STUDY OF LATERAL ROOT DEVELOPMENT DURING SALT STRESS IN ARABIDOPSIS

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SUMMARY

In a taproot system, the development of lateral roots (LRs) from the primary root (PR) is an essential factor in shaping the whole root system architecture. Under environmental stress, such as high salinity, the patterning of LRs along the longitudinal axis of PR and the post-emergence growth of LRs are disrupted. In this thesis, we describe the detailed analysis of these two aspects of LR development in Arabidopsis during salt stress.

When facing high salinity environment, PRs and LRs show distinct intrinsic programing in controlling growth. We report that salt stress induces an extended quiescence phase in post-emergence lateral roots (LRs) whereby the rate of growth is suppressed for several days before recovery begins. Quiescence is correlated with sustained Abscisic Acid (ABA) response in LRs and is dependent upon genes necessary for ABA biosynthesis, signaling and transcriptional regulation. Here we show that high salinity, an environmental stress widely impacting agricultural land, regulates growth of the root system through a signaling network operating primarily in the endodermis. Gibberellic Acid (GA) signaling, which antagonizes the ABA pathway, also acts primarily in the endodermis and we define the cross talk between these two hormones. The antioxidant genes, peroxidases, could be the potential targets of both ABA and GA signaling in the endodermis in controlling LR growth. Our results define the endodermis as a gateway with an ABA-dependent guard, which prevents root growth into saline environments.

Besides the post-emergent growth, the early LR patterning determination is also disrupted by salt stress. We reveal salt stress affects LR initiation through signaling to the primary root tip. The maintenance of LR pre-branch sites is largely disrupted during salt stress, indicated by the disrupted pattern of the auxin responsive reporter, *DR5::Luciferase*. This study provides the first evidence on the mechanism of early LR patterning disruption during salt stress.

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LIST OF ABBREVIATIONS AND SYMBOLS

Chemicals and reagents

ABA	Abscisic Acid
ACC	1-aminocyclopropane-1-carboxylic-acid
DEX	Dexamethasone
dH_2O	Distilled water
EtOH	Ethanol
GA	Gibberellic Acid
IAA	3-Indoleacitic acid
KCl	Potassium chloride
KOH	Potassium hydroxide
MEP	2-C-methyl-D-erythritol4-phosphate/1-deoxy-D-xylulose 5-phosphate
MES	2-(N-Morpholino)ethanesulfonic acid
MgSO4	Magnesium sulfate
NaCl	Sodium chloride
NPA	N-1-Naphthylphthalamidic acid
PAC	Paclobutrazol

Unites

°C	Degree celsius
bp	Base pairs

g	gram
hr	Hour
kb	Kilo base-pairs
kV	kilo volt
M	molar
min	minute
ml	Milliliter
mM	Millimolar
nm	Nanometer
nM	Nanomolar
rpm	Revolution per minute
w/v	Weight per volume
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar

Others

cDNA	Complementary DNA
COL	Collumella
DNA	Deoxyribonucleic acid
Epi	Epidermis

GFP	Green fluorescence protein
LR	Lateral root
LRC	Lateral root cap
LRP	Lateral root primordium
miR	MicroRNA
PBS	Pre-branch site
PCR	Polymerase chain reaction
PR	Primary root
Q-PCR	Quantitative PCR
RFP	Red fluorescence protein
RNA	Ribonucleic acid
RSA	Root system architecture
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of mean
T-DNA	Transfer DNA
TF	Transcriptional factor
YFP	Yellow fluorescence protein

Chapter 1 LITERATURE REVIEW

1.1 Root system development and regulation in Arabidopsis

In plants, root is the organ directly contacting soil. The root system architecture (RSA) determines the ability of plants to absorb water and nutrients as well as anchoring soil and support the whole plant. As model dicots, Arabidopsis root has a symmetric and well organized structure, which provides a very good platform to study the developmental programs in shaping the whole root architecture.

Lateral roots (LR) in Arabidopsis initiates from an inner tissue layer of the primary root (PR) called pericycle and the lateral root primordia (LRP) need to break through the primary root in order to form a mature LR. This organogenesis process has been found to be tightly regulated by phytohormones, which are small amount of chemicals that regulate plant development. Auxin, one of the most important growth hormones, has been found to play a very important role in this process.

Besides the patterning and organogenesis of LR and PR, RSA is also determined by the post-emergence growth of LR and growth of PR. This process is tightly regulated by environmental factors, like water and nutrients.

1.1.1 Arabidopsis primary root structure and development

Among the different organs of a plant, the root is intimately associated with the environment; it absorbs water and nutrients by directly contacting the soil. Plant roots have evolved the ability to sense a myriad of factors in their local environment and use this information to drive changes in growth direction (tropisms) and root

system architecture. Arabidopsis has a very simple root structure that is rotationally symmetric with a stereotypical tissue organization (Fig. 1). A small number of stem cells surrounding the quiescent center of the root tip produce daughter cells that divide, differentiate, expand and subsequently develop to all kinds of cell types (Scheres, 2002). Different cell layers have distinct stem cells, for example, Epidermis/Lateral root cap (Epi/LRC) initials, Endodermis/Cortex (End/Cor) initials, stele initials and collumella cell initials. Auxin, a phytohormone, is essential in distal patterning by defining the position of the stem cell niche, though the mechanism is still not well understood. Disruption of the normal function of stem cells can cause abnormal divisions or differentiation of cells in the root tip region causing altered root structures. An AP2 transcription factor family protein -- PLETHORA (PLT) is found to be auxin responsive and necessary for stem cell maintenance and root formation. The double mutant *plt1/plt2* has reduced number of stem cells, which results in a great reduction in primary root length, numerous lateral roots and abnormal divisions in the root tip (Aida et al., 2004; Galinha et al., 2007). To further maintain the radial patterning of different cell types, many crucial genes or genetic pathways need to be normally functioning. Two GRAS transcription factors: SCARECROW and SHORT-ROOT were shown to maintain the endodermis and cortex cell identities. In both *scr* and *shr* mutants, one ground tissue layer is missing (van den Berg et al., 1995), and in *shr*, only the cortex marker can be found, while in *scr*, both endodermal and cortex markers can be found in the single mutant cell layer (Di Laurenzio et al., 1996). Therefore, although both TFs promote the periclinal cell division, only *shr* is essential for cell specification. Further studies indicate that *shr* is required for the asymmetric

division responsible for the specification of ground tissues, and the ectopic expression of shr causes formation of extra ground tissue layers (Helariutta et al., 2000). Similarly, normal asymmetric cell division in Epi/LRC initials needs to be controlled in order to develop epidermis and lateral root cap cells. Two NAC domain transcription factors: FEZ and SMB were found to antagonistically control the division plane in the Epi/LRC initials and COL initials (Willemsen et al., 2008).

