *abi2* and *abi3* are sensitive to the inhibitory effect of high glucose levels on early seedling development, but *abi4* and *abi5* are resistant (Finkelstein, 1994; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). Our experiments comparing glucose, sorbitol and glucose plus sorbitol suggest that the effects of some of these mutations on later stages of seedling development are related to osmotic stress resistance and are not specific to glucose.

Our data also show that seedling growth of *spy-5* on glucose-free medium is much better than that of WT. It is possible that part of the stimulatory effect of glucose on seedling growth is via activation of the GA signaling pathway since GA is necessary for root and leaf growth. In the *spy* mutant the GA signaling pathway is on in the absence of the functional SPY gene product. Therefore in the *spy* mutant, glucose is not necessary

to activate the GA signaling pathway. The reason why *spy* grows better with than without glucose may be that only a part of the stimulatory effect of glucose on seedling growth is via relieving the inhibitory effect of SPY. Since SPY also functions in cytokinin signaling therefore it cannot be ruled out that glucose exerts it stimulatory effect via this pathway instead of or in addition to GA signaling pathway (Greenboim-Wainberg et al., 2005).

In conclusion, glucose can delay seed germination through a different pathway from sugars such as mannose. The effect of glucose delay on seed germination is via genes in both GA and ABA signaling pathways. Genes we tested, ABI3 and RGL2, may act via the same pathway in glucose delay of seed germination. We have also demonstrated that glucose, applied after germination, has a strong stimulatory effect on seedling growth and development. This effect is partially mediated via activation of GA and/or inactivation of cytokinin signaling pathways by relieving the inhibitory or the stimulatory effect of a key regulator, SPY. Therefore, exogenous glucose in moderate concentrations has opposite effects on plant growth and development depending on the developmental stage during which it is applied. The inhibitory and stimulatory effects of glucose are at least in part mediated via components of ABA and GA signaling pathways.

Materials and methods

Plant materials

Two ecotypes of *Arabidopsis thaliana* were used in this study: Columbia (Col) and Landsberg erecta (Ler). Various *Arabidopsis* mutants with altered ABA and GA signaling were obtained from Arabidopsis stock centers. *spy5, abi2-1, abi3-1, abi4-1* and

abi5-1 came from the Arabidopsis Biological Resource Center at Ohio State University, (http://www.arabidopsis.org/abrc/) and *rgl2-1* (SGT625, Lee et al., 2002) came from the Nottingham Arabidopsis Stock Center (http://arabidopsis.info/).

Germination, plant growth assays and RNA isolation

Before plating, seeds were sterilized in 6.25% Sodium hypochlorite for 3 min and rinsed three times with sterile distilled water. Seeds were planted on 0.5X Murashige and Skoog medium (0.5MS, Caisson Laboratories) solidified with 0.8% agar (Fisher Scientific) and containing varying concentrations of glucose, mannose or hormones. Filter sterilized hormone stock solutions were added after autoclaving to avoid the inactivation of hormones. α-D-Glucose and mannose were obtained from Acros Organics. Paclobutrazol (PAC), gibberellin (GA), and abscisic acid (ABA) were obtained from Sigma-Aldrich. Arabidopsis seeds were stratified for 3 d at 4°C in dark, then placed in a growth chamber with 70% humidity and under 18h light and 6h dark at 23°C to facilitate germination. Germination (based on radicle emergence from the seed coat) was scored every 6-12 h or daily depending on the experiment. Each plate contained 90–110 seeds. Every experiment was repeated two to three times. In each experiment different batches of seeds were used. However, in each experiment WT and mutant seeds used were collected at the same time from the same age plants grown under the same conditions. Although time course of germination differed in each experiment the trends in germination were the same for different batches of seeds. The data shown here is from one of these experiments. Plant growth was estimated by root length, number of leaves

and shoots weight at day 18. Seed germination and plant growth data were analyzed using Microsoft Excel 2000. For germination tests, seeds collected at the same or similar times were used and stored at 4°C before using.

For mRNA expression analyses seeds were imbibed in distilled water for 7 days at 4°C in the dark or imbibed in glucose or water for 72 hours in the dark at 4°C. Total RNA was extracted from these treated seeds for use in QRT-PCR. RNA extraction was performed as described previously (Vicient and Delseny, 1999).

Real time PCR

Total RNA (2 µg) was treated with a DNA-*free* kit (Ambion INC.) and tested with real time PCR for DNA contamination. The purified total RNA was used as a template to synthesize first-strand cDNA using a TaqMan Reverse Transcriptase kit (Roche) with oligo(dT) primers as per the manufacturer's instructions. Quantitative real time PCR using first-strand cDNA as a template was carried out using an ABI Prism 7000 Sequence Detector with TaqMan Universal PCR Master Mix (Roche). PCR reactions were carried out in a final volume of 50 µl using gene specific primers and probes in concentrations determined individually for each set of primers. Probes were modified with 6-FAM, reporter dye at 5'-end and TAMRA quencher at 3'-end. All oligo synthesis and modifications were done by Sigma-Genosys. Gene specific primers and probes used were as follows: APT1 forward 5'TGTTCCTTGCAACCGTCTTCT3', reverse 5'TGGTTGAACGGTGGTTTGAG 3', probe 5' CCACCACCGTGCTCCTCCTTCG3'; SPY forward 5'

GAGCTTGCTTTCCACTTTAATCCA 3', reverse 5' ATCAAGGTTGTCACGGTCTTTGTA 3', probe 5' TGCTGAGGCTTGCAACA ATTTGGGAGTAC3'; RGL2 forward 5' GGCTGCACAGTGGAGGATTC3', reverse 5' CGCGCTAGATCCGAGATGA3', probe 5' TGAAATCCGCTGGGTTTGACCCG 3'; ABI3 forward 5' CCATGGAAGACATCGGAACCT3', reverse 5' GGAGATACATCCTGCTTTTGTTGTT3', probe 5'TCGTGTTTGGAACATGCGCTACAGGT3'; ABI4 forward 5' TTCCGGTAACTAATTCGACTTCGT3', reverse 5' TTACACCCACTTCCTCCTTGTTC3', probe5' TCATCATGAGGTGGCGTTAGGGCA3'.

Thermocycler conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15s at 95°C and 1 min at 60°C. The mRNA level for each gene was determined using the standard curve method according to manufacturer's instruction (ABI Prism 7000 Sequence Detection System User Guide). APT1 (Adenosine phosphoribosyl transferase) transcript level in each sample was used as an internal control (Arroyo et al., 2003). The mean value from triplicate samples was used to calculate the transcript level. Results were analyzed using Microsoft Excel.

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Table 1. Relative mRNA levels

genotypes	Genes		
	ABI3	RGL2	SPY
abi3-1[a]		0.31 ± 0.12	1.21 ± 0.44
rgl2-1[a]	0.14 ± 0.04		1.80 ± 0.11
WT[b]	2601.4 ± 43.6	46.6 ± 3.7	
WT[c]		2.5 ± 0.1	

a) mRNA levels in mutant seeds/ mRNA levels in Ler seeds imbibed in H_2O at 4^oC for 7 days

b) mRNA levels in WT (Ler) seeds imbibed in glucose solution/ mRNA levels in WT seeds imbibed in H_2O at 4^oC for 3 days

c) mRNA levels in WT (Ler) seeds imbibed in ABA solution/ mRNA levels in WT seeds imbibed in H_2O at 4^oC for 3 days

Fig.1A. Time course of *abi3-1*, *rgl2-1*, *spy5* and WT (Ler) seed germination on 0.5MS. Points on the Z-axis are in hours after transfer of plates to the growth chamber.

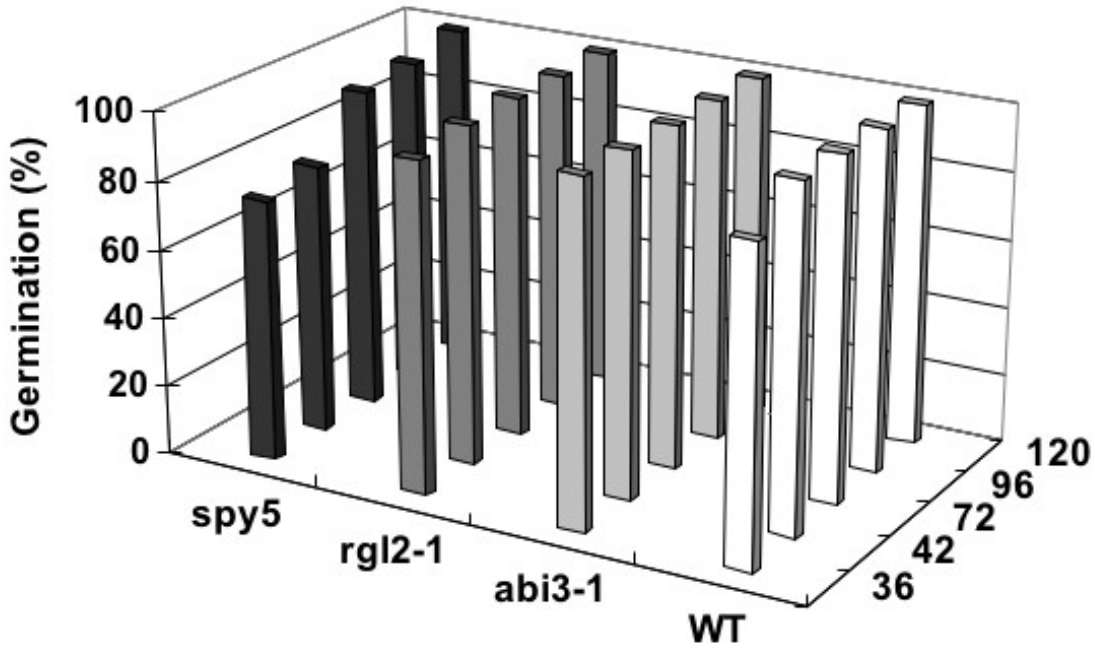


Fig.1B. Time course of *abi3-1*, *rgl2-1*, *spy5* and WT (Ler) seed germination 0.5MS containing 2.5% glucose. Points on the Z-axis are in hours after transfer of plates to the growth chamber.

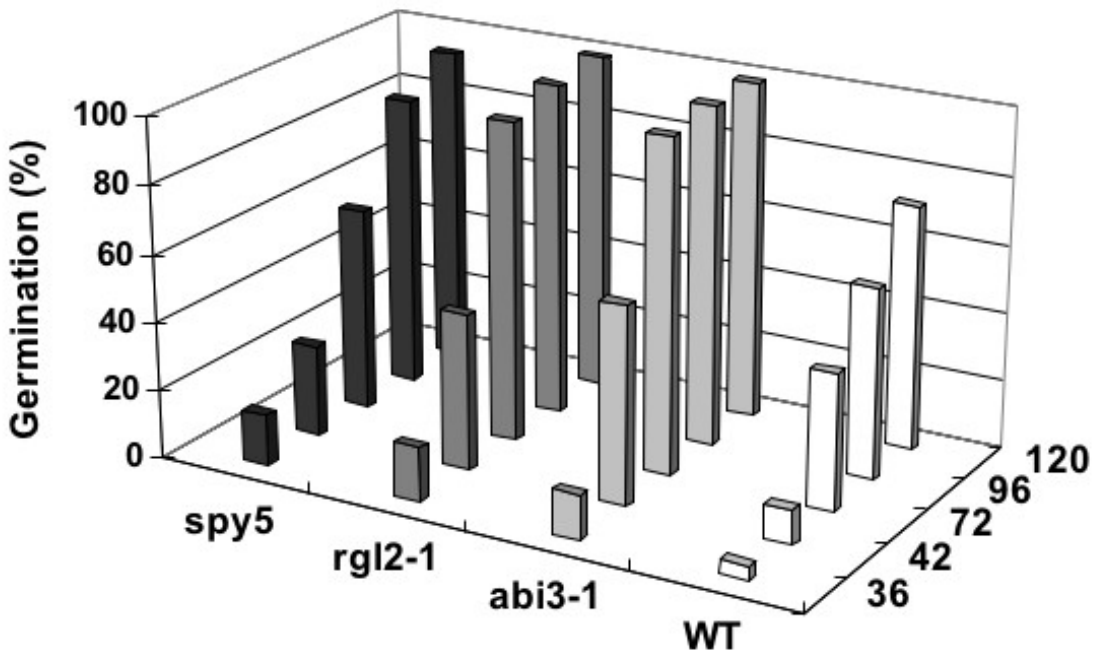


Fig.1C. Time course of *abi3-1*, *rgl2-1*, *spy5* and WT (Ler) seed germination on 0.5MS containing 2.5% glucose. Points on the Z-axis are in hours after transfer of plates to the growth chamber.

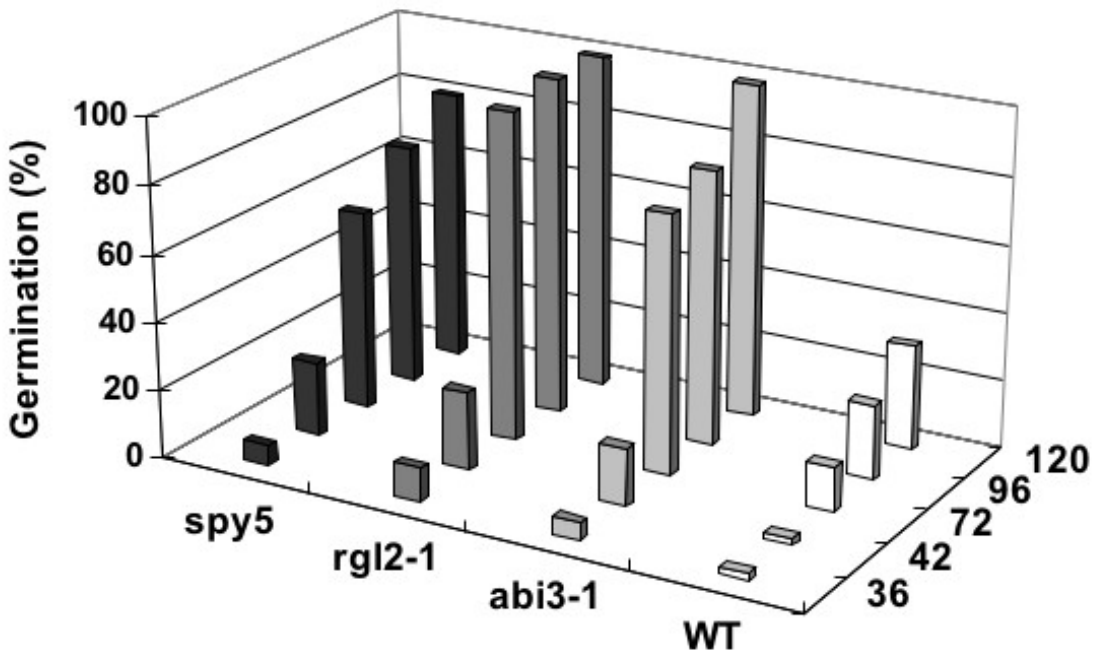


Fig. 2. Effects of mannose on seed germination. Percent germination of WT and mutants, 10 days after transfer to a growth chamber, on 0.5MS containing different concentrations of mannose. Concentration of mannose (mM) is indicated on the Z-axis.

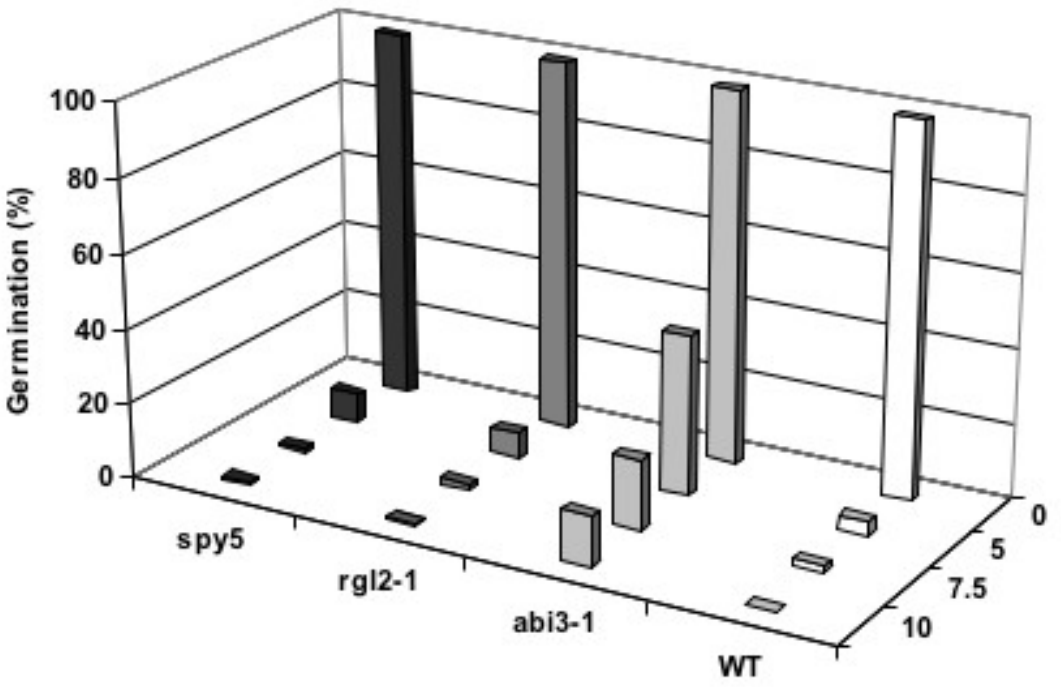


Fig. 3A. Percent germination of WT and mutants, on 0.5MS containing: 1 µM ABA. Time (days) after transfer to the growth chamber is indicated on the Z-axis.

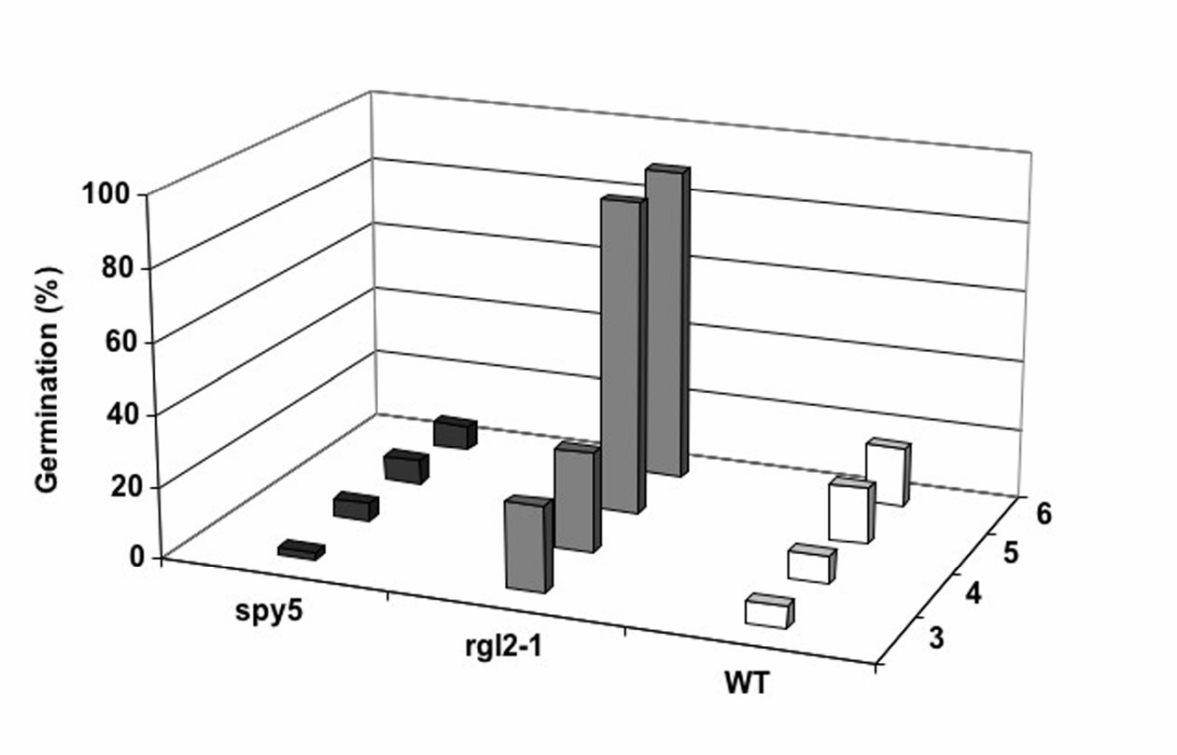


Fig. 3B. Percent germination of WT and mutants, on 0.5MS containing: 3µM ABA. Time (days) after transfer to the growth chamber is indicated on the Z-axis.