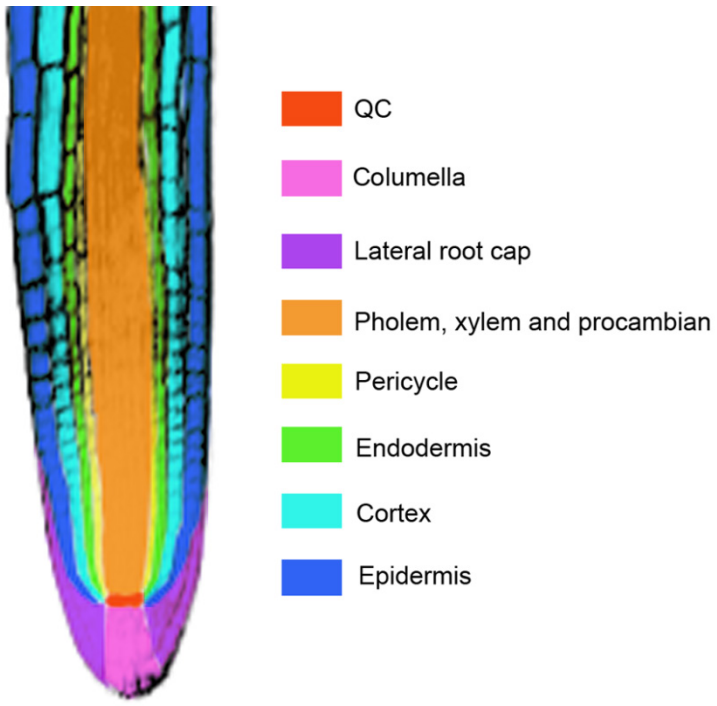

Figure 1. Diagram of Arabidopsis root structure.

Different cell types are labeled with false-colors. In the root tip, collumella and lateral root cap cells constitute the root cap. In the mature root region, from outer to inner cell layers, the radial root structure is composed of epidermis, cortex, endodermis, pericycle and the vascular tissues and procambian.

1.1.2 Organogenesis of lateral roots in arabidopsis

Different from the embryonic-emergence process of the primary root (PR), lateral roots (LRs) initiate from a cell layer embedded deep in the PR, pericycle, which is the outmost cell layer of the stele. The mature pericycle cells adjacent to xylem pole cells are able to differentiate and become LRP founder cells. The two adjacent founder cells will undergo nucleus migration toward the shared cell plane (De Rybel et al., 2010) and followed by an asymmetric cell division, which generated a cell file with two short and two long cells (Casimiro et al., 2001). This stage is also defined as LRP stage Ⅰ (Malamy and Benfey, 1997). Subsequently, this cell file will undergo three periclinal cell divisions to form stage Ⅱ, Ⅲ, and Ⅳ LRP. After that, both periclinal and anticlinal cell divisions will occur to develop stage Ⅴ, Ⅵ, and Ⅶ LRP, at which stage the LRP has broken through ground tissue and epidermis and is about to emerge from the PR. In order to form a mature LR after emergence, the outer cell layer will maintain 8-10 cells and elongate, while the inner cells will go through much more proliferation and finally form the apical meristem of LR (Malamy and Benfey, 1997) (Fig.2).

In order to determine the positioning and trigger the re-differentiation of the LR founder cells along the PR, a change in cell cycle status needs to happen in the specified pericycle cells. A cyclin dependent kinase, CDC2, has been found to be expressed in the LRP founder cells (Martinez et al., 1992), and in order for the founder cells to initiate a LRP, a G2-M phase transition needs to occur. Another gene, CYC1, is expressed in actively dividing cells; however, the overexpression of this

gene driven by the promoter of CDC2 fails to disrupt the normal patterning of LRP while the overall proliferation of both PR and LR increases (Doerner et al., 1996). This suggests that the determination process for the patterning of founder cells happens earlier than the change in cell cycle status, which is likely to be the result of LR initiation.

Besides the regulation in cell cycle, the regulatory roles of various plant hormones have been found to be important in LR patterning. Numerous studies have shown that auxin, as important growth hormone, plays essential roles in LR patterning. Those findings will be introduced in the following sections.

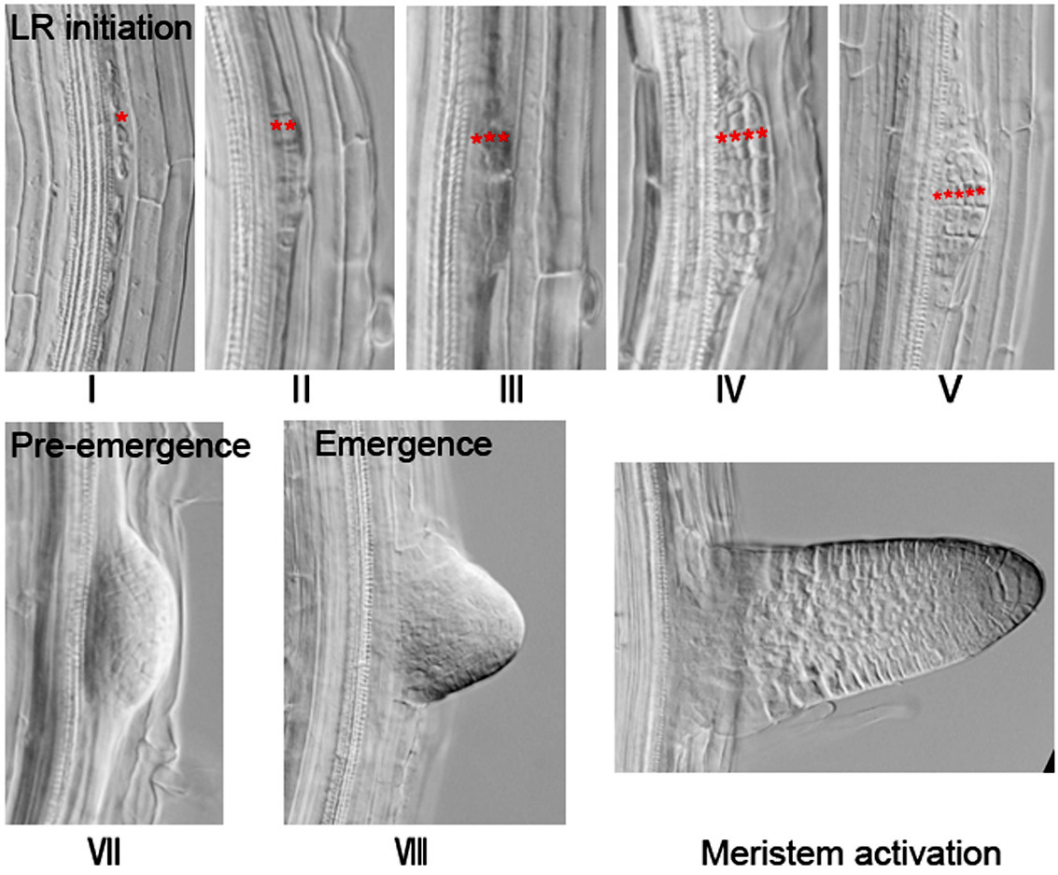

Figure 2. Process of Lateral root development.