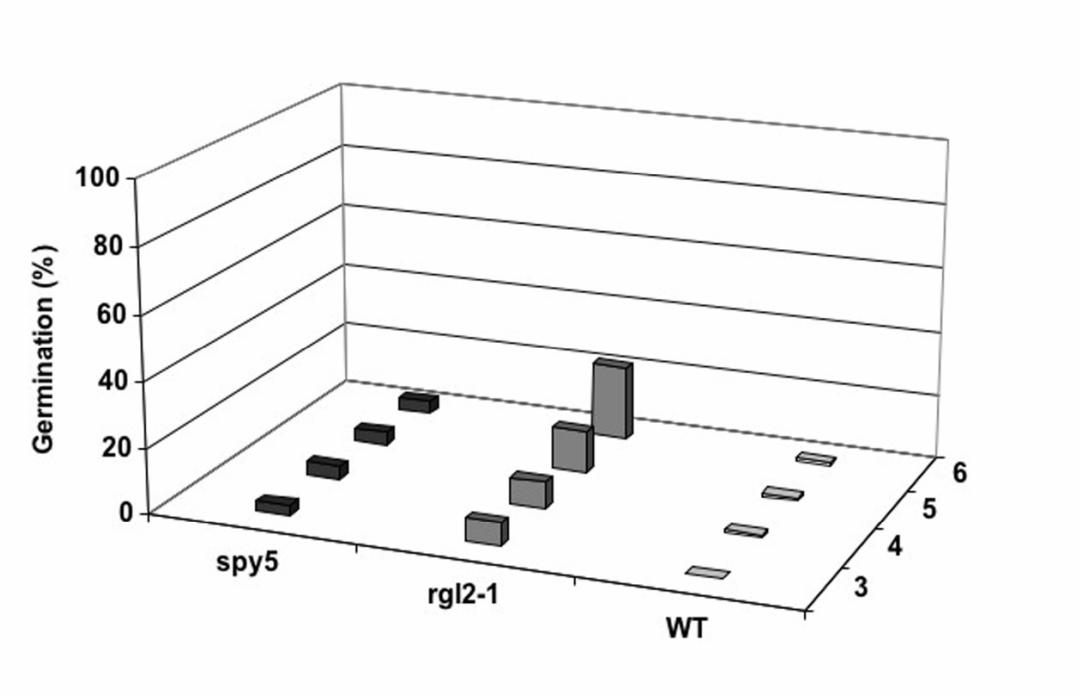


Fig. 3C. Percent germination of WT and mutants, on 0.5MS containing: 10^{-5} M PAC. Time (days) after transfer to the growth chamber is indicated on the Z-axis.

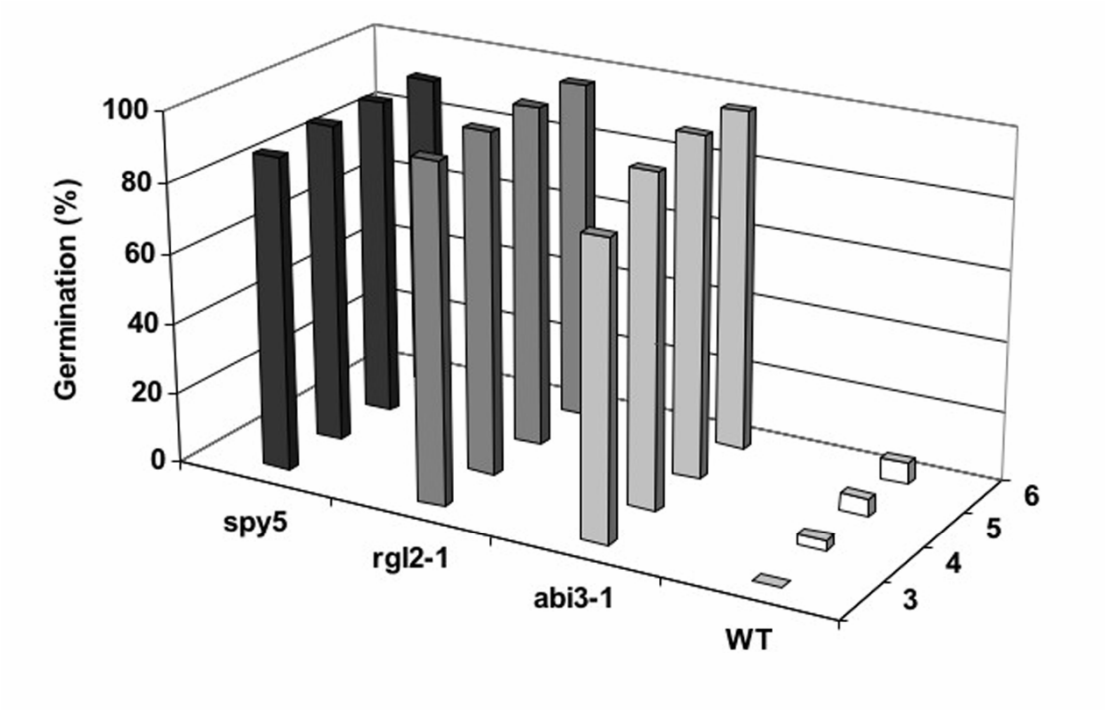


Fig. 3D. Percent germination of WT and mutants, on 0.5MS containing: 10^{-4} M PAC. Time (days) after transfer to the growth chamber is indicated on the Z-axis.

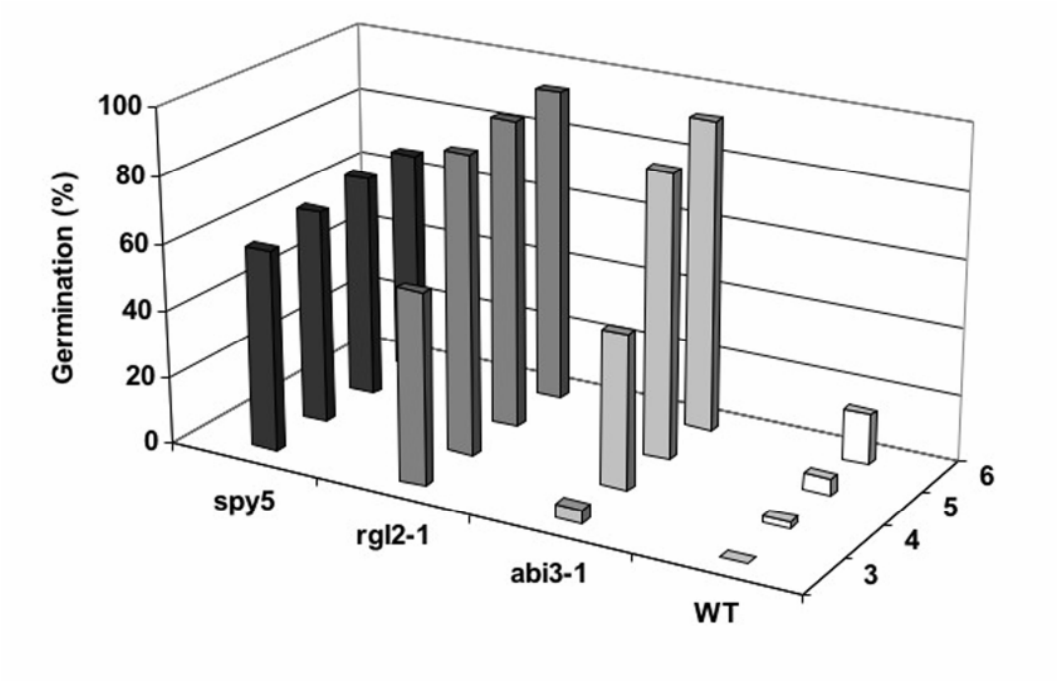


Fig. 4A-F. Comparison of glucose and sorbitol effect on the WT seedling development (A-C) and effects of glucose on GA signaling mutant seedling growth (D-F) analyzed on day 18 after the transfer to growth chamber. In the glucose plus sorbitol groups, 1.5% treatment is glucose alone, 2.5% treatment is 1.5% glucose plus 1% sorbitol, 5% treatment is 1.5% glucose plus 3.5% sorbitol, 7% treatment is 1.5% glucose plus 5.5% sorbitol.

Fig. 4A. Representative WT rosettes grown on various concentrations of glucose, sorbitol and glucose plus sorbitol, total concentrations are indicated above rosettes

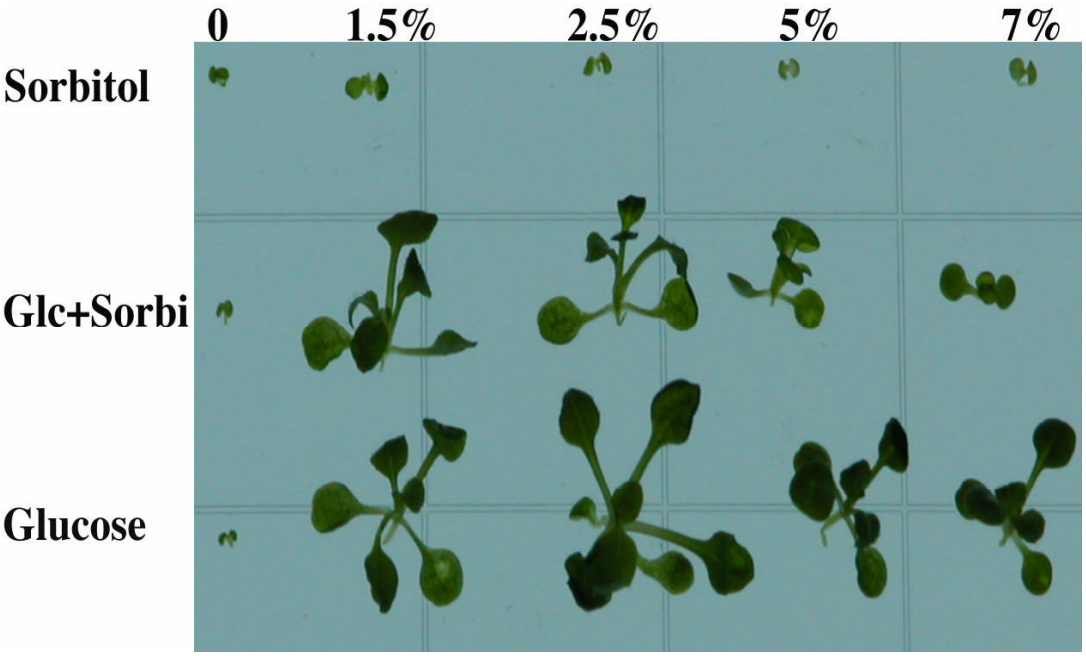


Fig. 4B. Root length of WT grown on various concentrations of glucose, sorbitol and glucose plus sorbitol.

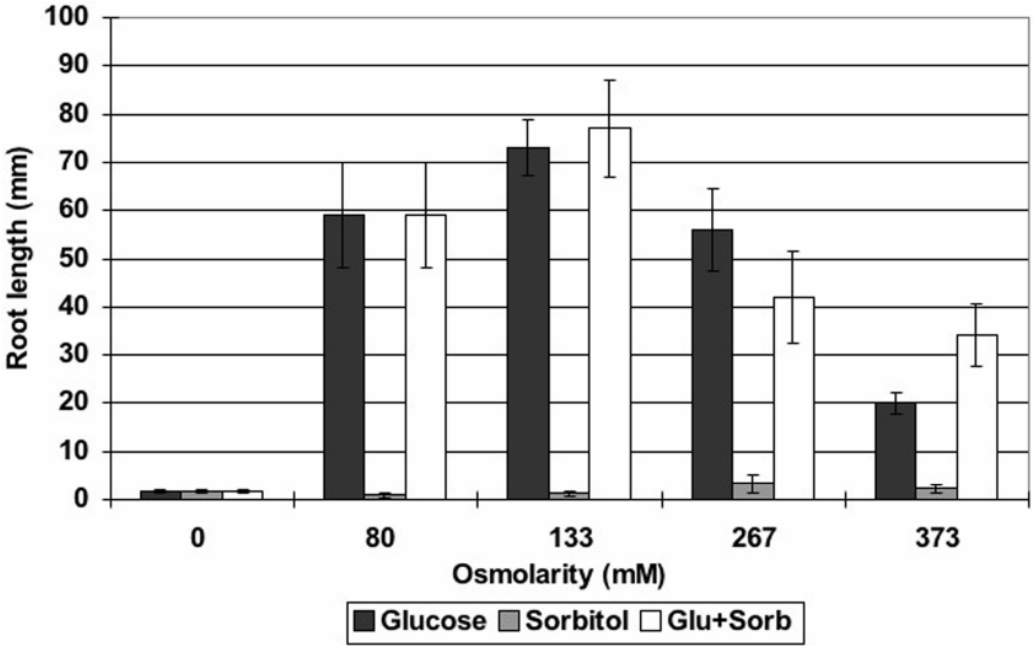


Fig. 4C. Shoot weight of WT grown on various concentrations of glucose, sorbitol and glucose plus sorbitol.

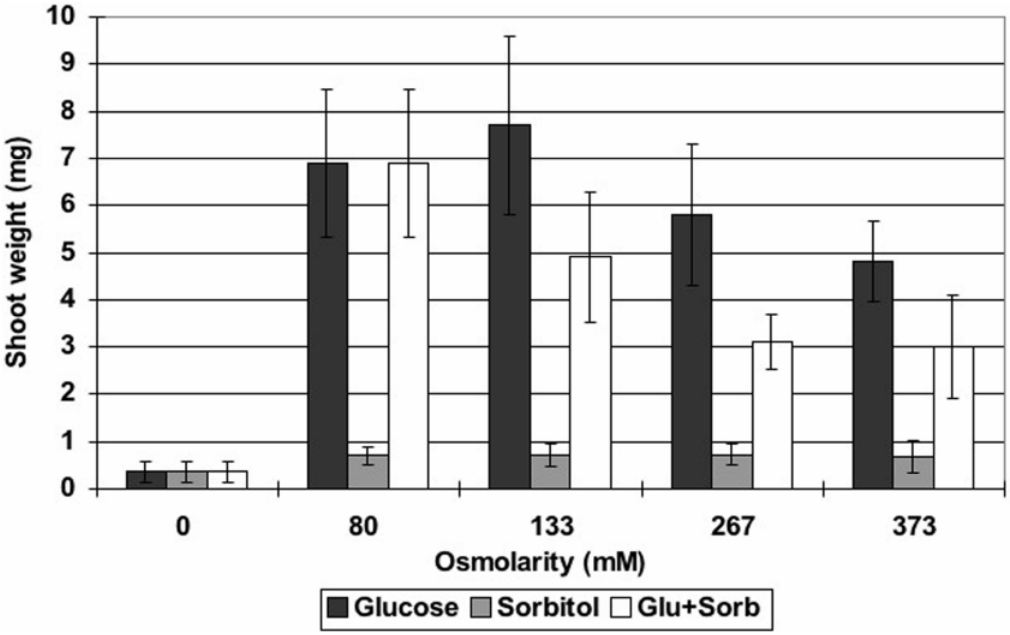


Fig. 4D. WT and GA signaling mutant rosettes grown on various concentrations of glucose. Glucose concentrations (%) are indicated above rosettes.

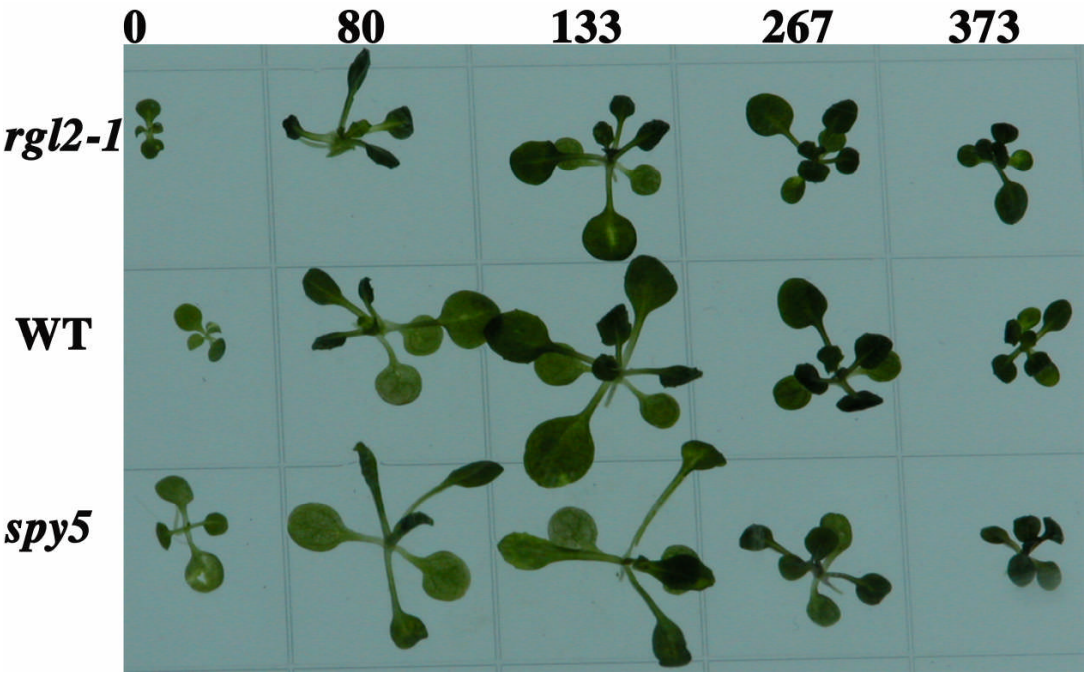


Fig. 4E. Root length of WT and GA signaling mutant grown on various concentrations of glucose.

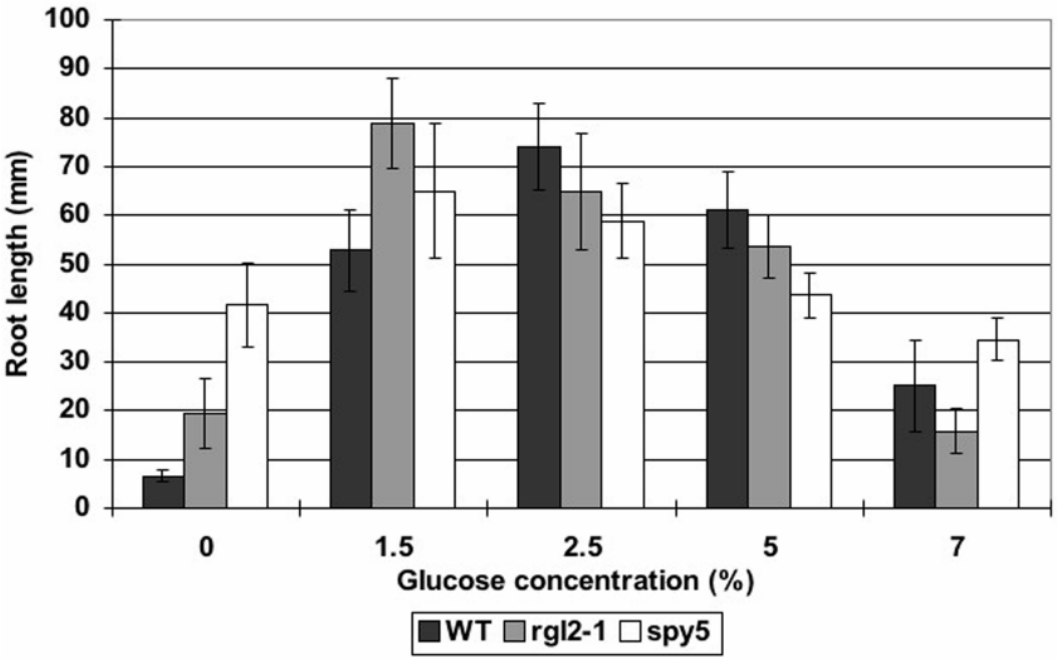


Fig. 4F. Shoot weight of WT and GA signaling mutant grown on various concentrations of glucose.

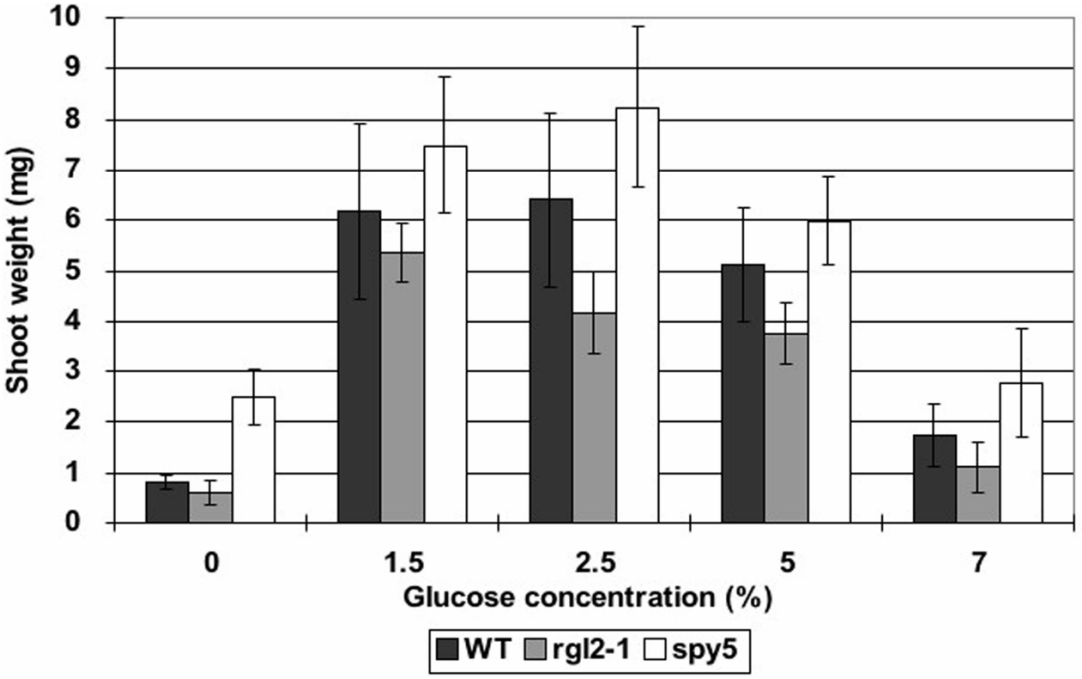
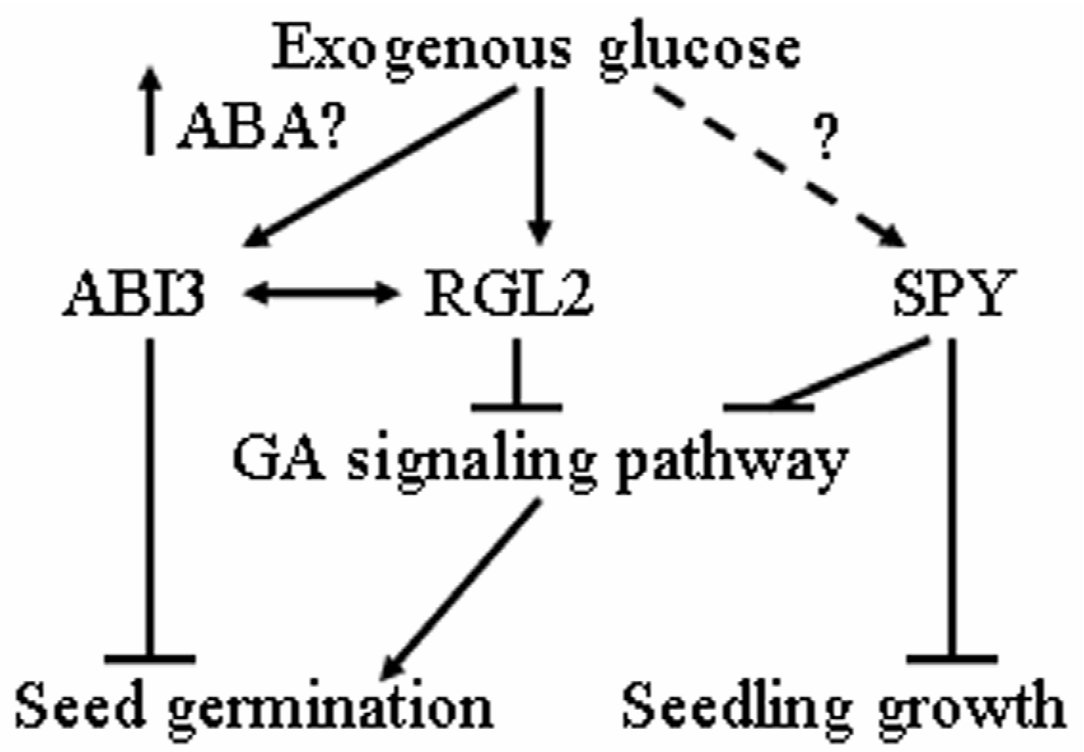


Fig. 5. Proposed model for interactions between glucose, ABA and GA signaling pathways during seed germination and later stages of seedling development. See text for details.



IV. PHYTOHORMONE SIGNALING PATHWAYS INTERACT WITH SODIUM CHLORIDE DURING SEED GERMINATION AND SEEDLING DEVELOPMENT

Abstract

Soil salinity is one of the most significant abiotic stresses limiting plant growth. The adaptation of plant cells to stress conditions involves triggering a network of signaling events. The plant hormone abscisic acid (ABA) regulates many important aspects of plant growth and development, and plays a critical role in stress responses. The process of seed germination is affected by salt and osmotic stress at least partially via the ABA signaling pathway. Several components in the GA signaling pathway are known to be involved in germination but have not been tested for their involvement in salt and osmotic stress. We examined the responses of two mutants in GA signaling pathway to salt and osmotic stress during seed germination and early seedling development. Two mutants in the ABA signaling pathway, previously demonstrated to be involved in seed germination under these stress conditions, were used as positive controls. Real-time PCR was employed to test the genes relative expression levels in several mutants and under various stress conditions to determine whether salt stress affects the seed germination via transcriptional control of the components in GA or ABA signaling pathways. This study suggested that different genes in ABA and GA signaling pathways are involved during different developmental stages under stress condition. There is also a suggestion on

possible crosstalk between different hormone signaling pathways under the salt and osmotic stress conditions.

Key words: Abscisic acid, *Arabidopsis*, Germination, Gibberellic acid, Salt signaling

Introduction

Abiotic stresses affect plant growth. Salinity in soil is one of the most significant abiotic stresses. The phytohormones such as abscisic acid (ABA) play a critical role in stress responses (Himmelbach *et al*., 2003). ABA content in plants under normal physiological conditions is very low. Osmotic stresses induce dramatically levels of ABA (Himmelbach *et al*., 1998). Certain salt-tolerant mutant plants have been identified that were unable to accumulate ABA after hyperosmotic stress treatment (Ruggiero *et al*., 2004). Several ABA-insensitive (*ABI*) and ABA-deficient (*ABA*) mutants have reduced sensitivity to salt and osmotic stress during seed germination in *Arabidopsis*. Quesada et. al reported that one of the salt-tolerant mutants was a null allele of the *ABI4* gene (Quesada *et al*., 2000). *ABI3*, *ABI4* and *ABI*5 encode transcription factors and they have been shown to regulate ABA responses in seeds and in various aspects of vegetative growth (Finkelstein *et al*., 1998; Finkelstein and Lynch, 2000; Signora *et al*., 2001; Brady *et al*., 2003). Salt stress responses of *aba* mutants after germination are complicated. For example, the *aba1* mutant is more sensitive to salt while *aba3* mutant shows the same sensitivity as WT during seedling development (Xiong *et al*., 2001). The salt tolerance of *abi* mutants in vegetative tissues has not been reported. The possible relation between stress tolerance and another important plant hormone GA has been studied in *Arabidopsis* (Magome *et al*., 2004). The *ddf1* (dwarf and delayed-flowering 1) is a novel *Arabidopsis* mutant deficient in GA biosynthesis. Transgenic plants overexpressing DDF showed increased tolerance to high-salinity stress (Magome *et al*., 2004). However, the levels of endogenous active GAs in those plants exposed to stress have not been determined.

RGL2 negatively regulates GA responses that primarily control seed germination (Lee *et al*., 2002; Tyler *et al*., 2004). In this report, we examine the response of mutants, *rgl2* and *spy* in GA signaling pathway, to salt and osmotic stress during seed germination and early seedling development. We confirm the previous findings about the involvement of two components of ABA signaling pathway, ABI3 and ABI5 in seed germination under salt and osmotic stress. We extend this analysis to the response of these mutants to the stress condition during early seedling development. We find that different genes in ABA and GA signaling pathways are involved at different stages of plant development under stress condition.

Results

Components of both ABA and GA signaling pathways are involved in inhibition of seed germination by exogenous sodium chloride or mannitol

We investigated the seed germination kinetics of several mutants involved in the ABA and GA signaling on plates containing various concentrations of sodium chloride or mannitol. The initiation of seed germination is demonstrated by radical emergence. We tested mutants in GA signaling pathways, *spy-5* and *rgl2-1* that are known to be involved in germination. We also used two mutants, *abi3-1* and *abi5-1*, in ABA signaling pathway that were previously found to be salt resistant (Brocard *et al*., 2002). Because not all the mutants are in the same genetic background we first tested WT seeds for degree of sensitivity to salt during germination. Wassilewskija (Ws) is more sensitive to salt than Landsberg erecta (Ler) during germination. Germination of Ler seeds on moderate

concentration of salt, 200 mM NaCl approached 95% on day 14 while only 1% of Ws seeds germinated (Fig. 1A). *rgl2, abi3* and *abi5* are all resistant to high concentration of salt during seed germination (Fig. 1A and 1B). The s*py* mutant is as sensitive as WT to NaCl (data not shown). Moderate concentration (250mM) of salt delayed seed germination of all the lines tested as shown in Figure 1B. Although the seed germination percentage of Ler is similar to those of *rgl2* and *abi3* on day 14 on 250mM salt plates (Fig. 1A), the seed germination kinetics of mutants are very different than the WT (Fig. 1B). The mutant seeds of *rgl2*, *abi3* and *abi5* show over 50% germination on day 4 as compared to 0% by WT (Fig. 1B). The germination of Ler on 250mM NaCl begins on day 5 and only approaches 33% on day 7. In contrast, the germination of *abi3* and *rgl2* is already above 90% on day 5. The germination kinetics of *rgl2* mutant seeds is similar to *abi3* seeds (Fig. 1B). High concentration (300mM) of sodium chloride also completely inhibited seed germination of *abi5* but not *abi3* and *rgl2*. This may be primarily attributed to different salt sensitivity of the background ecotypes of these mutants (Fig. 1). WS seeds do not germinate on salt above 200mM (Fig.1A).

The seeds of two ecotypes (Ws, Ler) have different responses to osmotic stress (mannitol) during germination. Ws is more sensitive to osmotic stress compared with Ler during germination (Fig. 1C). The mutants we tested, *rgl2*, *abi3* and *abi5*, are resistant to high concentration of mannitol during seed germination. Surprisingly, s*py* mutant seeds are also resistant to high concentration of mannitol during germination. Although the seed germination percentage of Ler was similar to those of *rgl2* and *abi3* on day 14 on 400mM mannitol plates, the seed germination kinetics of mutants are very different than

the WT (Fig. 1D). The germination of Ler on 400 mM mannitol begins on day 2 and only approaches 52% on day 4. The germination frequencies of *abi3* and *rgl2* are above 90% on day 4. The germination kinetics of *rgl2* mutant seeds is similar to *abi3* seeds (Fig. 1D). The inhibition of seed germination of *abi5* mutant was less severe than that of Ws (Fig. 1C). The seed germination of *abi5* approached 88% on 400mM mannitol plates while only 2% of Ws seeds germinated.

Our data suggest that both the ABA signaling pathway via ABI3 and ABI5 and GA signaling pathway via RGL2 are involved in salt and osmotic stresses inhibition and delay of seed germination. SPY in GA signaling pathway is only involved in osmotic stress inhibition.

Components of both ABA and GA signaling pathways have different functions during completion of seed germination and on seedling survival under salt stress

The completion of seed germination was demonstrated by the cotyledon emergence and the seedling survival was indicated by the presence of green cotyledons after 14 days in growth chamber. The two ecotypes used, Ws and Ler, have different response to salt stress during the completion of seed germination. Ws is more sensitive to salt stress as compared with Ler (Fig. 2A). However, all of cotyledons of Ws that do emerge remain green. Only some of Ler cotyledons remain green even at lower salt concentrations (Compare Fig. 2A and 2B). The *abi5* is highly resistant to salt stress during the completion of seed germination even at higher concentration of salt (250mM). Also, all of the *abi5* cotyledons remain green on lower to medium salt concentrations,

150mM and 200mM (Fig. 2A and 2B). However, only about 50% of *abi5* cotyledons remain green at higher salt concentration, 250mM (Fig. 2A and 2B). The trend of *rgl2* mutant response to salt stress during completion of seed germination and seedling survival is similar to that of *abi5* mutant. *rgl2* is resistant to salt stress during the completion of seed germination but only partially resistant to salt stress for seedling survival especially at salt concentration above 200mM (Fig. 2A and 2B). The *spy* mutant is indistinguishable from WT for completion of germination and seedling survival under salt stress (not shown). The *abi3* differs from WT and other mutants tested in its response to salt at later stages of seed germination. Although most of *abi3* seeds initiate germination (Fig. 1A and 1B), none of the seeds complete the process at high salt concentrations (Fig. 2A). The *abi3* seeds that complete germination at lower salt concentration, 150mM and 200mM, have much lower survival rate than even WT (compare Fig.2A and 2B).

We extended our analysis to the salt stress effects on later seedling development. In these experiments seeds were germinated on plates without salt and young seedlings were transferred to plates with 50mM and 75mM sodium chloride. The root lengths of *rgl2* and *abi5* mutant seedlings were not significantly different from those of WT seedlings on control plates without salt (Fig. 2C). There is also no significant difference between the root lengths of *rgl2* and *abi5* mutant seedlings and those of WT seedlings from day 1 to day 7 after transfer to plates with 50mM and 75mM sodium chloride (Fig. 2D and 2E, *abi5* data not shown). The root lengths of *abi3* mutant seedlings were significantly shorter as compared to those of WT seedlings from day 2 to day 7 after

transfer to plates with 75mM sodium chloride (Fig. 2E). There is a slight difference between root lengths of *abi3* mutants and WT on 50mM sodium chloride plates (Fig. 2D). These data suggest that ABI3 may provide protection from salt stress or it may decrease the sensitivity to salt stress once the roots emerge during later stages of seedling development. ABI5 and RGL2 do not seem to be involved in seedling growth under salt stress conditions.

Components of both ABA and GA signaling pathways have different effects on completion of seed germination and on seedling survival under osmotic stress conditions

All mutants that are resistant to osmotic stress, as shown by initiation of germination on various concentrations of mannitol (300mM, 400mM and 500mM) were tested for the germination completion and seedling survival (Fig. 3A). The *abi5* is highly resistant to osmotic stress during the completion of seed germination even at high (500mM) mannitol concentrations. The trend of *rgl2* and *abi3* mutant response to osmotic stress during completion of seed germination and seedling survival is similar to that of *abi5* mutant (Fig. 3A and 3B). The *spy* mutant is highly resistant to osmotic stress during the completion of seed germination even at highest mannitol concentration, 600mM, we used (Fig. 3A). All emerged mutant cotyledons except *spy* remain green on day 14 (Compare 3A and 3B). Although majority (above 80%) of *spy* seeds complete germination on all mannitol concentration used, only less than 20% of seedlings survives on 600mM mannitol (Fig. 3B).

Transcription of genes involved in salt stress sensing during seed germination

We have shown that ABI3, ABI5 and RGL2 are all involved in inhibition of seed germination under salt and osmotic stress conditions. All three of these genes are transcription factors regulating expression of downstream effectors of germination. Therefore we determined transcription levels of these genes on WT and mutant backgrounds under salt stress conditions.

To investigate the effect of ABI3, ABI5 and RGL2 on seed germination delay and inhibition by sodium chloride, we compared *ABI3*, *ABI5* and *RGL2* mRNA levels in WT seeds imbibed in 200mM sodium chloride to seeds imbibed in H_2O alone. *ABI3* mRNA level in seeds imbibed in salt is more than seventy-six times higher than that of H_2O treated seeds (Table 1). This indicates that sodium chloride induces *ABI3* gene expression at transcriptional level. The *ABI5* mRNA level in seeds treated with sodium chloride is more than forty-eight times higher than that of H_2O treated seeds (Table 1). The *RGL2* mRNA level in seeds treated with sodium chloride is more than one hundred and fourteen times higher than that of H_2O treated seeds (Table 1). These data suggest that sodium chloride delays and/or inhibits seed germination via transcriptional induction of all three genes tested: *ABI3*, *ABI5* and *RGL2*.

The expression levels of all genes we tested went down in mutant backgrounds under normal conditions. We analyzed the RGL2 transcription levels in *abi3* and WT seeds imbibed in H_2O at 4^oC in dark for 3 days. The relative RGL2 mRNA levels in *abi3* mutant seeds decreased four fold relative to WT seeds and the relative ABI5 mRNA levels in *abi3* mutant seeds decreased thirteen fold relative to WT seeds (Table 1). This

result suggests that ABI3 has a positive effect on the *RGL2* and *ABI5* gene expression. Since RGL2 belongs to the GRAS family of putative transcriptional regulators (Pysh *et al*., 1999), we also compared the *ABI3* and *ABI5* transcription levels in *rgl2* and WT seeds. *ABI3* mRNA levels in *rgl2* mutant seeds decreased by sixty six fold compared to those in WT seeds and *ABI5* mRNA levels in *rgl2* mutant seeds decreased by eleven fold compared to those in WT seeds (Table 1). This suggests that RGL2 may be involved in the activation of *ABI3* and *ABI5* expression. The mRNA levels of *RGL2* and *ABI3* in *abi5* mutant seeds also decreased compared to those in WT seeds. *RGl2* mRNA levels in *abi5* mutant seeds decreased by thirty one fold compared to those in WT seeds and *ABI3* mRNA levels in *abi5* mutant seeds decreased by twenty fold compared to those in WT seeds. These data suggest that ABI3, ABI5 and RGL2 may be involved in the crosstalk between the ABA and GA signaling pathways and that it may be accomplished by reciprocal transcriptional activation.

To see if salt effect on each genes expression is “direct’, we also looked at expression levels of each gene under salt stress in mutant backgrounds. The *RGL2* expression level in *abi3* mutant seeds is not induced by salt (Table 1A). In contrast, *RGL2* expression is induced 14 fold by salt in *abi5* background. The *ABI3* expression in *rgl2* and *abi5* seeds is only slightly induced by salt, 3 and 5 fold respectively. However, this level of induction is still significantly below the level of induction, 118 fold, in WT seeds. The *ABI5* expression level in *rgl2* mutant seeds is only slightly induced by salt, 2 fold, as compared to nearly 50 fold induction in WT. There is no induction by salt of *ABI5* expression in *abi3* mutant seeds (Table 1). All of the gene expression levels we

tested in the mutant seeds treated with salt are significantly lower than their expression in the WT seeds treated with salt. These data indicate that salt may induce expression of one or two of these genes which, in turn up-regulate each other.

Discussion

The salt stress consists of ionic and osmotic impact on plant cells. The mechanism of salt tolerance is very complex. Along with the primary ionic and osmotic signals, stress hormone, ABA working as the secondary signal is generated. Plant mutants defective in ABA and other hormone production or signal transduction have been used to study the plant hormones function in response to salt stress. Several ABA-insensitive mutants have reduced sensitivity to salt and osmotic stress during seed germination in *Arabidopsis* (Werner *et al*., 1995; Carles *et al*., 2002). Our results confirmed the previous studies that salt delays germination by activating the ABA signaling pathway via ABI3 and ABI5. The previous data showed that a gene in GA signaling pathway, *rgl2* is involved in germination (Lee *et al*., 2002). The involvement of GA signaling pathway in the salt and osmotic stress tolerance was demonstrated in our study. In this report we show that *rgl2* seeds can germinate on NaCl and mannitol concentrations higher than the control. This indicates that *rgl2* mutant is less sensitive to general osmotic stress and NaCl ion toxicity during germination than wild type. This result strongly suggests that the biological role of RGL2 is to prevent germination when osmotic pressure is too high. During germination, RGL2 would act also as a sensor for osmotic status and would be part of a growth repression mechanism during germination when the seed imbibes under

unfavorable conditions. All mutants were sensitive to KCl (not shown). It indicates that *abi3, abi5* and *rgl2* mutants are less sensitive to ion toxicity caused by Na^+, but not Cl^-.

Salt application has a pronounced effect on *ABI3, ABI5* and *RGL2* expression, Our data show that *ABI3, ABI5* and *RGL2* gene expression levels are induced by salt, although whether salt induces all genes "directly" remains to be determined. The products of all these genes are putative transcription factors. ABI5 is active throughout the plant's life and the highest expression induced by ABA and stress is at the transition from mature seeds to seedling development (Brocard *et al*., 2002). The high level of complexity of the crosstalk and interactions exist between the different regulatory genes and pathways. Our data show that *ABI3, ABI5* and *RGL2* transcription in *abi3, abi5* and *rgl2* mutant backgrounds was significantly lower, ranging between 4 and 67 fold, in all the mutant backgrounds as compared to WT in the absence of stress. This indicates that each of the three genes has a positive effect on the transcription of the other two under normal conditions. The transcription of *ABI5* is not induced by salt in *rgl2* and *abi3* mutants. Also, there is no induction of *RGL2* or *ABI5* in *abi3* mutant background under salt stress. The highest induction by salt in any of the mutant backgrounds was observed for the expression of *RGL2* in the *abi5* background. Theses results suggest that salt induction of *ABI5* expression may be accomplished via the induction of *RGL2* and/or *ABI3* expression first (Fig. 4). It appears that under salt stress *ABI3* and/or *RGL2* expression is induced first and later each of the three genes up-regulates the expression of the other two (Fig. 4). These data suggest a complex crosstalk between the ABA and GA signaling pathways that involves ABI3, ABI5 and RGL2 gene products. ABI3, ABI5 and

RGL2 act as major factors during germination because of their accumulation in the presence of salt. Figure 4 shows our model of a possible mechanism of the salt-induced delay and inhibition of seed germination. Salt leads to a dramatic increase in *ABI3* and *ABI5* gene expressions at the transcriptional level, which in turn activates the ABA signaling pathway resulting in inhibition of seed germination. The ABI3 and ABI5 gene products may also activate *RGL2* transcription and thus lead to inactivation of GA signaling pathway at the same time. Salt also up-regulates *RGL2* expression either directly or via ABI3 and/or ABI5 thus leading to inhibition of seed germination by turning off the GA signaling pathway. Our data also suggest that RGL2 may activate the ABA signaling pathway by up-regulating *ABI3* and *ABI5* transcription. Taken together, the salt tolerance of *rgl2* and *abi* mutants to Na^+ ion is relative to the disturbance of both ABA and GA signaling pathways. It indicates that both ABA and GA signaling pathways are involved in the effect of salt stress during seed germination.

Salt not only plays a role in germination but also in other aspects of the plant life cycle, such as seedling development. Quesada et al. reported that the salt tolerant mutants isolated by seed germination screening may not be resistant to salt during seedling or adult stages of development (Quesada *et al*., 2000). The sensitivity or tolerance of *abi* mutants to salt stress during the early seedling stage has not been reported. Our results indicate that the responses to salt during early seedling development in *abi3*, *abi5*, *rgl2* and *spy* are not the same. Although all the mutant plants were eventually killed by salt after long exposure to high salinity environment *abi5* and *rgl2* young seedlings have reduced sensitivity to ion toxicity induced by Na^+. Both of these mutants, *rgl2* and *abi5*,

completed germination at higher rates and survived longer than WT under salt stress conditions. On the other hand, the *abi3* young seedlings are resistant to osmotic stress but are hypersensitive to ion toxicity caused by Na^+. We speculate that Na^+, not Cl^-, causes these altered responses by mutants because all mutants are indistinguishable from WT when germinated and/or grown on KCl containing media (not shown). Our results indicate that the functions of ABI genes are different during early seedling development. ABI5 may prevent the initiation and the completion of seed germination under osmotic and salt stress. ABI3 seems to have different functions during different stages of seed germination under salt stress. It seems that ABI3 gene product together with ABI5 and RGL2 inhibits initiation of seed germination. However, *abi3* mutants seem to be hypersensitive to salt after germination has been initiated. This suggests that the normal ABI3 product may provide some kind of protection to young seedlings if the germination is initiated under high salt stress. RGL2 transcription is not detectable after the seed germination under non-stress conditions (Lee *et al*., 2002). Our data indicate that RGL2 together with ABI3 and ABI5 prevents initiation of seed germination. RGL2 like ABI5 also seems to inhibit all stages of seed germination under osmotic and salt stress. Also like ABI5, RGL2 seems to have no function in post-germination seedling development.