DIC images of various stages during LR development. Red asterisks mark single cell files within the LRPs.

1.1.3 Hormonal regulation of root development

Plant hormones are small amount of chemicals that play very important roles in various aspects of plant development. Auxin is an essential growth hormone that is involved in various organogenesis processes in plants, including shoot meristem establishment, leaf patterning, root stem cell maintenance, lateral root organogenesis and so on. Cytokinin and gibberellic acid are also positive growth regulators that control the balance between cell division and differentiation and promote cell elongation, respectively. There are also negative growth regulators such as Abscisic acid (ABA) and ethylene. They are mostly induced during stress environment such as water stress and salt stress and modulate various aspects of plant developmental processes. In the following sections, the signaling pathway and the regulatory roles of each plant hormone in root development, especially lateral root formation, will be discussed.

1.1.3.1 Auxin

Auxin is important for nearly all aspects of plant development. Firstly, there are two possible auxin biosynthesis pathways: Tryptophan (Trp)-dependent and Trp-independent. YUCCA family proteins were shown to be important in the Trp-dependent pathway (Zhao et al., 2001). Over-expression of YUCCA family proteins leads to an auxin-overproduction phenotype, while the *yuc* quadruple mutant fails to establish the basal-apical axis during embryogenesis and develops abnormal root meristem (Cheng et al., 2007). In Arabidopsis roots, patterning needs proper auxin

distribution and many auxin influx and efflux carriers play essential roles in this process. For instance, PIN family proteins function as auxin efflux carrier and the polar localization pattern directs auxin flow in the roots (Wisniewska et al., 2006). Furthermore, the specific expression pattern of different PIN family members in the root function together to maintain the auxin maxima and regulate different developmental processes. For example, the auxin gradient maintained by PIN proteins restricts the expression pattern of PLT, which specifies the root stem cell niche, and therefore defines the distal patterning of root tip (Blilou et al., 2005). Besides, the root response to gravity can also be regulated by auxin transporters such as the efflux carrier-PIN2/PIN3 and influx carrier-AUX1 (Swarup et al., 2005). Over the past decades, auxin signal transduction has been well-studied. The AUX/IAA proteins negatively regulate auxin signaling and are subject to degradation by the 26S proteasome following ubiquitination mediated by the E3 ubiquitin-ligase SCF^{TIR1} when auxin is present (Dharmasiri and Estelle, 2002). Mutations in the interaction site with SCF^{TIR1} stabilize the AUX/IAA protein and result in a gain-of-function phenotype.

Lateral root development determines the shaping of root system architecture. Many years of study have shown that auxin is involved in various aspects of lateral root development. In *Arabidopsis*, the auxin responsive reporter, *DR5::Luciferase*, oscillates with a rhythm of every 6 hours, which determines the position and spacing of possible LRPs. This determination sites is termed Pre-Branch Site (PBS). A constant number of PBS is found in the roots grown under various environmental conditions (Moreno-Risueno et al., 2010). This result suggests that auxin

accumulation and response is involved in very early determination stage before specification of founder cells, and this determination could not be affected by multiple environmental changes. At the same time, the localized accumulation of auxin in the pericycle cells specifies the identity of LR founder cells, while this identity is genetically separable with the first pericycle division (Dubrovsky et al., 2008). A gain-of-function mutation in IAA14 (*slr-1*), which dominantly suppresses auxin signaling, has also been found to greatly block the anticlinal cell divisions in the pericycle(Fukaki et al., 2002). Auxin responsive factors (ARFs) are transcriptional factors that are suppressed by AUX/IAA family protein. ARF7 and ARF19 have been demonstrated to act redundantly in promoting very early LR initiation through direct activation of LBD/ASL genes (Wilmoth et al., 2005; Okushima et al., 2007), and IAA14 is a direct suppressor of ARF7/ARF19 (Fukaki et al., 2005). Successively, BDL/IAA12-ARF5 also acts as a secondary module to regulate LR initiation as well as LR formation (De Smet et al., 2010). Recently, a GATA family transcriptional factor, GATA23, was found to be expressed in LR founder cells before first asymmetric division, and its expression pattern oscillates along the primary root, which highly correlates with the oscillated auxin response maximum. Interestingly, ectopic expression of GATA23 in the xylem pole pericycle cells induces irregular LR initiation, suggesting an essential role of GATA23 in determining founder cell identity as well as triggering first asymmetric cell division. IAA28 is found to be the regulator of GATA23, while five ARFs directly interact with GATA23, indicating a new pathway in LR early determination (De Rybel et al., 2010). In summary, the auxin regulated IAA/ARFs/LBD/GATA23 regulatory

module play essential roles in early founder cell determination and lateral root initiation processes.

In order to maintain proper spacing of LR along the PR, sophisticated auxin transportation system is necessary. Consistent with this hypothesis, it is found that the inhibition of auxin transportation by NPA treatment disrupts LR spacing as well as initiation processes (Casimiro et al., 2001). It has also been shown that the alternative patterning of LR along the PR is related to the gravitropic and waving response, and this patterning process is dependent on an auxin influx carrier, AUX1 (De Smet et al., 2007). Interestingly, 'fused LRs' has been observed in *pin2/3/7* triple mutants (Laskowski et al., 2008), suggesting the importance of sustained auxin circulation in determining LR position.

1.1.3.2 Cytokinin

Cytokinin interested people by its outstanding function in promoting cell division, differentiation and other physiological processes. In 2001, the ATP/ADP - isopentenyltransferase (AtIPT) gene family was shown to be important for cytokinin biosynthesis (Kakimoto, 2001; Takei et al., 2001). Plants respond to cytokinin using a two-component signaling pathway. First, members of the histine-kinase family was found to be the cytokinin trans-membrane receptor, for example, AHK4 (Suzuki et al., 2001) and CRE1 (Inoue et al., 2001). According to the current model, Type A and Type B ARRs are the two-component signaling systems that pass the cytokinin signal. Type A ARRs work as negative regulators of cytokinin signaling (Kiba et al., 2003;

To et al., 2004), while Type B ARRs function as transcription factors that positively regulate cytokinin signaling (Ishida et al., 2008). Besides, several cytokinin dehydrogenases (CKXs) were shown to regulate the degradation of cytokinin and therefore negatively regulate the signaling pathway (Werner et al., 2006).