In conclusion, several components of ABA and GA signaling pathways are involved in inhibition of seed germination under osmotic and salt stress conditions. In addition, it appears that one of the components of the ABA signaling pathway, ABI3, has an additional role in salt tolerance if seed germination is initiated under high salt stress conditions.

Materials and methods

Plant materials

Two ecotypes of *Arabidopsis thaliana* were used in this study: Landsberg erecta (Ler) and Wassilewskija (Ws). Various *Arabidopsis* mutants with altered ABA and GA signaling were obtained from *Arabidopsis* stock centers. *spy5, abi3-1* and *abi5-1* came from the *Arabidopsis* Biological Resource Center at Ohio State University, (http://www.arabidopsis.org/abrc/) and *rgl2-1* (SGT625, Lee *et al*., 2002) came from the Nottingham *Arabidopsis* Stock Center (http://arabidopsis.info/).

Germination, plant growth assays and RNA isolation

Before plating, seeds were sterilized in 6.25% Sodium hypochlorite for 3 min and rinsed three times with sterile distilled water. Seeds were planted on 0.5X Murashige and Skoog medium (0.5MS, Caisson Laboratories) solidified with 0.8% agar (Fisher Scientific) and containing varying concentrations of sodium chloride or mannitol. Sodium chloride was obtained from Fisher Scientific and mannitol was obtained from Sigma. *Arabidopsis* seeds were stratified for 3 d at 4°C in dark, then placed in a growth chamber with 70% humidity and under 18h light and 6h dark at 23°C to facilitate germination. Germination (based on radicle emergence from the seed coat) was scored daily for 3-14 days. Each plate contained 90–110 seeds. Every experiment was repeated two to three times. In each experiment different batches of seeds were used. However, in each experiment WT and mutant seeds used were collected at the same time from the

same age plants grown under the same conditions. Although time course of germination differed in each experiment the trends in germination were the same for different batches of seeds. The data shown here is from one of these experiments. Completion of seed germination was measured by cotyledon emergence by day 14. Plant survival was estimated by percentages of cotyledons remaining green at day 14.

For later seedling growth development assay, seeds were plated on 0.5MS solidified with 0.8% agar and plus 3% sucrose. *Arabidopsis* seeds were stratified for 3 d at 4°C in dark, then placed in a growth chamber with 70% humidity and under 18h light and 6h dark at 23°C to facilitate germination. Young seedlings were transferred to 50mM or 75mM sodium chloride plates at day 4 and plates were upside down. Root lengths were measured from day 1 to day 7 after transferred to salt plates. Seed germination, plant growth and plant survival data were analyzed using Microsoft Excel 2000. For germination tests, seeds collected at the same or similar times were used and stored at 4°C before use.

For mRNA expression analyses mutant seeds were imbibed in distilled water for 3 days at 4°C in the dark or WT/ mutant seeds were imbibed in stress solutions, hormone solutions or water for 3 days in the dark at 4°C. Total RNA was extracted from these treated seeds for use in QRT-PCR. RNA extraction was performed as described previously (Vicient and Delseny, 1999).

Real time PCR

Total RNA (2 µg) was treated with a DNA-*free* kit (Ambion INC.) and tested with real time PCR for DNA contamination. The purified total RNA was used as a template to synthesize first-strand cDNA using a TaqMan Reverse Transcriptase kit (Roche) with oligo(dT) primers as per the manufacturer's instructions. Quantitative real time PCR using first-strand cDNA as a template was carried out using an ABI Prism 7000 Sequence Detector with TaqMan Universal PCR Master Mix (Roche). PCR reactions were carried out in a final volume of 50 µl using gene specific primers and probes in concentrations determined individually for each set of primers. Probes were modified with 6-FAM, reporter dye at 5'-end and TAMRA quencher at 3'-end. All oligo synthesis and modifications were done by Sigma-Genosys. Gene specific primers and probes used were as follows: APT1 forward 5'TGTTCCTTGCAACCGTCTTCT3', reverse 5'TGGTTGAACGGTGGTTTGAG3', probe 5' CCACCACCGTGCTCCTCCTTCG3'; RGL2 forward 5' GGCTGCACAGTGGAGGATTC3', reverse 5' CGCGCTAGATCCGAGATGA3', probe 5' TGAAATCCGCTGGGTTTGACCCG 3'; ABI3 forward 5' CCATGGAAGACATCGGAACCT3', reverse 5' GGAGATACATCCTGCTTTTGTTGTT3', probe 5'TCGTGTTTGGAACATGCGCTACAGGT3'; ABI5 forward 5'GAGGTGGCGTTGGGTTTG3', ABI5 reverse 5'GGGCTTAACGGTCCAACCAT3', probe5'AGCGGGTGGACAGCAAATGGG3'

Thermocycler conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15s at 95°C and 1 min at 60°C. The mRNA level for each gene was determined using the

standard curve method according to manufacturer's instruction (ABI Prism 7000 Sequence Detection System User Guide). APT1 (Adenosine phosphoribosyl transferase) transcript level in each sample was used as an internal control (Arroyo *et al*., 2003). The mean value from triplicate samples was used to calculate the transcript level. Results were analyzed using Microsoft Excel.

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Table 1. Relative mRNA levels

Treatment/genotype	NaCl[a]	*rgl2*[b]	*abi3*[b]	*abi5*[b]	*rgl2*[c]	*abi3*[c]	*abi5*[c]
RGL2	114.5 ± 38.6	-	0.23±0.04	0.032±0.008	-	0.67±0.042	14.4±1.3
ABI3	76.4 ± 3.4	0.015±0.009	-	0.05±0.006	2.67±0.1	-	5.27±0.96
ABI5	48.7 ± 5.7	0.094±0.12	0.077±0.003	-	2.18±0.23	0.82±0.053	-

a. mRNA levels in *WT* seeds imbibed in salt solution/ mRNA levels in *WT* seeds imbibed in H_2O at 4^oC for 3 days.

b. mRNA levels in mutant seeds/mRNA levels in *WT* seeds imbibed in H_2O at 4^oC for 3 days.

c. mRNA levels in mutant seeds imbibed in salt solution/mRNA levels in mutant seeds imbibed in H_2O at 4^oC for 3 days

Table 1A. Relative mRNA levels

Treatment/genotype	NaCl[a]	*rgl2*[b]	*abi3*[b]	*abi5*[b]	*rgl2*[c]	*abi3*[c]	*abi5*[c]
RGL2	**+115**	-	**-4**	**-31**	-	**-1.5**	**+14**
ABI3	**+76**	**-67**	-	**-20**	**+3**	-	**+5**
ABI5	+49	**-11**	**-13**	-	**+2**	1	-

Fold increase marked as +number, fold decrease marked as –number.

a. mRNA levels in *WT* seeds imbibed in salt solution/ mRNA levels in *WT* seeds imbibed in H_2O at 4^oC for 3 days.

b. mRNA levels in mutant seeds/mRNA levels in *WT* seeds imbibed in H_2O at 4^oC for 3 days.

c. mRNA levels in mutant seeds imbibed in salt solution/mRNA levels in mutant seeds imbibed in H_2O at 4^oC for 3 days

Fig.1A. Percentage of WT and mutant germination on 0.5MS containing different concentrations of sodium chloride, 14 days after transfer to a growth chamber. Concentration of sodium chloride (mM) is indicated on the Z-axis.

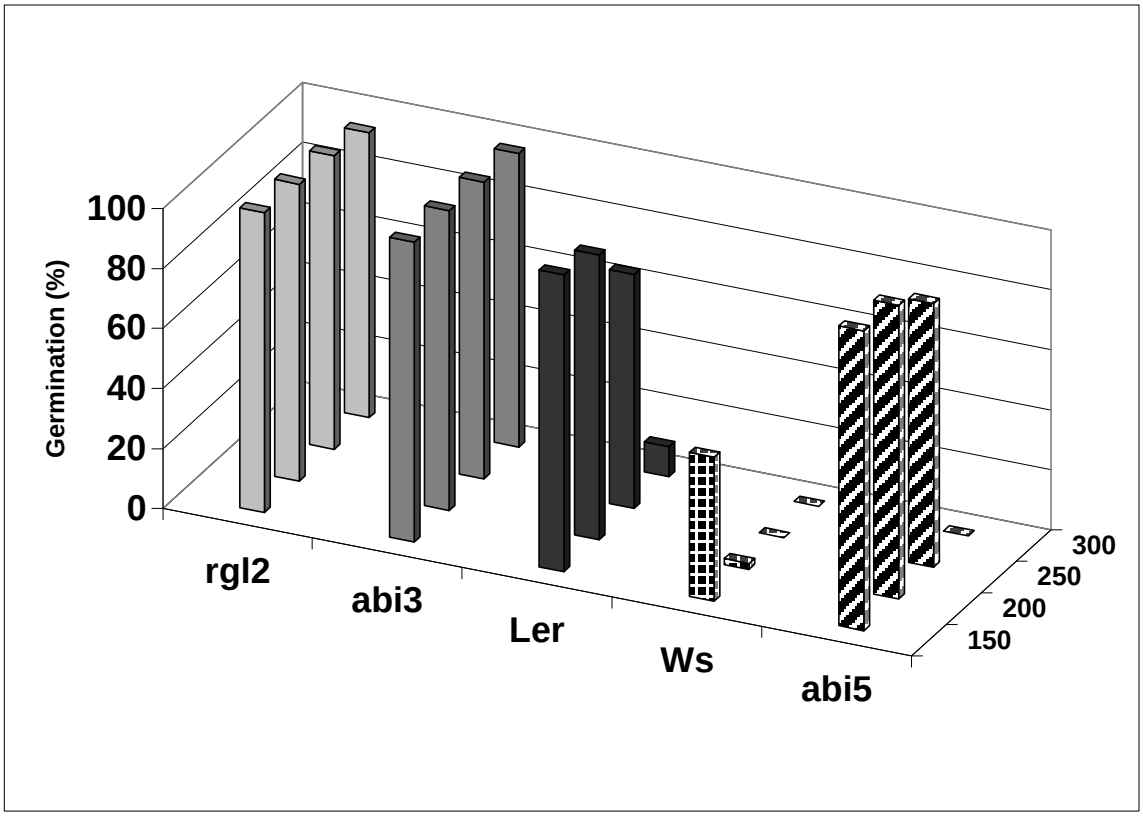


Fig.1B. Time course of *abi3-1*, *abi5-1*, *rgl2-1*, and WT (Ler and Ws) seed germination on 250mM sodium chloride. Time (days) after transfer to the growth chamber is indicated on the Z-axis.

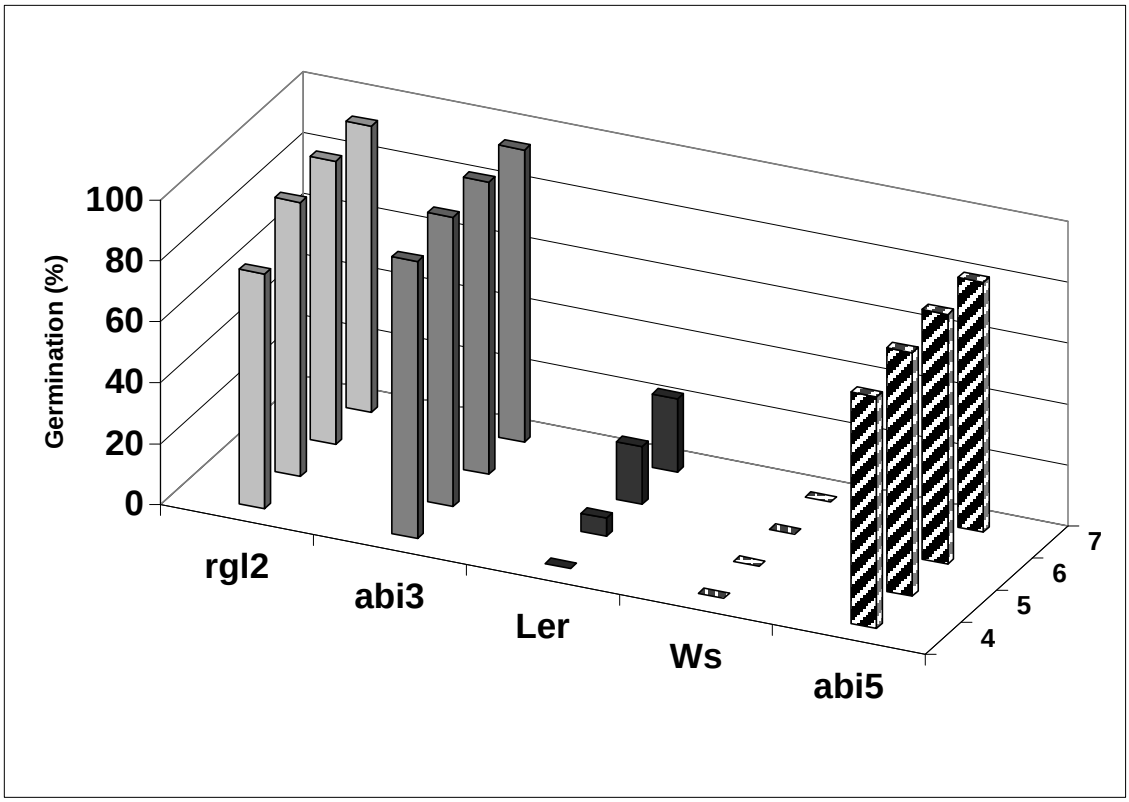


Fig. 1C. WT and mutant seed germination percentages, 14 days after transfer to growth chamber, on plates containing mannitol. Concentrations of mannitol (mM) are indicated on the Z-axis.

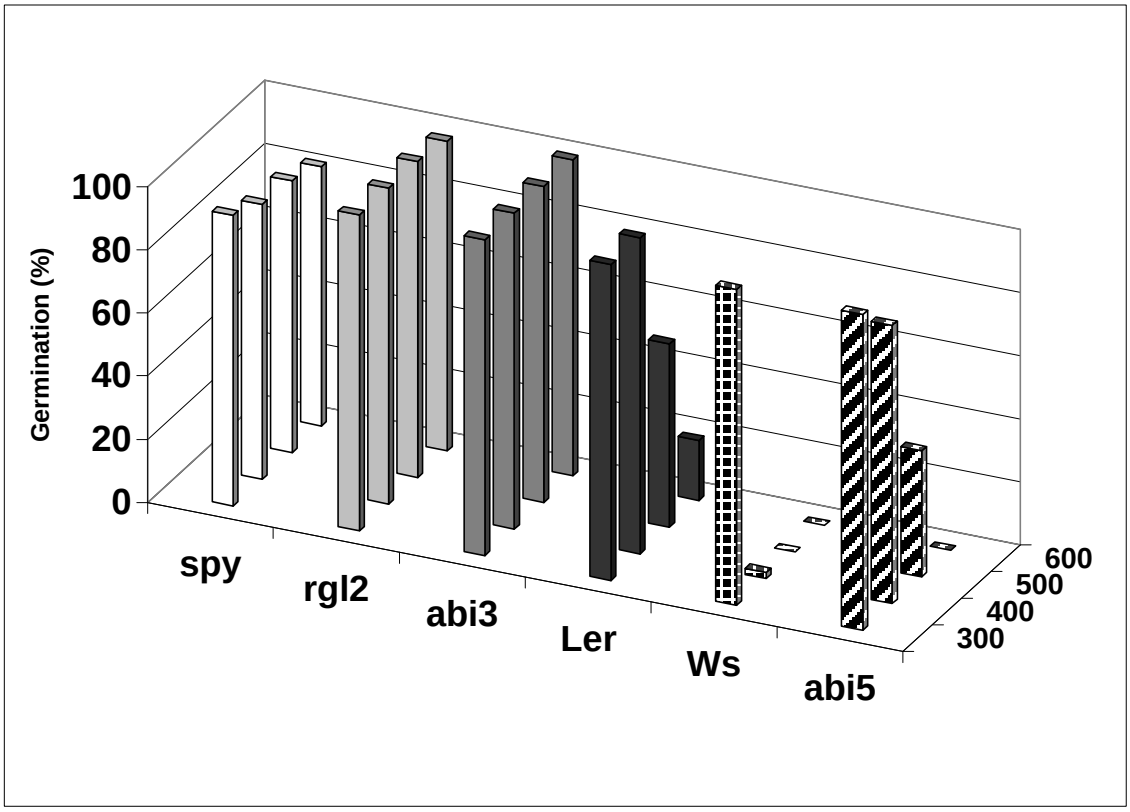


Fig. 1D. Time course of *abi3-1*, *rgl2-1*, and WT (Ler) seed germination on 400mM mannitol. Time (days) after transfer to the growth chamber is indicated on the Z-axis.

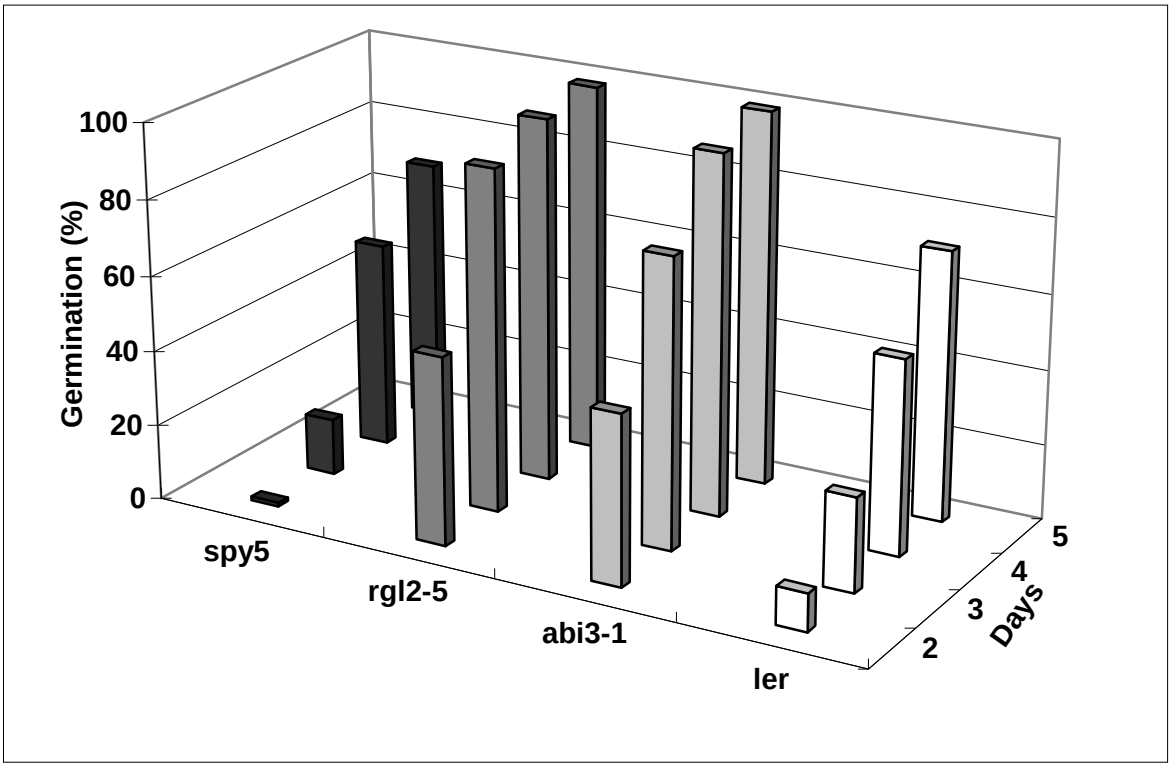


Fig. 2A. Completion of germination under salt stress. Percentage of WT and mutant cotyledon emergence, 14 days after transfer to a growth chamber, on 0.5MS containing different concentrations of sodium chloride. Concentrations of sodium chloride (mM) are indicated on the Z-axis.

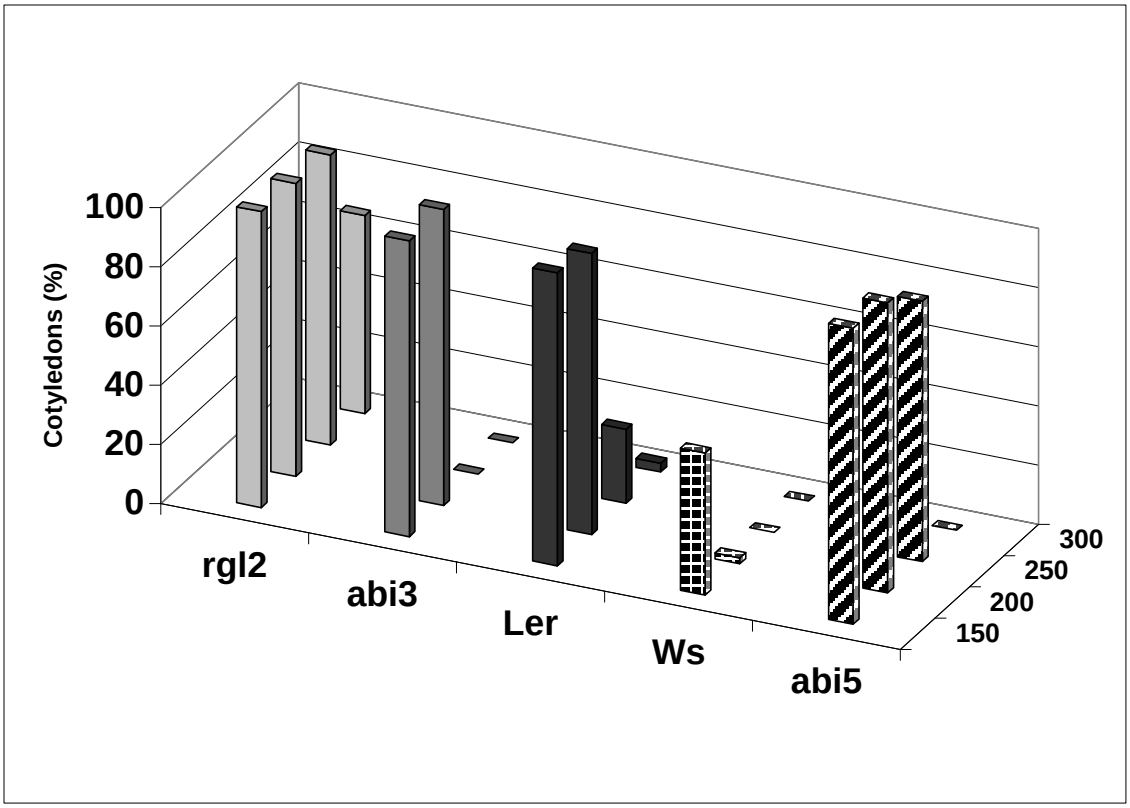


Fig. 2B. Percentage of WT and mutant seedling survival as determined by the presence of green cotelydons, 14 days after transfer to a growth chamber. Concentration of sodium chloride (mM) is indicated on the Z-axis.

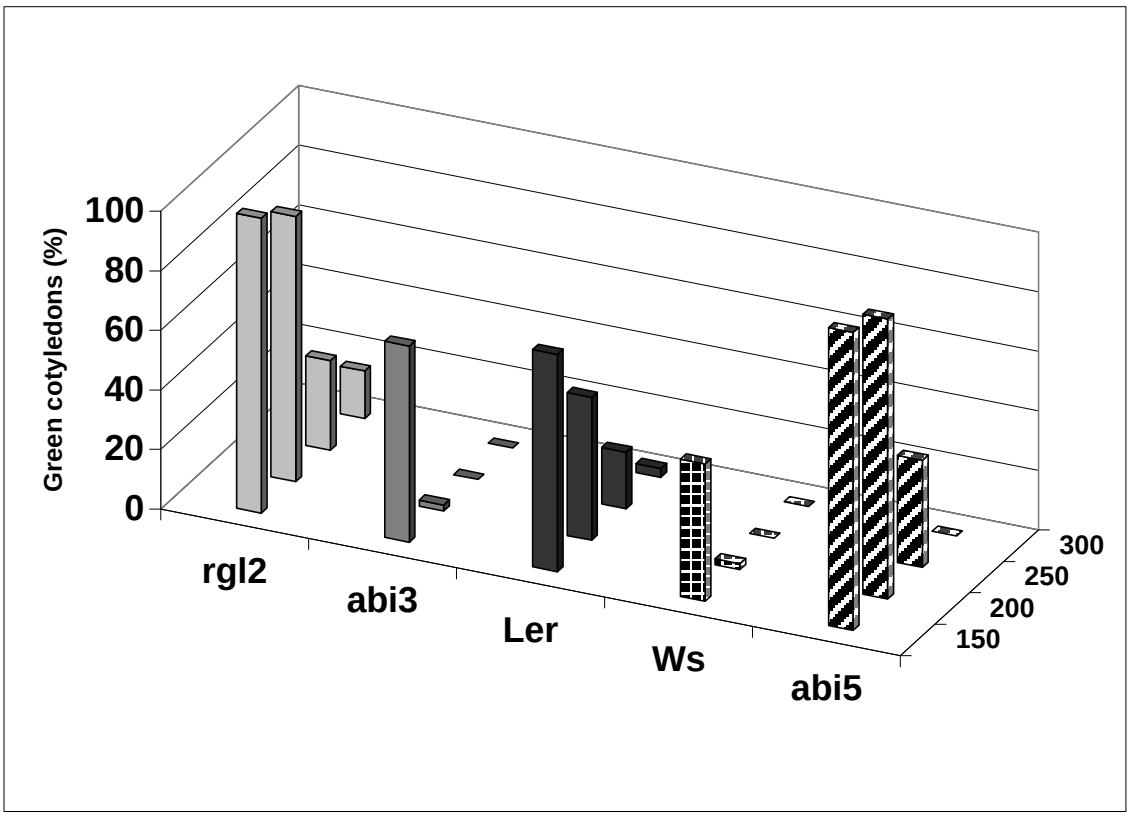


Fig. 2C. Root length of WT and mutant seedlings, on 0.5MS without salt. Time (days) after transfer to the growth chamber is indicated on the X-axis.

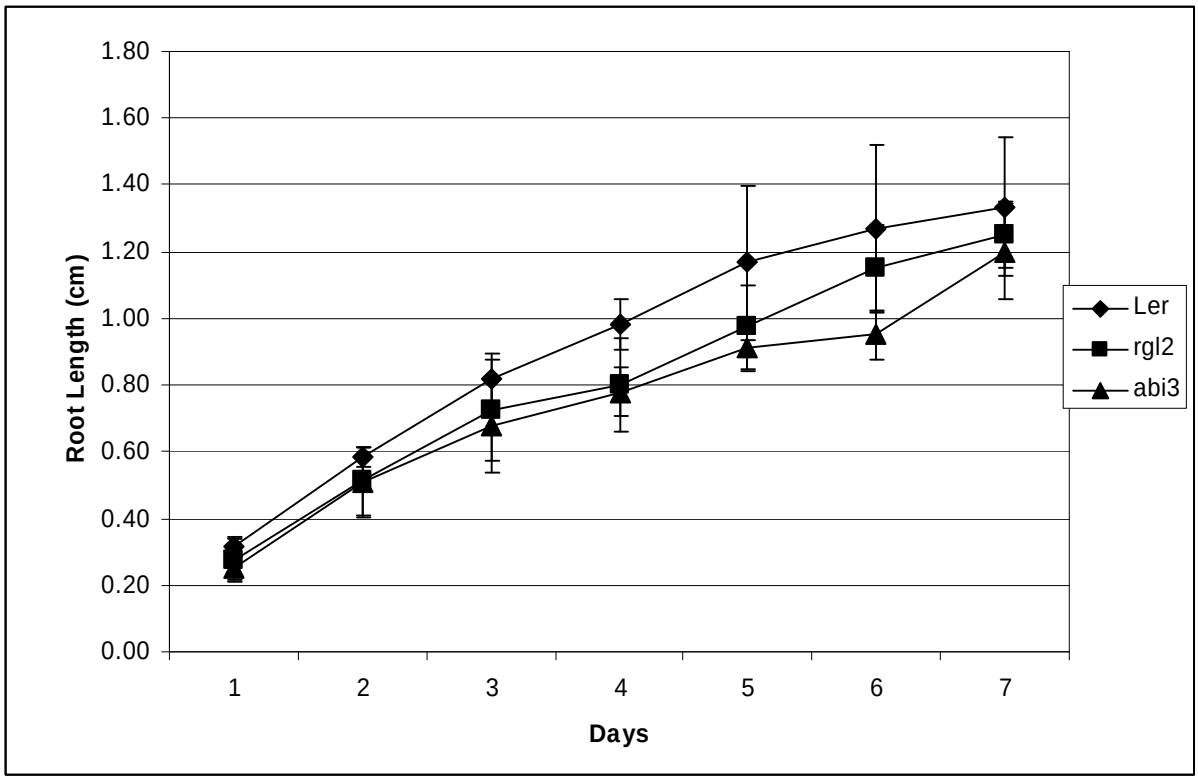


Fig. 2D. Root length of WT and mutants seedlings, on 0.5MS containing 50mM NaCl. Time (days) after transfer to salt plates is indicated on the X-axis.

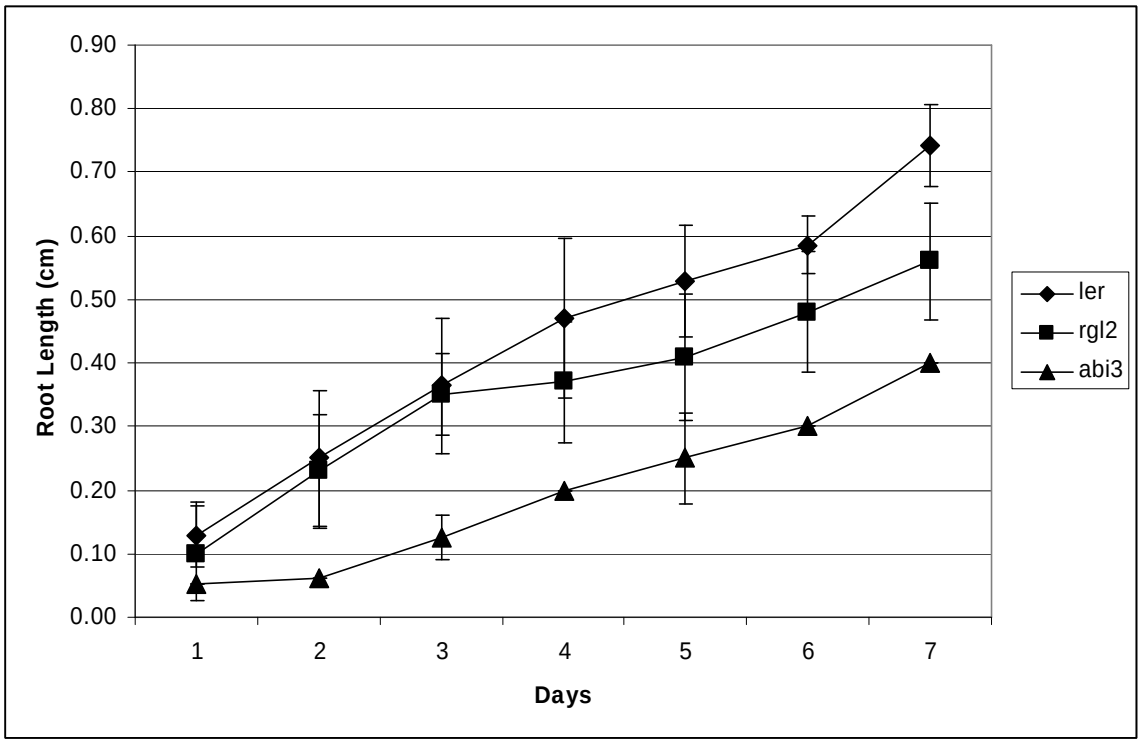


Fig. 2E. Root length of WT and mutants seedlings on 0.5MS containing 75mM NaCl. Time (days) after transfer to the salt plates is indicated on the X-axis.

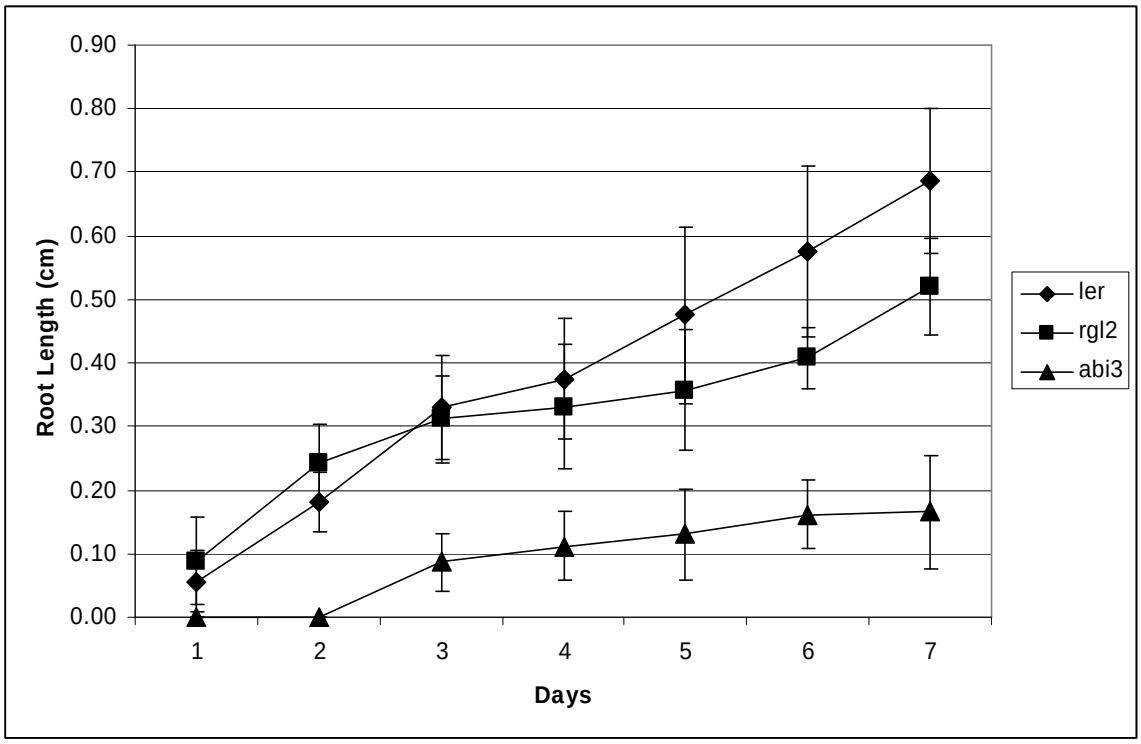


Fig. 3A. Completion of germination under osmotic stress. WT and mutant cotyledon emergence at day 14 after transfer to growth chamber on various concentrations of mannitol. Concentration of mannitol (mM) is indicated on the Z-axis.

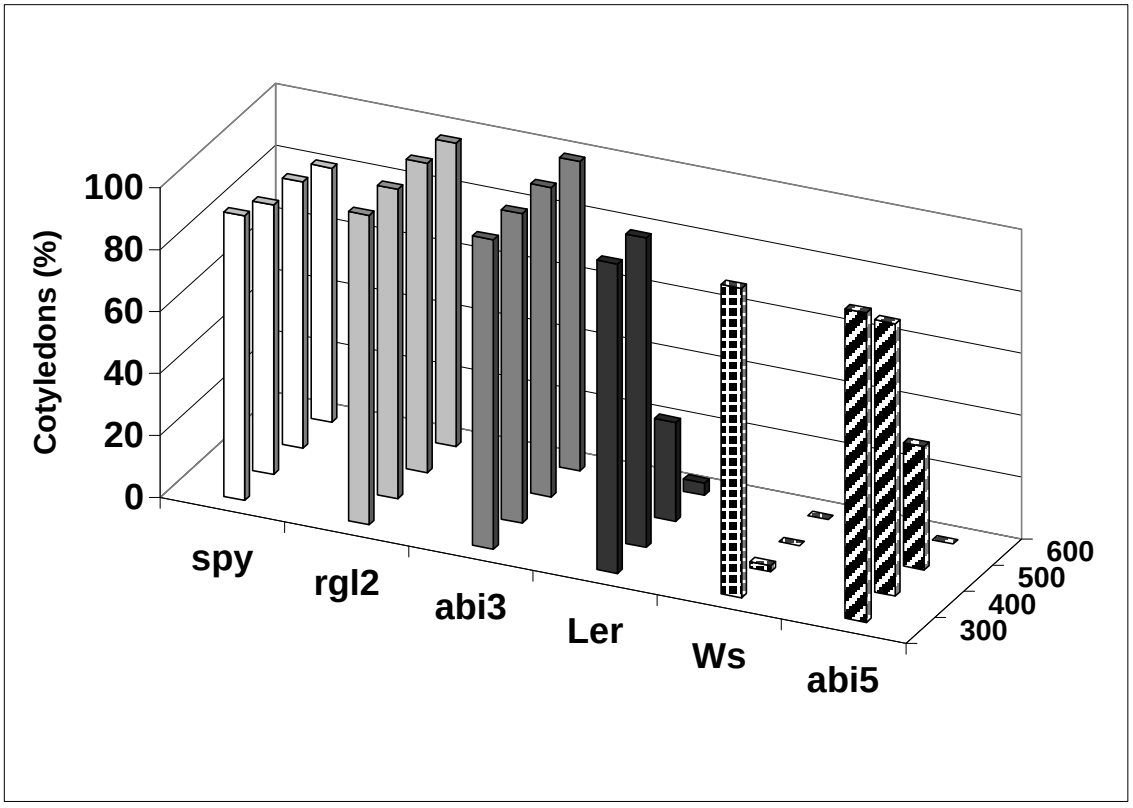


Fig. 3B. WT and mutant germinated seedling survival (presence of green cotyledon) at day 14 after transfer to growth chamber on various concentrations of mannitol. Concentrations of mannitol (mM) is indicated on the Z-axis.

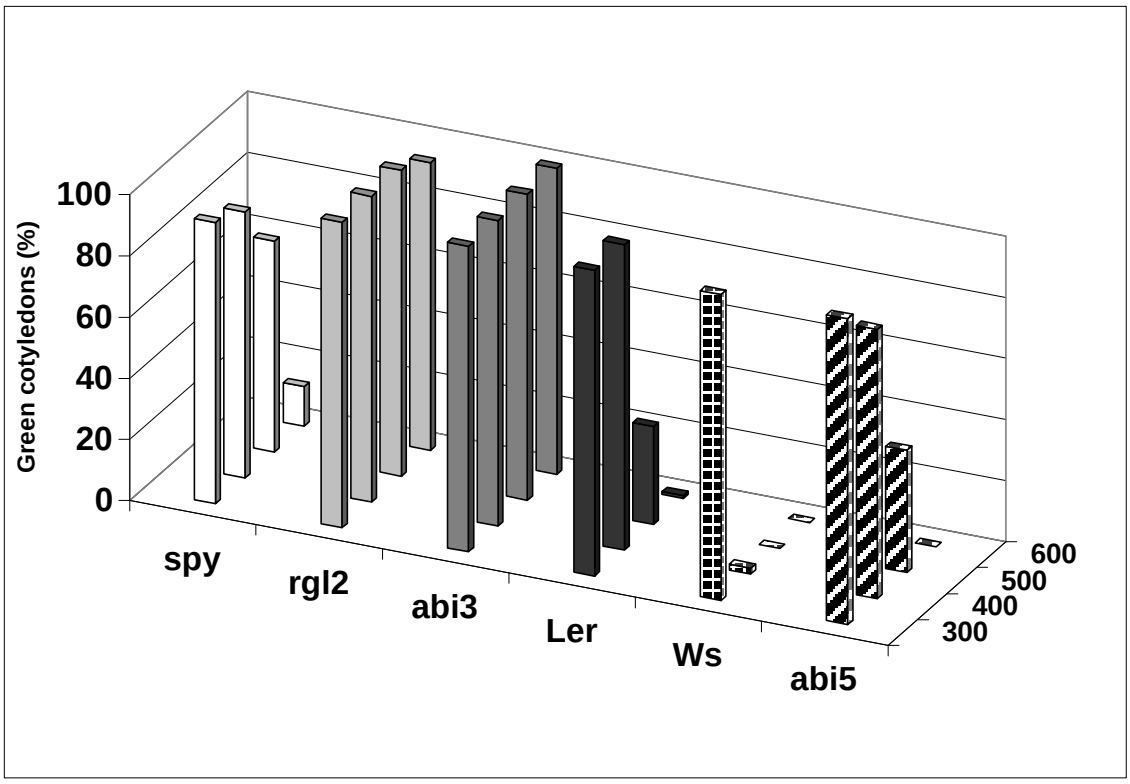
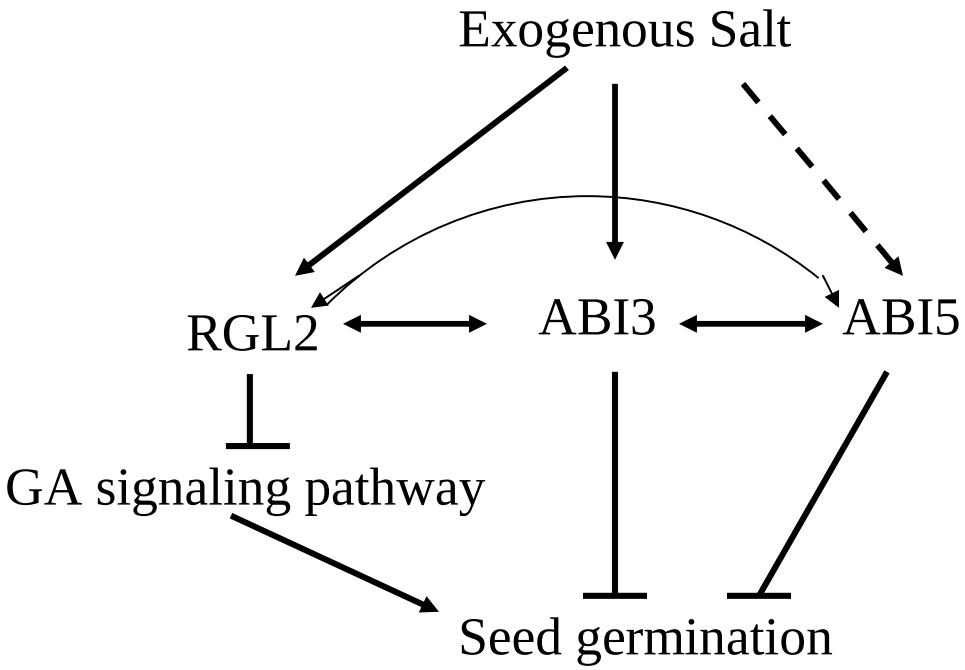


Fig. 4. Proposed model for interactions between salt, ABA and GA signaling pathways during seed germination. See text for details.



V. SEVERAL GENES IN GRAS FAMILY OF TRANSCRIPTION FACTORS ARE INVOLVED IN SATL STRESS TOLERANCE IN *ARABIDOPSIS*

Abstract

Exogenous salt inhibits seedling development when applied after germination. We have identified several mutants that are hypersensitive to salt during seedling development. Three genes in GRAS family, *SCARECROW* (*SCR*), *SHORT ROOT* (*SHR*) and *SCARECROW-LIKE 3 (SCL3)* are involved in the salt-induced inhibition of seedling development. Transcription of the *SCR* and *SHR* genes is up-regulated by salt. There are differences in stress tolerance between genotypes or differences in stress tolerance at different developmental stages of a single genotype. This study also demonstrated the interaction among GRAS family genes (*SCR*, *SHR* and *SCL3*) and components (*SOS1*, *SOS2* and *SOS3*) in SOS signaling pathway. The result indicated that *SCR* and *SHR* may play important roles in the salt-induced inhibition of seedling development via regulating transcription levels of SOS1, 2 and 3 in SOS signaling pathway.