In the Arabidopsis roots, cytokinin is involved in many developmental processes like meristem patterning, primary vascular tissue development, and lateral root formation. During most of these processes, cytokinin has an antagonistic effect to auxin. For instance, recent studies provide more evidence about the cell type-specific cytokinin regulation of root meristem size by balancing the proliferation and differentiation of the meristem cell. For example, the cytokinin biosynthesis mutants- *ipt*s have been found to have an enlarged meristem, and the cell type-specific expression of the CKX suggests that cytokinin functions at the meristem-elongation transition zone to control cell differentiation (Ioio et al., 2008).

At the same time, exogenous or over-production of cytokinin prevents lateral root initiation by potentially disrupting PIN expressions and thus auxin gradient in LR founder cells (Laplaze et al., 2007; Kuderová et al., 2008). In addition, cytokinin can also lead to cell cycle arrest in pericycle founder cells during LR initiation by blocking the transition of G2 to M phase, while the D-type cyclin could be induced by cytokinin in promoting post-emergent LR elongation (Li et al., 2006). The opposite roles of cytokinin in LR initiation and elongation are also found in rice (Rani Debi et al., 2005).

1.1.3.3 Gibberellic acid

Gibberellic acid promotes plant growth in many aspects, including seed germination, hypocotyl elongation, flowering, and root development. The biosynthesis of active GA needs several steps, generally from geranylgeranyl diphosphate (GGDP) to the active form- ent-Kaurene, which has been well-studied (Hedden and Kamiya, 1997). The GA-deficient mutants like *ga1-3* and *ga2-1* develop a dwarf phenotype and severe defects in seed germination (Ogawa et al., 2003). GA promotes growth through targeting the degradation of DELLA repressor proteins (Dill et al., 2001; Wen and Chang, 2002). The gain-of-function mutant *gai-1* prevents the protein from degradation and presents a similar phenotype as the GA-deficient mutants. Recently, by controlling the spatial expression of *gai-1*, it was found that GA signaling in endodermis is necessary for both root meristem size and primary root growth (Ubeda-Tomás et al., 2008; Ubeda-Tomas et al., 2009). Interestingly, GA was also found to be involved in ground tissue patterning by inhibiting middle-cortex cell proliferation (Paquette and Benfey, 2005) and this process might involve SCR and epigenetic regulation (Cui and Benfey, 2009). However, the role of GA signaling in LR development is less clear.

1.1.3.4 Ethylene

Ethylene is well-known by people for its function in regulating the triple response of plants and stimulating fruit ripening, but the function of ethylene is not restricted in the regulation of senescence and stress responses, but also plays very important roles in many aspects of plant development.

Ethylene is originally synthesized from methoinine and there are two major intermediates among this process: S-AdoMet, which is the precursor of the polyamine biosynthesis pathway, and ACC, which is the precursor of ethylene. The biosynthesis of ACC is the most rate-limiting step (Wang et al., 2002). ACC synthase has a big gene family and many ACSs have been identified previously (Sato and Theologis, 1989; Liang et al., 1995), and most of them have been found to function in a spatial and temporal-specific manner and respond to many environmental stimuli. Ethylene is perceived by the cell through a family of membrane localized receptors. Five receptors are known: ETR1, ETR2, ERS1, ERS2, which have histidine kinase activity, and EIN4, which has serine kinase activity (Chang et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998). CTR1 is a serine-threonine kinase and it can regulate ethylene signaling by interacting with the receptors (Gao et al., 2003). Downstream of CTR1, EIN2-a metal transporter, is essential for signal transduction. Recently there is evidence that EIN2 localize to the ER and can interact with ETR1 (Bisson et al., 2009). Downstream of EIN2, there are two classes of transcription factors that act at different steps. For example, EIN3 family proteins can be firstly stabilized by ethylene signaling and then regulate the downstream transcription factors such as the Ethylene Response Factor-class TFs (ERFs) (Stepanova and Alonso, 2005).

Ethylene is found to be involved in many developmental processes in roots, for instance, the root hair development. The epidermis of a root is specifically patterned by both hair cells and non-hair cells. Ethylene was found to promote the formation and patterning of root hairs by causing the differentiation at non-hair cell positions. In *etr1-1* mutant, there is less but ectopic root hair formation (Dolan and Roberts, 1995);

it is also known that ethylene affects root growth as well as root gravitropism, and studies have provided evidence that ethylene can function through affecting auxin biosynthesis and distribution in regulating root growth, specially cell elongation (Ruzicka et al., 2007; Swarup et al., 2007). Ethylene can also affect root gravitropic response through inducing the accumulation of auxin transport regulator-flavonoid (Buer et al., 2006); in rice, the formation of adventitious roots needs growing through epidermis and cuticles, and ethylene facilitates this process by inducing cell death in epidermis (Mergemann and Sauter, 2000).

In addition to primary root growth and patterning, ethylene is also involved in regulating lateral root development. High concentrations of ACC (ethylene biosynthesis precursor) inhibit LR initiation by acting at growing PR root tip, while low concentrations of ACC could promote LR initiation. This promotion effect is abolished in *arf7/arf19* mutant, suggesting an auxin dependent regulation of LR initiation (Ivanchenko et al., 2008). However, endogenous elevation of ethylene production by a mutation in E3 ligase family protein, XBAT32, suppresses LR production (Prasad et al., 2010), suggesting a basic negative role of ethylene in LR development. Impaired auxin transporter expression in various ethylene mutants also suggests that ethylene may suppress LR development through promoting auxin transportation (Negi et al., 2008).

1.1.3.5 Abscsic acid

ABA was found and named because of the accumulation during abscission, but it actually does not directly function in this process. There is a broad distribution of ABA and many biological processes, especially in the responses to stress, involve the activity of ABA. For example, ABA can be induced by osmotic stress and it is essential in controlling seed dormancy, stomata closure, and tolerance to high salinity.