Key words: Abscisic acid, *Arabidopsis*, Seedling development, Salt signaling, Salt-Overly-Sensitive (SOS) pathway.

Introduction

There are many environmental stresses that a plant may experience during its lifecycle such as extremes of temperature, drought and salinity. Soil salinity is one of serious environmental stresses reducing the productivity of plants. Soil salinity affects every stage of plant development from seed germination to mature plant growth. The growth of most plants is inhibited by the presence of high concentrations of soil sodium ions. Tolerance or susceptibility to salt stress is a very complex phenomenon. Most of the stress-signaling intermediates have not been identified. Because ABA and GA are key internal regulators of *Arabidopsis* plant growth it seems likely that salt exerts its inhibitory effects via the biosynthesis, degradation or signaling pathways for these hormones. Stress-responsive genes are regulated by both ABA-dependent and ABA-independent signaling pathways (Zhu, 2002). ABA biosynthesis is up-regulated by osmotic stress. The genetic studies on ABA-deficient mutants *los5/aba3* and *los6/aba1* of *Arabidopsis* indicated that ABA plays a crucial role in osmotic stress-regulated gene expression (Xiong *et al*., 2001; Xiong *et al*., 2002a). Our previous study showed that several genes in the ABA and GA signaling pathways are involved in the delay and inhibition of seed germination caused by salt stress.

Plant roots play important roles by taking up mineral nutrients from soil solutions. Plants have solute exclusion mechanisms to maintain low [Na^+] in shoots under salt stress based on anatomical mechanisms. Endodermal Casparian bands are highly effective to exclude charged solutes such as Na^+ (Steudle, 2000). Two main components of casparian bands are aliphatic and aromatic suberin. Aliphatic suberin is more hydrophobic and less

permeable to water. Caspariann bands prevent the passage of ions and big polar solutes. The endodermal tissue acts as an efficient barrier and builds up a Na^+ gradient between the root and the shoot.

The GRAS family (GIBERLLIC ACID INSENSITIVE (GAI), REPRESSOR of GA1 (RGA) and SCARECROW (SCR)) in *Arabidopsis* consists of at least 33 structurally related sequences (Bolle, 2004). The first GRAS family member isolated was SCR, which is involved in root and shoot radial patterning (Di Laurenzio *et al*., 1996; Wysocka-Diller *et al*., 2000). Another GRAS protein that is also involved in root and shoot radial patterning is SHORT ROOT (SHR) (Helariutta *et al*., 2000). The *scr* mutant seedling roots have only a single ground tissue cell layer in place of the two, cortex and endodermis, present in the WT (Di Laurenzio *et al*., 1996). This single remaining cell layer in *scr* roots expresses differentiated characteristics of both the cortex and the endodermis (Di Laurenzio *et al*., 1996, Wysocka-Diller *et al*., 2000). However, it is not clear whether this hybrid layer is fully functional as endodermis and cortex. In *shr* roots there is also a single ground tissue layer but this layer differentiates into cortex resulting in the complete deletion of root endodermis (Benfey *et al*., 1993; Helariutta *et al*., 2000). Given these structural defects in *scr* and *shr* root endodermis one might expect that these mutants would exhibit hypersensitivity to salt.

Another GRAS family member, *SCARECROW-LIKE 3 (SCL3)* has a tissue-specific expression pattern in the root similar to *SCR* (Pysh *et al*., 1999). Considering the expression pattern of *SCL3* as well as its sequence homology to *SCR* and *SHR* it seems likely that it may play a role in endodermal specification and/or function. *SCR* and *SCL3*

have been shown to be transcriptionally up-regulated by SHR (Levesque *et al.*, 2006). *SCL3* was suggested to be down-regulated by GA (Curtis *et al.*, 2005). However, phenotype of *scl3* has not been reported therefore its function was unknown.

Sodium is one of the nonessential ions in the soil. Sodium ions are abundant in saline soils. Regulation of cellular ion homeostasis during salinity stress is very important to increase salt tolerance of plants. The generation of a transient elevation of cytosolic Ca^{2+} and the subsequent activation of Ca^{2+} sensor protein expression and/or activity are the responses of plant cells to salt stress (Knight *et al.*, 1997). Much research has been directed toward understanding the molecular and cellular mechanisms of salt tolerance in plants, but most of the components and mechanisms involved in the plant's response to ionic stress remain unknown. However, the identification of components (SOS1, SOS2 and SOS3) in the Salt-Overly-Sensitive (SOS) pathway in *Arabidopsis* is a promising entry point for the elucidation of salt stress mechanism. The *sos1* (salt overly sensitive 1), *sos2* and *sos3* mutants were identified using a root-bending assay on NaCl-containing agar plates (Wu *et al.*, 1996; Zhu *et al.*, 1998). *SOS1* gene encodes the plasma membrane Na^+/H^+ exchanger (antiporter) (Shi *et al.*, 2000). *SOS2* gene encodes a serine/threonine type protein kinase which activity is required for salt tolerance (Liu *et al.*, 2000). The N-terminal kinase catalytic domain of *SOS2* is similar to that of the yeast Suc nonfermenting1 (SNF1) and AMP-activated (AMPK) kinases (Hardie, 1999). The N-terminal catalytic domain and the C-terminal regulatory domain of *SOS2* are essential for *SOS2* normal function. *SOS3* encodes a protein that acts as a Ca^{2+} sensor in the plant. The sequence of *SOS3* has a significant similarity with that of the calcineurin B subunit from

yeast and neuronal calcium sensors from animals (Liu and Zhu, 1998). SOS3 and SOS2 interaction has been shown in the yeast two-hybrid assay and the analysis of double mutant *sos3/sos2* suggested that *SOS2* and *SOS3* work in the same pathway (Halfter *et al*., 2000). *SOS2* and *SOS3* genes not only interact together but also regulate *SOS1* gene expression. *SOS1* gene expression level is low in wild type (WT) in the absence of salt but is up regulated, partially by SOS2/SOS3, during salt stress (Shi, 2000). SOS2 and SOS3 are also required for the activation of SOS1 Na^+/H^+ exchanger. Quintero *et al* (2002) reconstituted the SOS system in yeast cells and found that the SOS2/SOS3 kinase complex promoted the phosphorylation of SOS1. The co-expression of SOS1, SOS2 and SOS3 enhanced the salt tolerance of a yeast mutant that lacked Na^+ transporters. SOS2 protein activity partially phosphorylated SOS1 independently of SOS3, but SOS2 protein alone was not as efficient as the SOS2/SOS3 kinase complex for activation of SOS1 in vivo (Quintero, 2002). In addition, the increased salt tolerance in plants may be induced by the co-overexpression of SOS1, 2 and 3 (Guo *et al*., 2004). These results suggest that SOS1-3 all function in the same pathway to protect plants form salt toxicity.

In this study, we analyzed three alleles of *scl3* in order to deduce its function. We found that SCL3 is not required for normal radial patterning or for plant growth and development in the absence of environmental stress. SCL3 is important for salt tolerance during germination and seedling development. We have also looked at *scr* and *shr* responses to salt stress. We found that *SHR, SCR* and *SCL3* genes may all be involved in response to salt stress by interacting with each other and by affecting the expression of the components of the SOS pathway.

Results

SCL3, a component of GRAS family is important for conferring resistance to salt stress

We have obtained from the Arabidopsis stock center seeds of the T-DNA insertion line (*salk_002516)* potentially containing a T-DNA insert within the coding region of the *SCL3* gene. We called this line *scl3-1*. We tested *scl3-1* mutants for seed germination and the subsequent growth and development under various conditions. The *scl3-1* plants are indistinguishable from WT plants when grown on agar plates with or without sucrose or when grown in soil (not shown). The phenotypic differences between *scl-3* line and WT were observed only under salt stress.

In one set of experiments, two *scl3-1* lines (generated from two original seeds from the stock center) were tested for post germination responses to salt stress. WT and *scl3-1* seeds were germinated on agar plates without salt in the media. Newly germinated seedlings were then transferred to plates containing 120 mM NaCl. The roots of *scl3-1* seedlings showed three different phenotypes under salt stress conditions (Fig. 1A). Approximately a quarter of the *scl3-1* seedlings had long roots of similar length to WT controls (Fig. 1A – phenotype 3). The remaining seedlings, three quarters of the total, had roots much shorter than WT suggesting salt hypersensitivity. The root growth of approximately two thirds of shorter root seedlings showed partial sensitivity to salt stress. These seedlings had roots of an intermediate length (Fig. 1A - phenotype 2), between the WT length and the very short ones. The remaining *scl3-1* seedlings, approximately a quarter of the total, had very short roots (Fig. 1A - phenotype 1). These results suggest that the *scl3-1* lines used in these experiments are heterozygous for the mutation causing

salt sensitive phenotype. The partial salt sensitivity of half of the seedlings suggests incomplete dominance of this allele. To demonstrate that the salt oversensitivity is related to *SCL3* gene function we tested if the phenotype co-segregates with the T-DNA insertion in the *SCL3* gene.

Genomic DNAs were extracted from individual *scl3-1* seedlings that had different phenotypes under salt stress conditions (Fig. 1A). Primers were designed and synthesized for PCR analysis on genomic DNAs to detect the presence or the absence of T-DNA within the *SCL3* gene. For the detection of wild-type *SCL3* and mutant *scl3-1* allele, PCR reactions were performed using scl3F/LBb1 (*SCL3* forward/T-DNA), scl3R/LBb1 (*SCL3* reverse/T-DNA) and scl3F/scl3R (*SCL3* forward and reverse) primer combinations. Amplification with *SCL3* primers alone indicates the absence of T-DNA insertion in the sequence between these primers. Amplification with any combination of one *SCL3* primer, forward or reverse, and a T-DNA primer indicates the presence of T-DNA insert in or near the *SCL3* locus. Thus, the only amplification products in WT are generated with *SCL3* primers alone. We obtained a 1Kb product with SCL3 primers alone using template DNA from plants with phenotype 3 (Fig. 2B) and plants with phenotype 2 (not shown). Amplification product was generated with Scl3R/LBb1 primer combination in template DNA from plants with phenotype 2 (not shown) and phenotype 1 (Fig. 2B). It suggested that the partial salt sensitivity is caused by one copy of *scl3-1* allele and that plants with intermediate length roots (phenotype 2) are heterozygous for *scl3-1*. There were no amplification products with *SCL3* primers alone when we used DNA from plants with phenotype 1 (Fig. 2B). The presence of T-DNA containing *SCL3* allele and the

absence of wild-type *SCL3* allele in plants with very short roots (phenotype 1) indicates that these plants are homozygous for *scl3-1*. PCR fragments amplified with scl3R/LBb1 primer combinations were used for sequencing to identify the precise insertion point of T-DNA in *scl3-1* allele. Sequencing analysis indicates that T-DNA is inserted in the *SCL3* coding region and the T-DNA insertion point is at position 543 just 306 bases downstream of the start codon (Fig. 2A). The data shows that if the *scl3-1* derived mRNA was translated a truncated protein would be produced (Fig.2C). The truncated protein would be composed of only 102 normal amino acids, out of the normal 482, fused to two new ones encoded by the T-DNA (Fig. 2C). Because this represents less than a quarter of the normal SCL3 product this suggests that the truncated protein is not functional.

In order to further confirm the function of SCL3 in salt tolerance, we analyzed phenotypes of other potential alleles of *SCL3, scl3-2* and *scl3-3,* under salt stress. The seedling growth of *scl3-2, scl3-3* and WT is shown in Fig. 1C. The *scl3-2* and *scl3-3* mutant seedlings have similar phenotype to that of WT seedlings when grown on control plates without salt (not shown). *scl3-2* and *scl3-3* did not show the same level of sensitivity to salt as *scl3-1* allele. *scl3-2* mutant showed partial sensitivity to salt stress (Fig 1C). The phenotype of *scl3-3* mutant is similar to WT when grown on 120mM NaCl. To demonstrate the relationship between plant phenotypes under salt stress and T-DNA insertion positions, genomic DNAs were extracted from individual *scl3-2* and *scl3-3* seedlings. To detect the presence of T-DNA in *scl3-2* and *scl3-3* the same primer combinations were used as for *scl3-1* described above. The PCR diagnostic test results are shown in Fig. 2D. The 1Kb amplified fragments were detected with scl3F/scl3R

primer pair with both *scl3-2* and *scl3-3* DNAs. It indicated that there is no T-DNA in either *scl3-2* or *scl3-3* allele between the forward and the reverse primer sequences. In addition, the *SCL3* forward primer (scl3F) combined with one of the T-DNA primers (LBb) were the primer pair that yielded amplification products with DNA from both alleles. An amplification product about 1.5 Kb in length was detected in DNA template from *scl3-3* plants with scl3F/LBb primer combination. The size of the fragment suggests that T-DNA insertion point in *scl3-3* is located beyond the coding region of *SCL3* gene (Fig. 2A). An amplification product detected with DNA template from *scl3-2* plants with scl3F/LBb1was larger than 1.5 K indicating that the T-DNA insertion in *scl3-2* is even further downstream than the one in the *scl3-3*. PCR fragments amplified with DNA templates from *scl3-2* and *scl3-3* plants with scl3F/ LBb1 primer pairs were used for sequencing to identify the precise insertion point of T-DNA in each *SCL3* allele. The sequence analysis indicates that the T-DNA insertion points in both *scl3-2* and *scl3-3* alleles are within the 3' UTR. The T-DNA insertion point of *scl3-3* is at base position 1753 and that of *scl3-2* is at 1920 (Fig. 2A). Although, both alleles contain inserts beyond the coding region of *SCL3* gene they differ in their phenotype. The T-DNA in *scl3-2* is even further downstream than the insert in the *scl3-3*. However, *scl3-2* is a stronger allele. The T-DNA insertion in *scl3-2* may interfere with a polyadenylation signal that may lead to transcript instability in this allele. The finding that the position of T-DNA in each *scl3* allele correlates well with the phenotype confirms the conclusion that *SCL3* gene is involved in salt tolerance.

Two other genes in GRAS family, *SCR* and *SHR* are involved in root development and have similar root expression patterns to SCL3 (Pysh *et al*., 1999). To see if SCR and/or SHR may be involved in salt tolerance we tested the mutants for salt sensitivity. The roots of *scr1* and *shr1* mutants are much shorter than those of WT even on control plates. The *scr1* and *shr1* mutant roots stop growing after achieving lengths of up to 2cm and 1cm respectively. Therefore, it is difficult to compare the root growth of *scr1* and *shr1* mutants and those of WT directly. WT, *scr1* and *shr1* seeds were germinated on agar plates without salt in the media. Newly germinated seedlings were then transferred to plates containing 120 mM NaCl. The root growth of *scr1* and *shr1* seedlings under salt stress is not significantly different from the two mutant seedling growth on control plates containing no salt (data not shown). These findings suggest that *scr1* and *shr1* are not hypersensitive to salt stress.

Transcription of genes involved in plant salt tolerance

The mutants of three genes, *SOS1, SOS2* and *SOS3* in SOS signaling pathway have salt hypersensitive phenotypes. We have identified another gene involved in salt tolerance. The *scl3* mutants show salt hypersensitive phenotypes similar to *SOS* mutants. In our study, we also analyzed *scr* and *shr* mutants that have the defects in root architecture affecting the structure of endodermis. However, in spite of these defects *scr* and *shr* do not seem to be salt hypersensitive. To investigate potential roles of SOS1, SOS2, SOS3, SCL3, SCR and SHR in salt tolerance we analyzed their expression under stress conditions and on mutant backgrounds by Real Time PCR. First, we compared

mRNA levels corresponding to all six genes in WT 10-day roots imbibed in 200mM NaCl to roots imbibed in H_2O alone for 12 hours. We tested transcription levels of these genes in three different WT ecotypes, Ws, Col and Ler. The expression levels of all genes except SCR are similar in these three different ecotypes. SCR expression levels increased six fold in Ler background and twelve fold in Ws background under salt stress (Fig. 3A). The expression of only two genes, SCR and SHR, are induced by salt under conditions used in our experiment. SCR mRNA level is up-regulated by salt stress at least five fold depending on the ecotype (Fig. 3A). SHR mRNA level increases at least four fold in the presence of salt. This indicates that sodium chloride induces SCR and SHR gene expressions at the transcriptional level. SOS1, SOS2, SOS3 and SCL3 gene expression levels did not change in the WT roots under salt stress as compared to WT roots under non-stress conditions. Our SOS1, SOS2 and SOS3 expression data is inconsistent with data from previous studies that showed that SOS1, SOS2 and SOS3 expression in the roots is up-regulated by salt stress (Liu *et al*., 2000; Shi *et al*., 2000; Gong *et al*., 2001). This discrepancy may be due to the differences in experimental conditions used in the two studies. We imbibed roots for 12 hours in 200mM salt instead of 6 hours. Our data suggest that salt may inhibit root growth via transcriptional induction of both *SCR* and *SHR* genes.

SCR, SHR and SCL3 gene products are transcription factors. It is possible that they regulate the expression levels of genes that are directly involved in salt signaling pathway. SOS1, SOS2 and SOS3 in SOS signaling pathway may be their potential targets. The *SCL3* transcription level may also be regulated by SCR or SHR since *scl3* mutants

show salt hypersensitive phenotypes similar to *SOS* mutants. To investigate the interactions among these genes under normal conditions, we analyzed the gene expression levels in the mutant backgrounds under non-stress conditions. The *SOS1*, *SOS2* and *SOS3* transcription levels were analyzed in *shr*, *scr*, *scl3* and WT 10 days old roots after roots imbibed in H_2O at 4 °C in the dark for 12 hours. There were no significant differences of *SOS1*, *SOS2* and *SOS3* gene expressions in *shr*, *scr* or *scl3* mutant backgrounds under normal conditions (Fig. 3B). The *SCR, SHR* and *SCL3* transcription levels in mutants are similar to WT in the absence of stress (Fig. 3B). This suggests that there is no interaction among these genes under normal conditions.

To further investigate the potential interactions among *SCR, SHR* and *SCL3* genes and/or their potential role in the regulation of *SOS1, SOS2, SOS3 and SCL3* expression we compared transcription levels in mutants and WT under salt stress conditions by Real Time PCR. The *shr*, *scr*, *scl3* and WT 10-day roots were imbibed in 200mM sodium chloride at 4 °C in the dark for 12 hours. The transcription levels of *SOS1* were most significantly altered in the three mutants in response to salt. The *SOS1* expression is twelve fold higher in *shr* and two fold higher in *scr* relative to WT roots under salt stress. In contrast, the expression of *SOS1* in *scl3* is four fold lower than in WT roots (Fig. 3C). These results suggest that SHR and perhaps SCR negatively regulate the *SOS1* gene expression under salt stress. However, SCL3 has an opposite effect and seems to positively control the *SOS1* transcription. The *SOS2* mRNA levels in *shr* and *scr* increased five-fold relative to WT (Fig. 3C). The *SOS3* mRNA levels in *shr* and *scr* increased ten-fold and five-fold respectively as compared to WT (Fig. 3C). These results

suggest that SHR and SCR also down-regulate *SOS2* and *SOS3* expression under salt stress. SHR may regulate *SOS2* and *SOS3* gene expression indirectly through up-regulation of *SCR*. The expression of *SCR* in *shr* background is seven fold lower under salt stress (Fig. 3C). There were no significant differences in *SOS2* and *SOS3* expression in *scl3* mutants. These results suggest that SHR and SCR may down-regulate *SOS2* and *SOS3* gene expression under salt stress but SCL3 is not involved in regulation of SOS2 and SOS3 gene expression. The *SCL3* expression is increased five fold in *shr* background suggesting that SHR down-regulate *SCL3* expression under salt stress.

Components of GRAS family may be involved in seed germination under salt stress

The *shr1* and *scr1* mutants have abnormal root architecture but do not seem to be hypersensitive to salt during seedling development. The *scl3* mutant is hypersensitive to salt during seedling development. We investigated the initiation of seed germination kinetics of these mutants on plates containing intermediate concentrations of sodium chloride. The initiation of seed germination is demonstrated by radical emergence. We tested mutant *scl3-1* that shows a similar phenotype as the WT plant under the normal conditions. Germination of wild type (WT) and *scl3* seeds in sodium chloride-free control plates approached 100% after 72 h (Fig. 4A). However, *scl3-1* mutant is hypersensitive to the moderate concentration of salt during seed germination (Fig. 4). Germination of WT approached 93% on day 7 on 200mM salt plates while only 74% of *scl3* mutant seeds germinated (Fig. 4A). The dramatic increase of germination frequency of Col begins on day 3 but the germination frequency of *scl3* increases dramatically 2 days later on

200mM NaCl (Fig. 4A). These data indicate that *scl3* mutant is more salt sensitive than Col during germination. The mutant seeds of *shr1* and *scr1*, all have a significantly higher germination rate than WT on 200mM NaCl plates (Fig. 4B). Germination of *scr1* and *shr1* approached 71% and 82% respectively on day 7 on 200mM salt plates while only 25% of Ws seeds germinated. Our data suggest that all three GRAS family genes, *SCR1, SHR1* and *SCL3* are involved in seed germination under salt stress.

Discussion

SCR and SHR are transcriptional regulators belonging to GRAS family (Bolle, 2004). Both proteins are localized to root endodermis where they form heterodimers and regulate transcription of target genes (Cui *et al*., 2007). The *scr* and *shr* mutants have defects in root architecture affecting the structure and possibly the function of endodermis. The defective endodermis of *scr* and *shr* may affect Na^+ transport between root and shoot. Given these structural defects of *scr* and *shr* mutants we expected that they would exhibit hypersensitivity to salt. However, our results suggest that *scr* and *shr* are not salt hypersensitive after germination and that they may be slightly salt resistant during germination. These findings suggest that there must be some mechanism to compensate for the presence of the defective endodermis in these mutants.

DELLA proteins in GRAS family are all repressors of GA signaling pathway (Zentella *et al*., 2007). These proteins may also integrate signals from other hormone signaling pathways and environmental cues to inhibit seed germination and restrict seedling growth (Achard *et al*., 2003, 2006). SCL3 is in the GRAS family but it is not a

DELLA protein. The function of SCL3 has not been reported. There is evidence for the expression of *SCL3* being controlled by both GA and DELLA transcription regulators (Zentella *et al*., 2007). Our study identified the function for SCL3 in plant salt stress tolerance. We have shown that *scl3* mutant alleles are salt hypersensitive both during germination and also during seedling growth. *SCL3* has a tissue-specific expression pattern in the root similar to *SCR* (Pysh *et al*., 1999). Therefore, it was suggested that SCL3 may play a role in the specification and/or function of root endodermis (Pysh *et al*., 1999). However, our data indicates that SCL3 may not have a function in root development and/or growth under non-stress conditions.

Our data indicates that SCR, SHR and SCL3 are all involved in salt tolerance. These three proteins are transcriptional regulators. Therefore their primary function is to regulate the transcription of downstream targets that would have a direct function in stress response. Genes that are associated with abiotic stress response can be divided into four groups based on their gene product function: osmolyte biosynthesis, antioxidant protectants, protection of cell integrity and ion homeostasis (Nakajima *et al*, 2001; Valliyodan and Nguyen, 2006). Regulation of cellular ion homeostasis is important for plant salt tolerance. The Salt-Overly-Sensitive (SOS) pathway in *Arabidopsis* has been well-characterized (Chinnusamy *et al*., 2004). The components in SOS pathway, SOS1, SOS2 and SOS3, can transduce a salt stress-induced Ca^{2+} signal to reinstate cellular ion homeostasis (Zhu, 2002). In our study we investigated a possibility of an interaction between SCR, SHR and/or SCL3 transcription regulators and the components of SOS pathway in salt stress responses of seedling roots. Our results suggest that salt may inhibit

root growth by inducing *SHR* and/or *SCR* gene expression first. Salt may up-regulate *SCR* expression both directly and/or via SHR. Our data on *SOS1, SOS2* and *SOS3* transcription in *shr* and *scr* mutants under salt stress indicate that SHR and SCR down-regulate transcription of *SOS1*, *SOS2* and *SOS3*. These results may explain why *shr* and *scr* mutant seedlings do not have salt-sensitive phenotypes in spite of the defective root morphology. The endodermal casparian bands in *shr* and *scr* mutants are missing and/or are defective thus the movement of solutes such as Na^+ may not be effectively controlled by the abnormal endodermis. This defect is compensated for by the overexpression of SOS genes in *scr* and *shr* backgrounds under stress conditions. In the absence of SCR and/or SHR *SOS* gene expression is no longer repressed. Additional SOS1, SOS2 and SOS3 proteins in mutants can transduce a salt stress signal and reinstate cellular ion homeostasis to provide the protection from salt. In conclusion, the protection provided by SOS gene products compensates for the endodermal defects in *scr* and *shr* roots. So the phenotypes of *shr* and *scr* mutants under salt stress are similar as under non-stress conditions. The function of SCL3 in salt tolerance can be partially explained by our results linking SCR/SHR/SCL3 and SOS expression under salt stress. SCL3 expression seems to be down-regulated by SHR. In addition, SCL3 up-regulates SOS1 expression. Combining all these data together suggests that SCL3 mediates salt tolerance by increasing SOS1 transcription. Our data also, indicates that SHR down-regulates SCL3 under salt stress thus indirectly leading to decreased SOS1 transcription. Thus, in *shr* background SOS1 may be overexpressed because there is no repression by SHR and/or there is stimulation by overexpressed, no longer repressed by SHR, SCL3. These data are

in agreement with previous findings that SHR directly controls *SCR* transcription and that SHR controls *SCR* and *SCL3* transcription via binding to their promoters (Levesque *et al*., 2006). Our data suggests a very complex mechanism of gene expression regulation by SCR/SHR and SCL3 which is supported by recent finding that SCR is required for SHR to regulate some of its targets (Cui *et al*., 2007).

Based on the expression data of GRAS and SOS genes we have built a working model for the gene interaction under salt stress. Figure 6 depicts our model. According to this model, salt induces *SHR* and *SCR* gene expression either directly or indirectly or both. SHR also up-regulates *SCR* and down-regulates *SCL3*. In addition, SHR and/or SCR in turn down-regulate *SOS1*, *SOS2* and *SOS3* gene expression. SCL3 stimulates SOS1 expression. The levels of SCR, SHR and SCL3 products result in the regulation of SOS pathway via repression and/or induction of transcription of its components.

Our most recent findings indicate that *scr1* and *shr1* mutant seeds are resistant to salt stress during germination. In addition, SCL3 may also have a function during germination under salt stress. The *scl3* seeds are salt hypersensitive. Further experiments are required to investigate the role of GRAS genes during germination.

Materials and methods

Plant materials

Three ecotypes of *Arabidopsis thaliana* were used in this study: Columbia (Col), Wassilewskija (Ws) and Landsberg erecta (Ler). Various *Arabidopsis* mutants were obtained from *Arabidopsis* stock centers. *scl3-1(salk_002516), scl3-2(salk_023428),*

scl3-3(salk_099576), *35s::scr*, *abi3-1* and *abi5-1* came from the *Arabidopsis* Biological Resource Center at Ohio State University, (http://www.arabidopsis.org/abrc/) and *rgl2-1* (SGT625, Lee *et al*., 2002) came from the Nottingham *Arabidopsis* Stock Center (http://arabidopsis.info/).

Germination and root growth assays

Before plating, seeds were sterilized in 6.25% Sodium hypochlorite for 3 min and rinsed three times with sterile distilled water. Seeds were planted on 0.5X Murashige and Skoog medium (0.5MS, Caisson Laboratories) solidified with 0.8% agar (Fisher Scientific) and containing varying concentrations of salts or 75mg/l kanamycin. Filter sterilized Kanamycin stock solution was added after autoclaving to avoid inactivation of the antibiotic. *Arabidopsis* seeds were stratified for 3 d at 4°C in dark, then placed in a growth chamber with 70% humidity and under 18h light and 6h dark at 23°C to facilitate germination. Germination (based on radicle emergence from the seed coat) was scored daily. Each plate contained 90–110 seeds. Every experiment was repeated two to three times. In each experiment different batches of seeds were used. However, in each experiment WT and mutant seeds used were collected at the same time from the same age plants grown under the same conditions. Although time course of germination differed in each experiment the trends in germination were the same for different batches of seeds. The data shown here are from one of these experiments.

Seeds were planted on 0.5X Murashige and Skoog medium (0.5MS, Caisson Laboratories) solidified with 0.8% agar (Fisher Scientific) and 3% sucrose and were

covered with a piece of biomembrane. The biomembrane with four-day-old seedlings were transferred to 0.5MS medium containing 0.8% agar and indicated NaCl concentrations. The plates were inverted and placed on racks vertically. The root lengths were measured at day 12 after transferring to salt plates. Seed germination and root growth data were analyzed using Microsoft Excel 2000. For germination tests, seeds collected at the same or similar times were used and stored at 4°C before using.

DNA isolation and PCR

Genomic DNA was extracted from 2-week-old seedlings of several *scl3* allels mutants. The DNA isolation method is as following:

PCR analysis of T-DNA insertions was performed using left border (LB) primer and SCL3 forward and reverse primers. The primers sequences were: LBb1 of pBIN-pROK2 for SALK lines 5'GCGTGGACCGCTTGCTGCAACT3' (http://signal.salk.edu/tdnaprimers.html); SCL3 forward 5'GCCTTCAATGGTGGCTATGT3', reverse 5' CCGAAGAGCATCTTCTCCAC3'. Thermocycler conditions were as following: stage 1 has 10 cycles with 1 min at 94°C, 1min at 58°C and 2min at 72°C, stage 2 has 30 cycles with 20seconds at 94°C, 30 seconds at 58°C and 2min at 72°C, stage 3 has 1 cycle with 10min at 72°C. After confirmation of T-DNA insertion and insertion direction in *scl3-1*, a PCR amplification product with the following primers was sequenced (Genomics & sequencing laboratory, Auburn University). SCL3 reverse 5' CCGAAGAGCATCTTCTCCAC3' paired with left border reverse 5'GCTTGCTGCAACTCTCTCAG3'. Thermocycler conditions were the

same as shown above. Two PCR amplification product from *scl3-2* and *scl3-3* with the SCL3 forward and LBb1 primer pairs were sequenced. T-DNA primers were designed with program from http://signal.salk.edu/tdnaprimers.html and other primers were designed with program on http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.

RNA isolation and Real time PCR

For mRNA expression analyses ten-day-old seedlings were imbibed in 0.5MS medium or 0.5MS medium with 200mM NaCl for 12 hours in the dark at 23°C. Shoots and roots were separated after imbibition. Total RNA was extracted from these treated shoots and roots with RNApure kit (Genehunter Corporation) for use in QRT-PCR. RNA extraction was performed as described in the instruction of the manufacture (Genehunter Corporation).

Total RNA (2 µg) was treated with a DNA-*free* kit (Ambion INC.) and tested with real time PCR for DNA contamination. The purified total RNA was used as a template to synthesize first-strand cDNA using a TaqMan Reverse Transcriptase kit (Roche) with oligo (dT) primers as per the manufacturer's instructions. Thermocycler conditions were 10min at 25°C, 30min at 48°C, 5min at 95°C and hold temperature is 0°C. Quantitative real time PCR using first-strand cDNA as a template was carried out using an ABI Prism 7000 Sequence Detector with TaqMan Universal PCR Master Mix (Roche). PCR reactions were carried out in a final volume of 50 µl using gene specific primers and probes in concentrations determined individually for each set of primers. Probes were modified with 6-FAM, reporter dye at 5'-end and TAMRA quencher at 3'-

end. All oligo synthesis and modifications were done by Sigma-Genosys. Gene specific primers and probes used were as follows: APT1 forward 5'TGTTCCTTGCAACCGTCTTCT3', reverse 5'TGGTTGAACGGTGGTTTGAG 3', probe 5' CCACCACCGTGCTCCTCCTTCG3'; SCL3 forward 5' GCGGGTGCGCAGTAATTT 3', reverse 5' TTCCTGCATCTCCAAGCTGAT 3', probe 5' TGGCAAGATCGACCTCTATACTCGG 3'; SOS1 forward 5' ACCACTTTCCTTTTGACAGAAACG3', reverse 5' TCAGCAGGTCCTAGCTCCTCAT 3', probe 5' AGGCCTTACGAGCGTTTCAAGATCTAGGAGA3'; SOS2 forward 5' CAAGACAAGGCTCGAGGGATTA3', reverse 5' GCCACCTCGTAAATCTCTATCACA3', probe 5' CTTCGATCAAGGCCGGACAGTTAGCTG3'. SOS3 forward 5' GCGAAATGGAGTGATCGAGTTT3', reverse 5' GCGCGCTTGGATGGAA3', probe 5' TGAATTTGTCCGGTCCTTAGGTG3'. SHR forward 5' GGAGCAATCTTGGAAGCAGTAGAC3', reverse 5'CGGCCATTGAGTGCAAAAC3', probe 5' CAAAGATCCACATCGTTGACATAAGCTCCA3'. SCR forward 5' ATGCACCACCGCAACCA3', reverse 5' CTCCGCCGTATTTGTTTGGA3', probe 5' AGACAGTGACGGCCACTGTTCCC3'. Thermocycler conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15s at 95°C and 1 min at 60°C. The mRNA level for each gene was determined using the standard curve method according to manufacturer's instruction (ABI Prism 7000 Sequence Detection System User Guide). APT1 (Adenosine phosphoribosyl transferase) transcript level in each sample was used as an internal

control (Arroyo *et al*., 2003). The mean value from triplicate samples was used to calculate the transcript level. Results were analyzed using Microsoft Excel.

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Fig. 1A. Three root phenotypes of seedlings from heterozygous *scl3-1*. 12-day seedlings grown on 120mM NaCl are shown for each phenotype category. Phenotype 1: shortest root seedlings; Phenotype 2: intermediate root seedlings; Phenotype 3: WT length root seedlings.

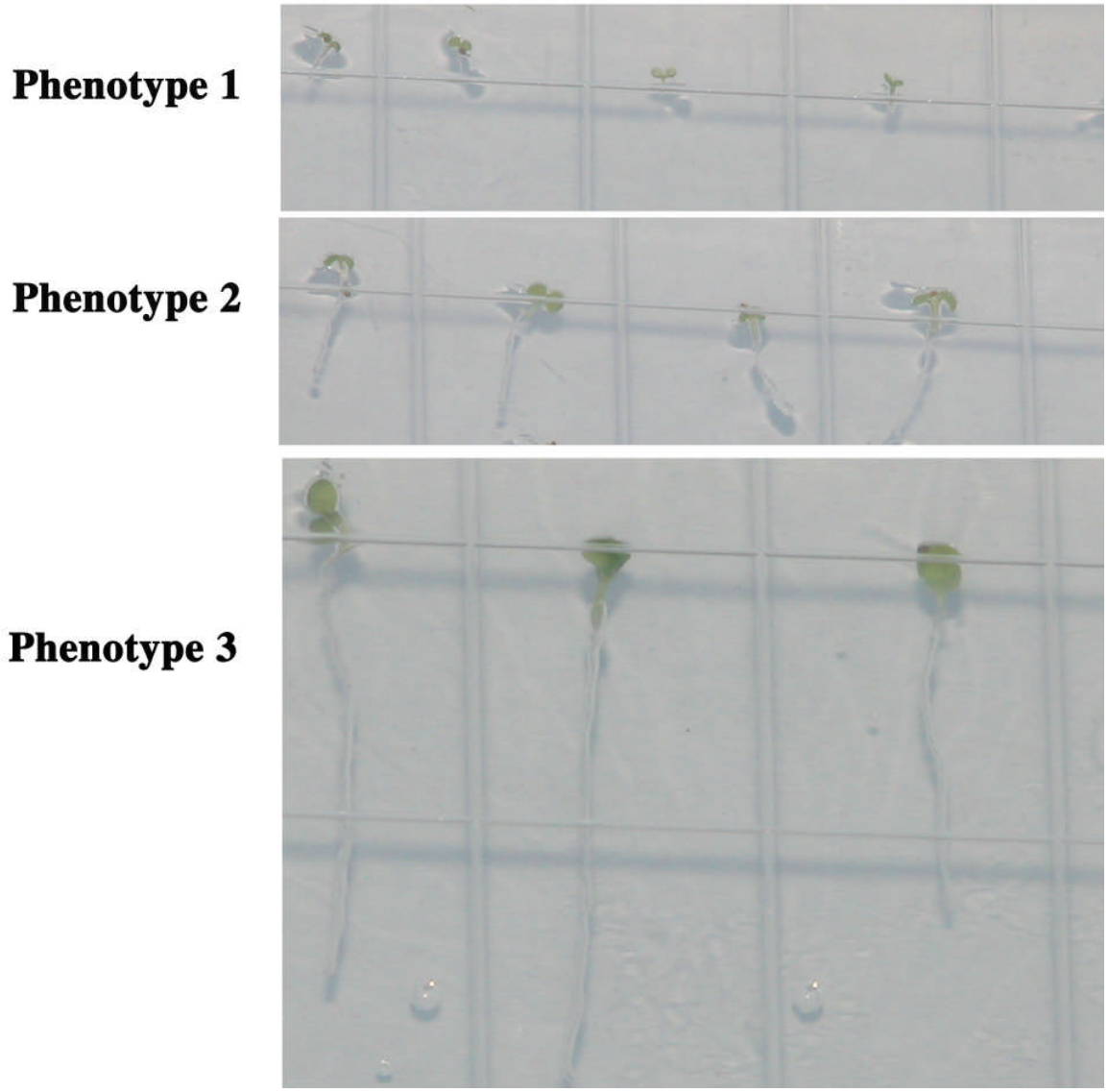


Fig. 1B. The root growth of two potentially heterozygous *scl3-1* lines (*scl3*-D and *scl3*-E) 12 day seedlings on 120 mM NaCl.

Phenotype 1: *scl3-1* seedlings with phenotype of salt sensitivity.

Phenotype 2: *scl3-1* seedlings partially sensitive to salt.

Phenotype 3: *scl3-1* seedlings have the same phenotype as WT.

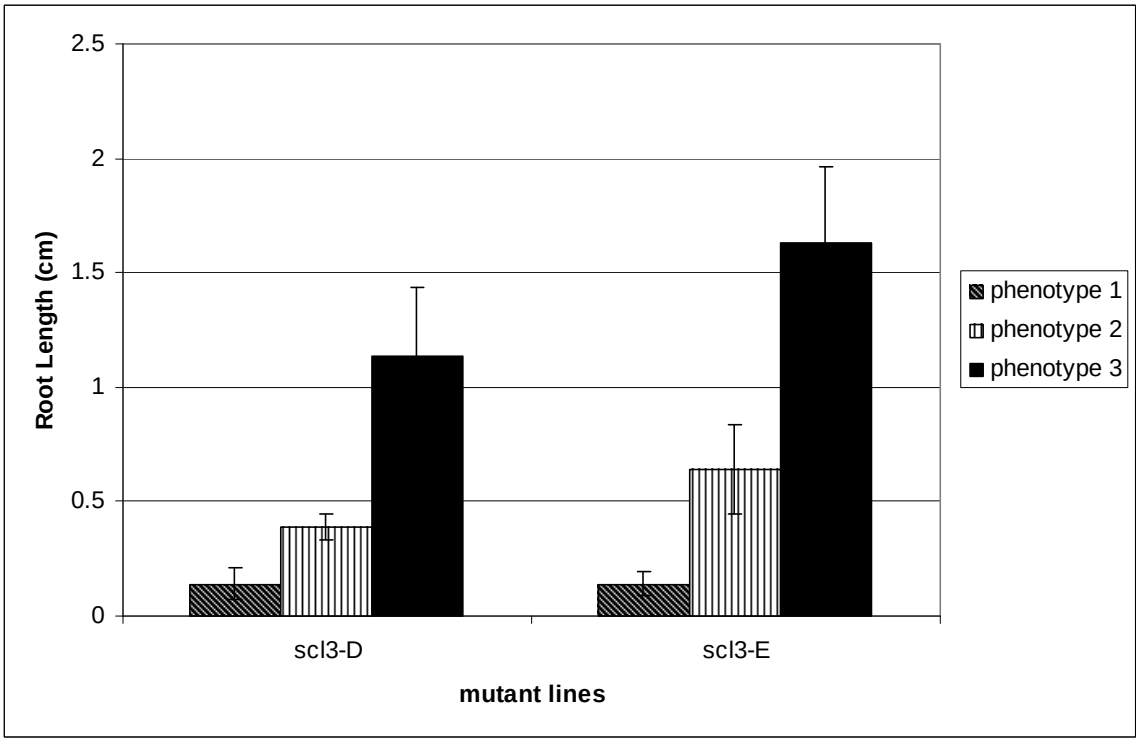


Fig. 1C. Comparison of representative WT, *scl3-2* and *scl3-3* mutant 12-day seedlings on control and 120mM NaCl plates

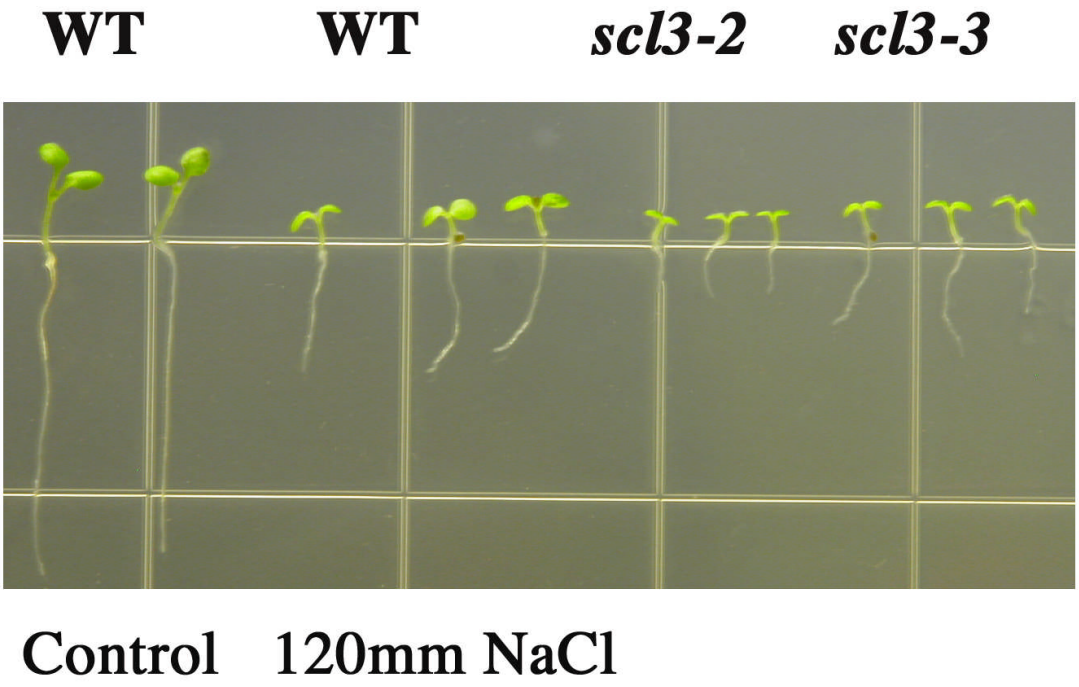


Fig. 2A. Schematic representation of SCL3 locus. 1. The entire transcription unit is shown. The number s indicated positions in mRNA. The box represents the coding region of SCL3. The T-DNA insertion sites are indicated by the open triangles. Arrowheads denote positions and orientation of primers used for PCR reactions that yielded amplification products in at least on DNA sample.