ABA is derived from a very complex biosynthesis pathway. Generally it is formed by cleavage of C_{40} carotenoids originating from the MEP pathway, and the first precursor of ABA biosynthesis is zeaxanthin. The whole pathway needs the activities of several enzymes: zeaxanthin epoxidase (ZEP), violaxanthin de-epoxidase (VDE), neoxanthin synthase (NSY) and an isomerase during early steps. A family of 9-cis-epoxycarotenoid dioxygenases (NCED) catalyze the cleavage of the long chain to C_{15} molecule-Xanthoxin, which is then converted by a short-chain alcohol dehydrogenase (ABA2) into abscisic aldehyde, which is oxidized into ABA by an abscisic aldehyde oxidase (AAO3) (Nambara and Marion-Poll, 2005). However, what perceives ABA signaling is a mystery for a long time until recently there are both studies on structure (Melcher et al., 2009; Santiago et al., 2009) and molecular signaling (Ma et al., 2009; Park et al., 2009) of ABA proving that the PYR/PYL family protein can directly interact with both ABA and PP2C and work as ABA receptors. By directly interacting with ABA receptors the protein phosphatase 2C (PP2C) family has been shown to negatively regulate ABA signaling, and the over-expression of PP2C leads to ABA insensitivity (Kuhn et al., 2006). Downstream of PP2C, there are the kinase family-SNRKs, especially SNRK2, which can be inactivated by PP2C (Umezawa et al., 2009; Vlad et al., 2009) and function in regulating ABA or drought responses and gene

expression (Fujita et al., 2009; Mizoguchi et al., 2010). The kinase family can then regulate downstream genes by controlling the activity of a number of transcription factors. For instance, ABI3, ABI4 and ABI5 are well-characterized transcription factors and are involved in many ABA regulated processes (Nambara et al., 2002).

ABA is involved in root development in many aspects. First, exogenous ABA inhibits seed germination in a dose dependent manner (Garciarrubio et al., 1997), and many ABA related genes have been shown to be involved in this process. Water deficiency or ABA treatment also inhibits root growth (Sharp, 2002). It is also found that ABA restrains root hair growth, while ABA-insensitive mutants like *abi1, abi2, abi3* are insensitive to this regulation (Schnall and Quatrano, 1992). ABA is also an important regulator of plant tropism. There is evidence that ABA negatively regulates root gravitropism in an auxin-dependent manner (Han et al., 2009). However, ABA treatment has also been found to diminish hydrotropic response while the roots present a strong gravitropic response (Ponce et al., 2008).

ABA also plays important roles in modulating root architecture. For instance, low exogenous ABA inhibits lateral root development in a specific stage after emergence and before meristem activation, which could be slightly rescued by *abi1-1* and *abi3-1* mutants. This regulation could be independent of auxin signaling (De Smet et al., 2003). ABA is also well studied to be involved in nitrate suppressed LR elongation. *abi4* and *abi5* mutants which are defective in ABA signaling show resistance to nitrate treatment in the number of visible LR as well as LR total length (Signora et al., 2001).

1.1.4 Effect of nutrients in root system architecture

Besides all kinds of internal hormonal signaling regulation, nutrient supply in the soil is necessary for root system architecture development and plant survival. Studies have demonstrated that spatial variation in nutrient conditions of the surrounding media can have important effects on the resultant RSA. Different from endogenous auxin regulation, availability of nutrient supply seems primary regulates LR elongation instead of patterning process. High concentrations of nitrate, for example, can primarily inhibit LR elongation through competition with auxin transportation, and by affecting mitotic activity (Zhang and Forde, 1998; Okushima et al., 2011), while localized nitrate patch will stimulate LR elongation within the nutrient patch (Zhang and Forde, 1998). However, the growth rate of LR could be inhibited after exiting the nitrate patch (Linkohr et al., 2002). Localized iron, on the other hand, promotes LR growth by triggering auxin accumulation in root apices through the regulation of gene expression of the auxin influx carrier AUX1 (Giehl et al., 2012). Intrinsic differences in the environmental response programs of PRs and LRs have also been observed. Phosphate promotes PR and inhibits LR growth (Linkohr et al., 2002), though the mechanistic basis for these differences are unknown.

1.2 High salinity stress in plants.

As we know, around 71% of the planet is covered by ocean, which is saline water. Thus, high salinity soil becomes one of the most severe global agriculture stresses. As a result, understanding how plants respond to salt stress and the mechanisms for

tolerating high salinity environment became important research topics. In the following sections, the physiological consequence of salt, natural variation in salt tolerance, signaling pathway as well as tolerance mechanism and hormone regulation during salt stress environment will be discussed in detail.

1.2.1 What is salt stress?

Salt stress is caused by excessive amount of sodium chloride in the environment, and the accumulation of NaCl in a plant cell leads to toxicity, which is mainly caused by both osmotic stress and ionic stress.

1.2.1.1 Osmotic stress

Osmotic stress in plants refers to the situation that limited water availability restrains plant growth (Zhu et al., 1997). When the environment surrounding the roots contains more soluble molecules, like NaCl, the osmotic stress to the plant root is increased and the turgor pressure of the cell against the cell wall is reduced. Other environmental stresses, such as cold stress, also induce osmotic stress by imitating the available water molecules. Osmotic stress greatly prevents plants from efficient water uptake from the environment, reduces the ability of plant cell wall to extent (Iraki et al., 1989), while the reduced turgor pressure might lead to plasmolysis, which reduces the rigidity of plants. Furthermore, the deficiency of water may lead to reduced growth of both leaves and roots (Kawasaki et al., 1983). Many biological processes are also disrupted or slowed down. Photosynthesis is the primary one, which can be a

result of reduced photosynthetic enzyme activity under high osmotic stress (Kaiser and Heber, 1981).

1.2.1.2 Ionic stress

Distinct from dehydration, ionic stress is specific to salt stress. The accumulation of Na^+ inside the cells will lead to toxicity, and it was found that the disturbed balance of K^+/Na^+ is an essential reason. First, the excessive amount of NaCl will lead to a competition between Na^+ and K^+ transportation into the cells due to their similar chemical properties, which induces the loss of K^+/Na^+ balance (Rubio et al., 1995). Second, it was also reported that K^+ is important in maintaining the activities of many enzymes inside the cell, while the excessive Na^+ will cause toxicity to many enzymes. Therefore, the ratio of K^+/Na^+ contribute to the ability of plants to tolerate salt stress (Shabala and Cuin, 2008; Luan et al., 2009).

In summary, by inducing both osmotic stress and ionic stress, salt affects many physiological changes in plant cells and subsequently inhibits the growth of the plant, and eventually leads to the wilting and death.

1.2.2 Variation in adaption to salt stress

Due to the variation in the ability of plants to tolerate salt stress, plants can be categorized into two groups, glycophytes and halophytes. Halophytes can tolerate and survive on 200 mM or higher concentrations of salt while glycophytes, which include most crops and plant species, will die (Waisel, 1973).

1.2.2.1 Halophytes

It has been found that for halophytes, structures called salt glands can help to secrete excess salt ions that are taken up from the environment so as to maintain the salt balance in leaves (Thomson et al., 1969). In addition, study of amino acid content in halophytes and glycophytes suggests that proline accumulates in halophyte at a much higher level than glycophytes, and the level of proline can be induced by salt treatment (Stewart and Lee, 1974). Although many studies provide information about factors that contribte to salt tolerance the underlined mechanisms in halophytes are still largely unknown (Flowers and Colmer, 2008).