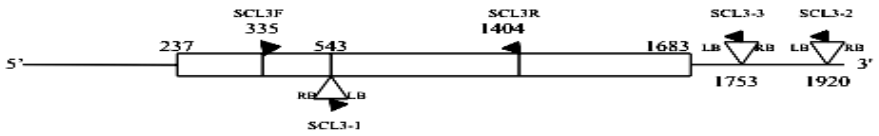


Fig. 2B. PCR diagnostic tests for the presence of T-DNA in *scl3-1* line. Scl3-1 DNA s from phenotype 1 and phenotype 3 we used. PCR reactions were performed using *scl3*F/scl3R, *scl3*F/LBb and *scl3*R/LBb primers, respectively.

a. *scl3-1* with phenotype 3 (WT looking)

b. *scl3-1* with phenotype 1

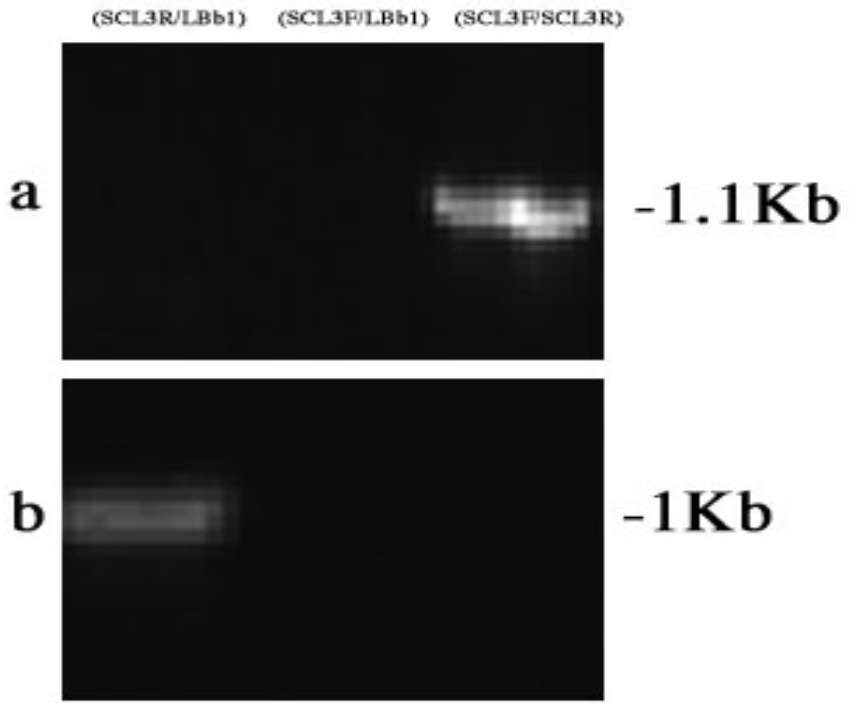


Fig. 2C. Truncated protein translated from *scl3-1* mutant

Fig. 2D. PCR diagnostic tests for *scl3-2* and *scl3-3* plants. For the detection of wild-type SCL3 and mutated *scl3-2*, *scl3-3* alleles, PCR reactions were performed using *scl3*F/scl3R, *scl3*F/LBb and *scl3*R/LBb primers, respectively.

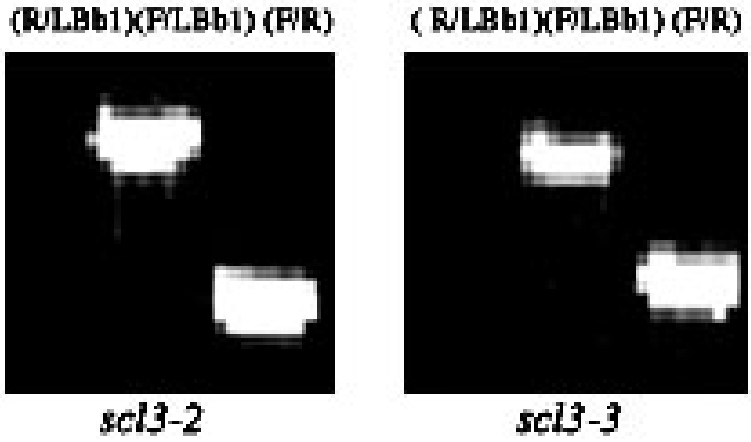


Fig. 3A. Induction of gene expression by salt. mRNA levels in 10 day WT roots imbibed in 200mM NaCl / mRNA levels in roots imbibed in H_2O alone at 4^oC for 12 hours

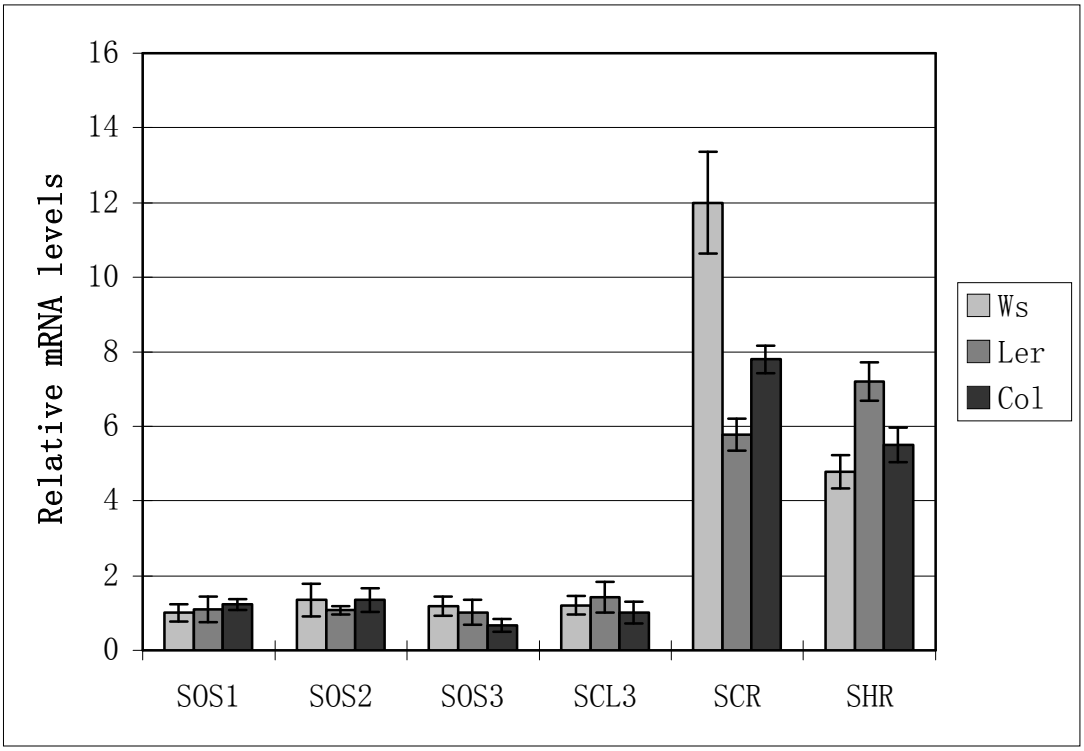


Fig. 3B. Real time PCR. mRNA levels in 10 day mutant roots/ mRNA levels in 10 day WT roots imbibed in H_2O at 4^oC for 12 hours. X-axis shows names of genes, Y-axis shows the genes relative mRNA levels in mutant roots.

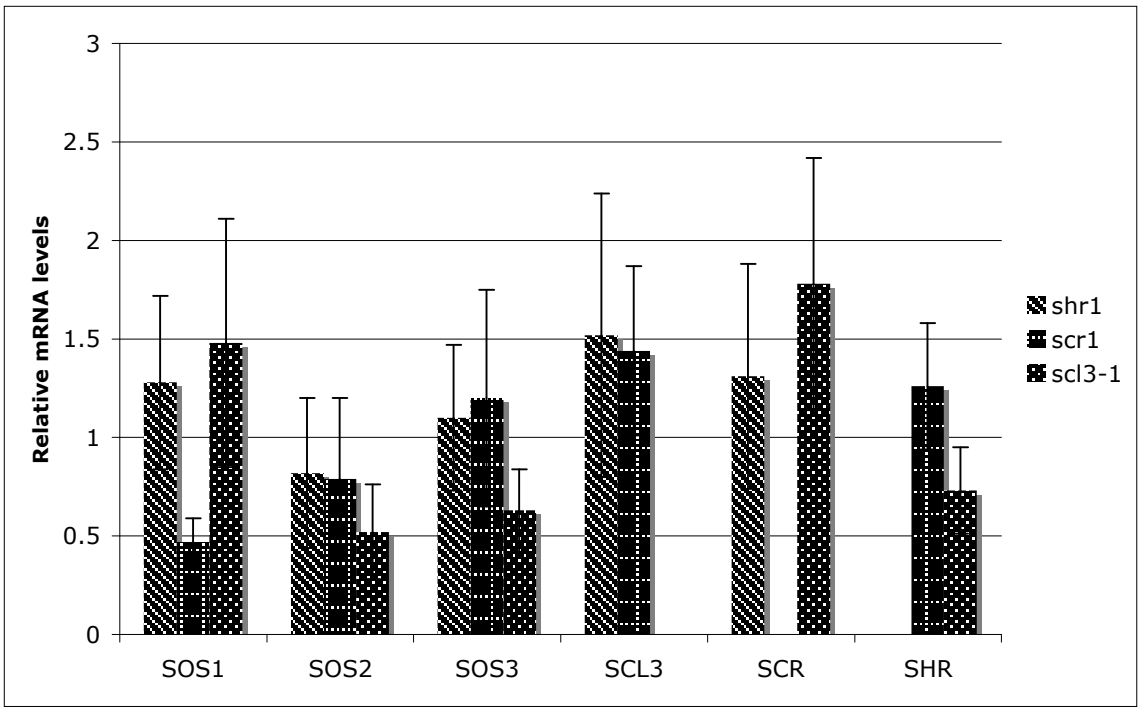


Fig. 3C. mRNA levels in 10 day mutant roots/ mRNA levels in 10 day WT roots imbibed in 200mM NaCl at 4^{o}C for 12 hours. X-axis shows names of genes, Y-axis shows the genes relative mRNA levels in mutant roots.

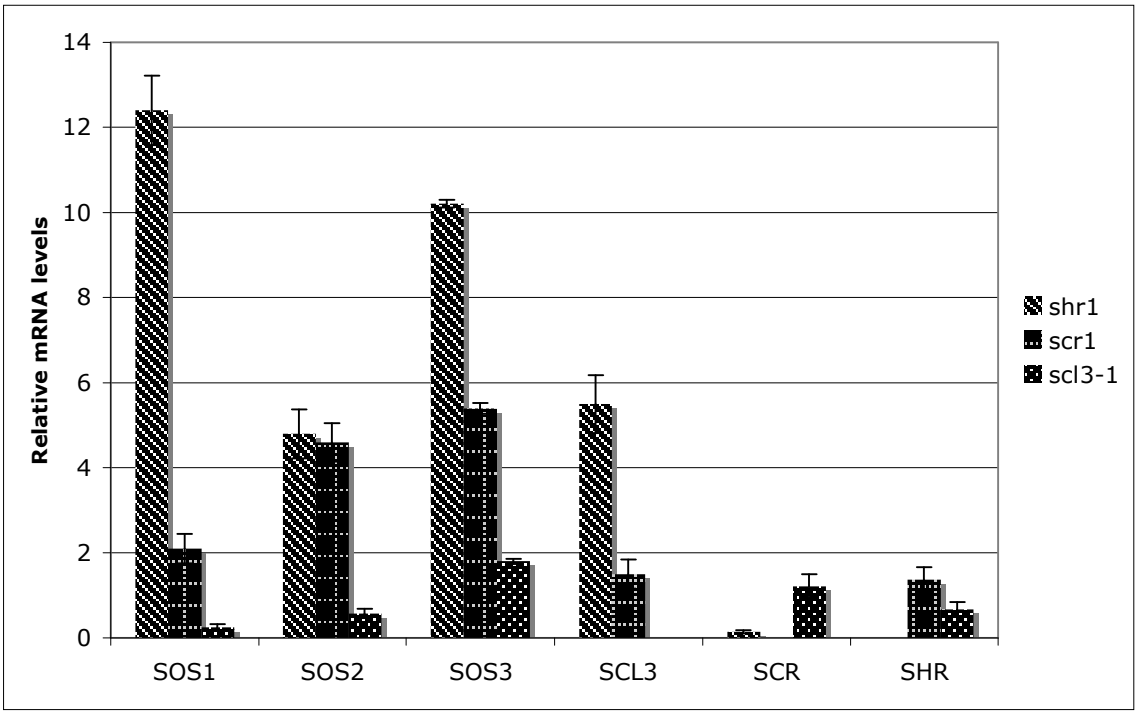


Fig. 4A. Time course of WT and scl3 mutant seed germination on 0.5MS or 0.5MS containing 200mM NaCl. Points on the Z-axis are in days after transfer of plates to the growth chamber.

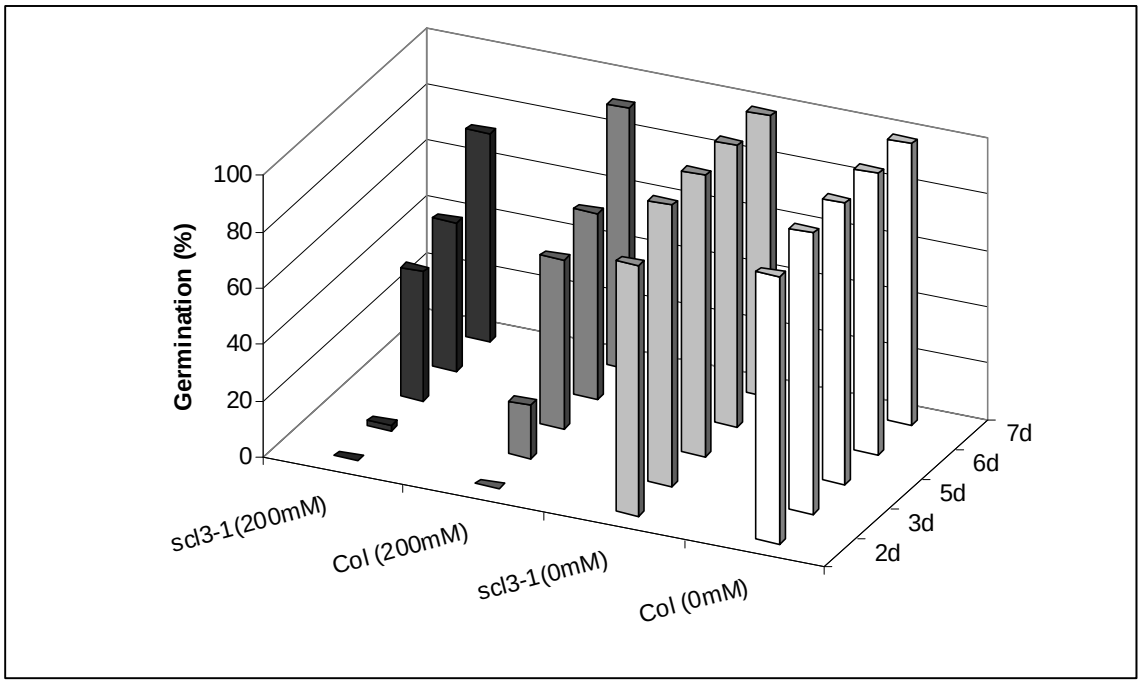


Fig. 4B. Percentage of WT and mutants seeds germination on 0.5MS or 0.5MS containing 200mM NaCl at day 7 after transfer of plates to the growth chamber.

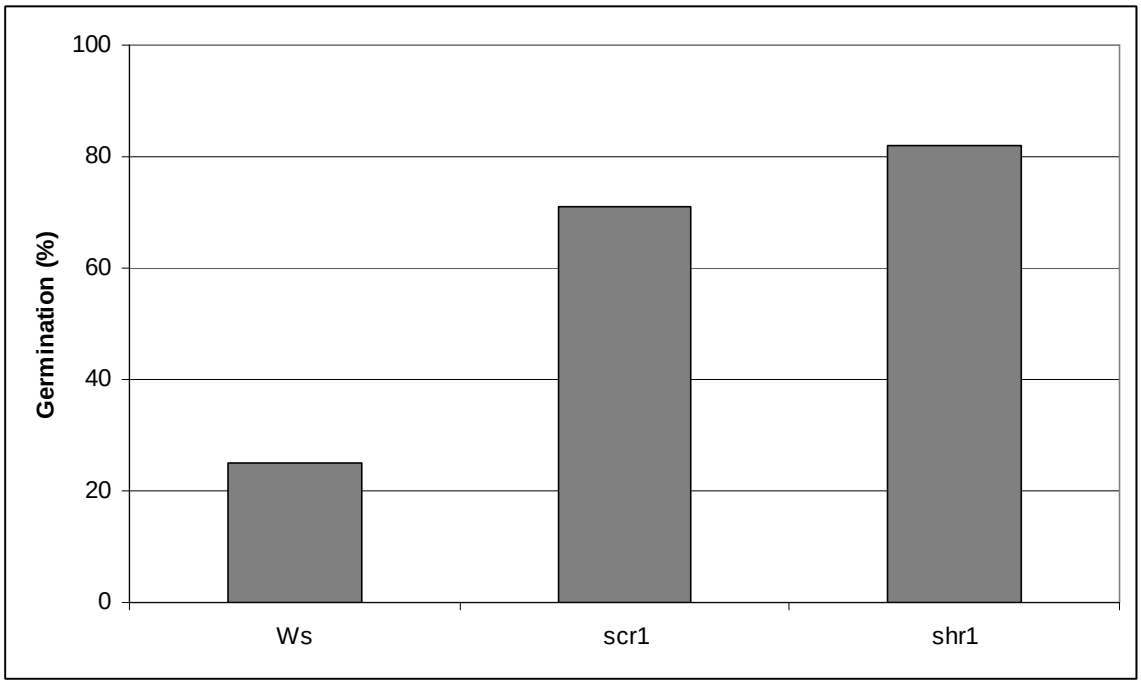
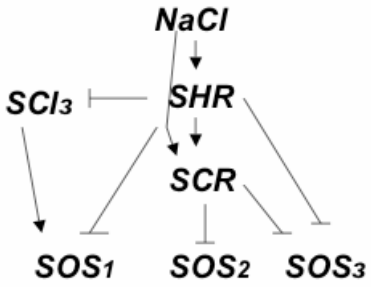


Fig. 5. Proposed model for interactions between SOS signaling pathway and transcriptional regulators in GRAS family under salt stress.



VI. CONCLUSIONS

My dissertation research focused on several genes in phytohormones pathways and GRAS family are involved in sugars and salt stress effect on plant development. The major results of my project are described in the following:

In the glucose effect on seed germination project:

1. Glucose can delay seed germination through a different pathway from sugars such as mannose.

2. The effect of glucose delay on seed germination is via genes RGl2 and SPY in GA signaling pathway and ABI3 in ABA signaling pathway.

3. ABI3 and RGL2 may act via the same pathway in glucose delay of seed germination.

4. Glucose, applied after germination, has a strong stimulatory effect on seedling growth and development. This effect is partially mediated via activation of GA and/or inactivation of cytokinin signaling pathways by relieving the inhibitory or the stimulatory effect of a key regulator, SPY.

5. Exogenous glucose in moderate concentrations has opposite effects on plant growth and development depending on the developmental stage during which it is applied. The inhibitory and stimulatory effects of glucose are at least in part mediated via components of ABA and GA signaling pathways.

In salt stress effect on seed germination and early seedling development project:

1. Salt delays germination by activating the ABA signaling pathway via ABI3 and ABI5 and repressing the GA signaling pathway via RGL2.

2. The crosstalk and interactions exist among the different regulatory genes and pathways. There are cross talks between the ABA and GA signaling pathways and that ABI3, ABI5 and RGL2 gene products are involved in this process.

3. *Abi3*, *abi5* and *rgl2* mutants are resistant to osmotic pressure during the seed germination. It indicates that both ABA signaling pathway via ABI3 and ABI5 and GA signaling pathway via RGL2 are involved in osmotic sensing during the seed germination.

4. The functions of ABI genes are different during early seedling development. ABI5 but not ABI3 may act as a negative regulator of ABA signaling pathway to adjust the ion cell balance during early seedling stage.

5. Both ABA signaling pathway via ABI3 and ABI5 and GA signaling pathways via RGL2 are involved in the early seedling tolerance to osmotic stress.

6. Genes in the ABA signaling pathway showed the different functions during the different stages of plant growth under salt stress. *abi3* mutant seedlings are sensitive to the salt stress but *abi5* mutant seedlings are similar as the WT seedling under the salt treatment.

In the GRAS family gene function project:

1. *scl3* mutant alleles are salt hypersensitive both during germination and also during seedling growth.

2. *SCR* and *SHR* genes in GRAS family are involved in the salt inhibition of seed germination.

3. Salt stress inhibits root growth by inducing SHR and SCR gene expression at the transcriptional level directly or indirectly or both, which in turn down-regulate SOS1, SOS2 and SOS3 gene expressions. This results in down-regulation of SOS pathway via components in SOS pathway.

4. SCL3 expression seems to be down-regulated by SHR. In addition, SCL3 up-regulates SOS1 expression. SCL3 mediates salt tolerance by increasing SOS1 transcription.

It is the first time that the function of *SCL3* gene in GRAS family has been identified under the stress condition. The function of *SHR* and *SCR* genes under the stress condition is a unique finding in this report. The crosstalk among phytohormones, glucose and salt signaling pathways via several genes has been discussed. Different genes in the different signaling pathways have different effect on different stages of plant growth. The future work is still needed because of the complexity of the network among various pathways.

Publications of my dissertation research:

Kun Yuan and Joanna Wysocka-Diller. 2006. Phytohormone signaling pathways interact with sugars during seed germination and seedling development. *Journal of Experimental Botany* 57(12): 3359-67 **.**