1.2.2.2 Glycophytes

Unlike halophytes, glycophytes are more sensitive to salt stress. Although they might not have as strong adaptation mechanisms to salt as halophytes, their sensitivity to salt allows us to explore the changes inside the cell environment in order to further investigate the salt tolerance mechanisms. For instance, it was found that in glycophytes, the toxicity effect mainly comes from the accumulation of Na^+ in leaves. The built-up ions in the cytoplasm of leaf cells will inhibit enzymes activity and lead to senescence (Munns and Passioura, 1984; Flowers and Yeo, 1986). Salt accumulation is regulated by Na^+ transporters including the initial entry into the roots trough some non-selective cation channels or high affinity K^+ transporters (Shabala et al., 2007), and the transfer from root to shoot, including a Na^+ transporter, HKT1 (Davenport et al., 2007).

1.2.3 Study of salt signaling and tolerance in Arabidopsis.

As a model organism, *Arabidopsis* has many advantages for studying salt tolerance. For example, the short life span, completed genomic sequence, large amount of mutant stocks and large number of research groups worldwide. In addition, from the perspective of salt sensitivity, *Arabidopsis thaliana* as well as most crops are glycophytes and present high sensitivity to salt stress, and the study on *Arabidopsis* could potentially provide information on improving the tolerance of crops to salt by using genetic engineering methods (Møller and Tester, 2007). To support this point, studies have shown that the over-expression of the Na^+ transporter-AtNHX1, in other plant species, for example the common buckwheat, conferred enhanced salt tolerance (Chen et al., 2008).

The study of salt tolerance or salt signaling pathways in *Arabidopsis* can be mainly divided into four categories: 1) Na^+ transporters, 2) proline accumulation and regulation, 3) transcriptional factors or elements that are essential in regulating salt responses, 4) global transcriptomic studies.

Firstly, the SOS (*Salt-Overly-Sensitive)* pathway mediates salt tolerance by regulating ion transporters on membrane. There are mainly three SOS family members that are working together to regulate salt responses. SOS1 is a Na+/H+ exchanger on plasma membrane that pumps NaCl back into soil, and the over-expression of SOS1 confers enhanced salt tolerance (Shi et al., 2002). SOS2 was identified as a protein kinase, and it can directly interact with SOS3, a $Ca2^+$ sensor, and SOS3 activates SOS2 in a $Ca2^+$ dependent manner (Halfter et al., 2000). Furthermore, SOS2 and SOS3 function

together to regulate the activity of SOS1 (Qiu et al., 2002). When the SOS genes are co-expressed in plants, their tolerance to salt increased as the Na^+ content decreased (Yang et al., 2009). Besides, the Na+/H+ exchangers on vacuole membrane like AtNHX1 have been well documented to be involved in transporting the NaCl into vacuole, which compartmentalize salt and protect cells from toxicity. Furthermore, AtNHX1 was also found to be regulated by the SOS pathway (Qiu et al., 2004) and the overexpression of AtNHX1 confers increased salt tolerance in plants (Chen et al., 2008).

Secondly, besides the well-studied ion transporters, an amino acid—proline, was found to be induced by salt and important in maintaining the ability to tolerate salt stress. P5CS1 (Pyrroline-5-Carboxylate Synthase 1) controls the production of proline (Yoshiba et al., 1995), and the overexpression or the inhibition of P5CS1 degradation were also found to enhance salt and cold tolerance (Nanjo et al., 1999), while the *p5cs1* mutant has reduced level of salt tolerance (Székely et al., 2008). Besides the biosynthesis of proline, a small nat-siRNA was also found to target the 3' region of P5CDH (pyrroline-5-carboxylate dehydrogenase) and mediate the cleavage of P5CDH mRNA, which lead to the accumulation of proline (Borsani et al., 2005).

Thirdly, the ability of plants to tolerate salt can also be regulated transcriptionally. For example, two cis-elements, DRE and ABRE, have been well studied to enable the response to salt stress and ABA, while the mutated reporter shows altered response to salt (Yamaguchi-Shinozaki and Shinozaki, 1994); DREB2 (Drought Response Element Binding transcription factor 2) was also found to be involved in drought and

the salt stress response pathway (Nakashima et al., 2000), and the over-expression of DREB2 in Arabidopsis induces down-stream genes that contain DRE and thus presents stronger resistance to salt stress (Chen et al., 2007).

Fourth, although many components have been shown to be important in salt tolerance, the temporal regulation of their transcriptional level and the changes induced by those genes inside the plant cells are still less clear. By studying genomic-scale expression profile in Arabidopsis, we could potentially discover novel target genes and perform evaluations on them (Denby and Gehring, 2005). Furthermore, the cell type-specific cell sorting and microarray on arabidopsis roots not only provides information about the temporal regulation, but also on how the salt responses in root is spatially regulated (Dinneny et al., 2008).

1.2.4 Roles of plant hormones during salt stress

As described above, hormones are important growth regulators in plants. They are also found to be secondary signals during plant response to stress environment.

Ethylene is long recognized as a stress hormone. Upon salt stress, ethylene production as well as many ethylene-related genes can be up-regulated (Achard et al., 2006; Ma et al., 2006b). Genetics studies also provide evidence that many components involved in ethylene signaling also play important roles in salt response. For example, over-expression of an ethylene receptor homolog-NTHK1 (in tobacco) enhances plant sensitivity to salt stress, which is similar to the gain of function mutation *etr1-1* in Arabidopsis, while ACC can counteract this process (Cao et al., 2007). Similarly, the

ethylene insensitive mutant, *ein3-1*, shows enhanced salt sensitivity while the constitutively responding mutant, *ctr1-1*, is salt stress resistant and shows enhanced survival (Achard et al., 2006).

ABA is well-known to be involved in salt stress responses. Firstly, ABA levels can be enhanced upon salt treatment and interestingly, root confers higher sensitivity to salt compared to shoot (Jia et al., 2002). Many ABA biosynthesis genes can also be induced by salt (BARRERO et al., 2006). Secondly, ABA signaling components are involved in salt responses, for example, an ABA induced transcriptional factor ATMYB44, promotes stomata closure and confers salt tolerance (Jung et al., 2008). Two ABA responsive elements, DRE and ABRE can also be integrated during salt stress and regulate the expression of salt responsive genes (Nakashima et al., 2000; Narusaka et al., 2003). Interestingly, it was found from the microarray data that ABA signaling is induced by salt stress throughout different cell layers (Dinneny et al., 2008). Thus, whether the ABA signaling is spatially regulated and has cell type-specific functions is still unclear.

As a plant hormone that mainly promotes growth, GA was found to counteract the effect of high salinity. For example, GA(3) treatment could enhance water up-take and seed germination rate under salt treatment in sweet beets (Jamil and Rha, 2007), and in wheat plant, priming by GA(3) changes the ion status as well as hormone homeostasis during salt stress and enhances the tolerance to salt (Iqbal and Ashraf, 2010). Over-expression of the GA responsive gene, *GASA4*, Improves plants responses to salt stress, which could be through salicylic acid biosynthesis pathway

(Alonso-Ramírez et al., 2009). Furthermore, it was found that as a negative regulator of GA response, DELLA family proteins can restrain plant growth and the stabilization of DELLA proteins can improve plant survival on salt stress (Achard et al., 2006). However, the possible roles of GA in different cell types of root in regulating salt responses are still largely unknown.

In summary, plant hormones are highly involved in plant responses to salt stress. However, the cell type specificity of their regulation as well as temporal dynamics is less well understood.

1.3 Cell type specific study and approaches

A multicellular organism is composed of various organs that function differently. In each organ type, cells are differentiated to be distinct cell layers which possess their own identities and functions. In *Arabidopsis*, the root has radial symmetric and simple structure, and most importantly, it is the organ which can mostly and rapidly perceive environmental changes. Thus, it provides a good platform to investigate the cellular level of signaling in controlling plant development as well as the interactions between environmental changes and various cell types. In the following sections, the function of specific cell layers in root development and stress responses, as well as various approaches in understanding these aspects of biology, will be discussed.

1.3.1 Cell type mediated root development

In section 1.1, we have discussed the patterning process of various cell layers in Arabidopsis root. Here, we will look more into the unique structure traits and biological functions of different cell types.

Epidermis is the outmost cell layer which directly contacts with environment such as soil. Part of the epidermis can develop a specialized extension structure, root hair, which enlarges the whole root surface area in order to efficiently uptake water and nutrients from the soil. This type of epidermal cell is termed hair cell, which is distinguished from the non-hair cell (Duckett et al., 1994). Sophisticated studies have been done to understand the genetic control of this patterning process. GLABRA2, a HD-zip transcriptional factor, is specifically activated in the non-hair cells (Masucci and Schiefelbein, 1996), and the *gl2* mutant activates ectopic hairs in the non-hair cells (Cristina et al., 1996), suggesting a negative role of *GL2* in root hair development. Another protein, CAPRICE, has been found to be positive regulator of root hair. Although CPC also has a preferential expression pattern in the non-hair cells, the protein is able to move to adjacent cell types to suppress the expression of *GL2* (Wada et al., 2002). The distinct localization and function of genes ensures the correct patterning of root hair, which is important in modulating the capacity of taking up water and nutrients from soil. Besides root hair development, epidermis specific signaling is also found to be important in other aspects of root development. For example, brassinosteroid, a plant hormone, is found to control root meritstem size through functioning in epidermis. The epidermis specific expression (driven by *GL2*)

of *BRI1* rescues the reduced meristem size in *bri1* mutant (Hacham et al., 2011), suggesting a non-autonomous regulation of root development. Epidermis is also a key site for root gravitropism regulation through auxin signaling (Swarup et al., 2005).

Another example for cell type specific regulation of root development is the endodermis. Endodermis is a thin cell layer beneath epidermis and cortex cell layers. It has a unique structure called casparian strip. The lignin and suberin enriched structure seals adjacent cell walls and provides a barrier for the vascular tissues to prevent diffusion of solutes from the environment (Nagahashi et al., 1974). The specific patterning process determined by SCR/SHR module is discussed in section 1.1. Similar to the epidermis, endodermis is also able to incorporate hormonal signaling in controlling root development. Gibberellic acid (GA), which regulates cell elongation in plants, has been found to primarily act at endodermis in promoting primary root elongation (Ubeda-Tomás et al., 2008). These results clearly suggest that distinct genetic regulatory modules exist in specific cell types to maintain the unique root morphology and functions.

1.3.2 Cell type-mediated responses to environment stresses

During environmental changes, plants need to adjust their own internal signaling in order to adapt unfavorable conditions. Thus, to understand the responsive mechanism of plants to environmental changes is crucial. Interestingly, recent studies have reported that different cell types in the root have distinct transcriptional profile in response to various stresses.

As an essential nutrient for growth, nitrogen supplement is important in plant development. In response to exogenous nitrogen treatment, different gene clusters are differentially regulated in specific cell types of *Arabidopsis* root (Gifford et al., 2008). In this study, a microRNA, miR167, has been shown to be repressed by nitrogen in pericycle and lateral root cap cells and it mediates the regulation of lateral root formation during nitrogen treatment. This study highlights the integration of unique cell types and environmental cues in regulating certain aspect of root development. Besides nutrient supply, abiotic stresses have also been found to trigger cell type specific transcriptional responses in *Arabidopsis* root. For instance, under NaCl treatment, most differentially regulated genes are enriched in single cell layers and the biological functions enrichment of those genes could correlate with the unique phenotypic changes occurring in specific tissue layers during salt stress (Dinneny et al., 2008). For example, cell wall modification genes are found to be down-regulated by salt in epidermis, where root hair morphology has significant changes during early salt treatment. At the same time, the controlled over-expression of a salt transporter, *HKT1;1*, in the vascular tissues confers salt tolerance by reducing the transportation of Na+ to the shoot (Møller et al., 2009), while interestingly, constitutive expression of this gene cause hypersensitive plants in response to salt and higher Na+ accumulation in the shoot, which could be due to the excessive uptake of sodium from the media through outer tissue layers of the root. This result indicates the importance of cell type specific engineering in promoting salt tolerance.

1.3.3 Cell type-specific study approaches used in this study

To evaluate the spatial function of hormones, we need to spatially control the expression of the mutant genes that suppress hormone signaling. An enhancer trap trans-activation system, GAL4-VP16/UAS, has been developed (Haseloff, 1999) and widely used recently to mis-express genes.

GAL4 is a transcription activator in yeast, and it can bind to the UAS (Upstream Activation Sequence), while VP16 is a virus protein. The GAL4/VP16 hybrid protein activates the transcription of the gene downstream of UAS efficiently. In the first T-DNA, UAS-GFP is put within the same construct of GAL4/VP16 and transformed into plants to trap enhancers. The lines that have cell-type-specific GFP expressions can be used as drivers in our study. At the same time, the gene of interest is made to be controlled by UAS and transformed into plants. The expression of the gene of interest will keep silenced until GAL4/VP16 is present, so when genetic crosses are made between the GAL4 and UAS lines, the gene of interest will be expressed in specific cell types. Therefore, to use the enhancer trap system allows us to understand how specific genes or signaling components function in specific cell types during salt response.

In this study, we mainly use six different enhancer trap lines that have expression in different regions including root cap region, epidermis, cortex, endodermis, pericycle and stele (Fig. 9). Meanwhile, to disrupt hormone signaling, especially ABA signaling in this study, a dominant mutation in PP2C phosphatase, *abi1-1*, was ectopically expressed in various cell types in order to suppress ABA signaling in a

tissue specific manner. As a PP2C, ABI1 negatively regulates ABA signaling, and the single mutation ABI1 G180D constitutively represses ABA signaling by disrupting the interaction with PYR proteins (Park et al., 2009). Subsequently, the phenotype of the trans-activated lines can be inspected during salt stress.

To complement this approach, we also used various promoters, which have been found to be expressed in specific tissue layers, to drive the expression of *abi1-1*. For example, *SCARECROW* has a unique expression pattern in the endodermis (Di Laurenzio et al., 1996).

1.4 Objective and significance of this study

As described in the previous sections, root system architecture is regulated by intrinsic hormonal signaling as well as environmental factors, such as high salinity. However, there are still several aspects of detailed investigations lacking:

1) The specificity of salt response in different root types;

2) The dynamics of developmental suppression during salt stress;

3) Involvement of hormone signaling in regulation of root system architecture under salt stress;

4) The tissue specificity of hormone signaling under salt stress environment.

To further understand the above mentioned gaps in salt response, we performed the detailed analysis of the effect of salt on shaping the root system architecture. In this thesis, two major topics including the spatial and temporal regulation of salt stress on post-emergence lateral root growth as well as the effect of salt on early patterning process of lateral roots are discussed in Chapter 3 and Chapter 4, respectively.

From the first part of this research, we reveal the differential growth regulation of primary root (PR) and lateral root (LR) under salt stress and the essential roles of Abscisic Acid (ABA) during this process. Significantly, an inner tissue layer, endodermis, was found to act as ABA signaling target cell type to inhibit LR growth under salt stress. This result suggests the importance of certain cell identity in integrating environmental cues and hormone-regulated root development. Our results also suggest a general 'quiescence' in plant growth is necessary when facing extreme environmental changes in order to allow further growth recovery.

At the same time, we have demonstrated for the first time that high salinity is able to disrupt the early patterning process of LR through the primary root tip. Our results also suggest that this effect of salt on LR patterning could be the result of reduced auxin circulation and greatly altered distribution in the PR. This study provides a very detailed description of the effect of salt stress on various stages of LR development and direct evidence that, salt stress results in a lack of auxin maintenance in the PR, which eventually leads to the skewed distribution of LR patterning. In summary, this thesis provides detailed information on dynamics of salt regulation of LR

development, which could shed light on the further understanding of the mechanisms for plant root system regulation in response to environmental changes.

Chapter 2 MATERIAL AND METHODS

2.1 Plant materials

Arabidopsis thaliana ecotypes Columbia (*Col-0*), Landsberg erecta (*Ler*), and Wassilewskija (*Ws*) were used in this study. Mutants, *abi1-1*(Armstrong et al., 1995), *abi2-1*(Leung et al., 1997), *abi3-1*(Nambara et al., 1995), *della quadruple*(Tyler et al., 2004), and *RGA::GFP:RGA* (Achard et al., 2006) and the *SCR::gai-1-GR*(Ubeda-Tomás et al., 2008) transgenes are in the *Ler* background. The *abi4-1*(Finkelstein et al., 1998), *aba2-1*(Léon‐Kloosterziel et al., 1996), *aba2-sail* (SAIL_407_E12), *nced2*(Toh et al., 2008), *nced3*(Ruggiero et al., 2004), *nced5*(Toh et al., 2008), *nced9*(Toh et al., 2008), *fus3-3*(Keith et al., 1994), *era1-1*(Cutler et al., 1996), mutations and the *UAS::abi1-1* and *RAB18::GFP*(Kim et al., 2011), *DR5::luciferase* (Moreno-Risueno et al., 2010), *AUX1:YFP* (Swarup et al., 2004), *PIN2:GFP* (Blilou et al., 2005), *SCR:GFP* (Brady et al., 2007), *miR390a::GFP* (Marin et al., 2010), *GATA23::GUS* (De Rybel et al., 2010) and *CYCB1;1:GUS* (Colón-Carmona et al., 1999) transgenes are in the *Col-0* background. The *abi5-1* (Finkelstein, 1994) mutation is in the *Ws* background. *GAL4-VP16/UAS* enhancer trap lines *J3411*, *J0951*, *J2812*, *J0571*, *Q2500*, *Q0990*, *J0121* (Haseloff, 1998) are in the *C24* background.

2.2 Plant growth conditions

Seeds were surface sterilized by washing them in a 95% EtOH solution for 5 minutes followed by a 5-minute wash in a 20% Bleach, 0.1% Tween-20 solution. Seeds were then rinsed in sterile dH_2O four times and stored in water for 2 days at 4°C.

Sterilized seeds were grown on sterile 1% Agar media containing 1X Murashige and Skoog nutrients (MSP01-50LT, Caisson), 1% sucrose and 0.5 g/l MES, adjusted to pH 5.7 with KOH (termed "standard media"). Seedlings were grown for 6-7 days before transfer to standard media supplemented with NaCl or other chemicals for 4-5 days. Supplements include sodium chloride (NaCl, Sigma-Aldrich), Abscisic Acid (ABA, Sigma-Aldrich), 1-aminocyclopropane-1-carboxylic-acid (ACC, Sigma-Aldrich), Gibberellic Acid (GA, Sigma-Aldrich), Paclobutrazol (Sigma-Aldrich), Dexamethasone (Dex, Sigma-Aldrich), 3-Indoleacitic acid (IAA, Sigma-Aldrich). The position of the root tip was marked at the time of transfer to distinguish the two regions of the root, A and B. Growth of seedlings was performed in a Percival CU41L4 incubator at a constant temperature of 22°C with long-day lighting conditions (16 hours light and 8 hours dark). Plates were partly sealed with parafilm (Alcan Packaging) on 3 sides while the top of the plate was sealed with micropore tape (3M) to allow for gas exchange. This was important for allowing a sufficient number of lateral roots to grow for observation.

2.3 Transgene construction

2.3.1 Gene fragment cloning and plasmid constructions

The *UAS::abi1-1* construct was generated by mobilizing the *abi1-1* cDNA (with N-terminal triple myc tag) in plasmid pDONR207 (gift from J. Leung) into the plasmid pUAS-KWG (Karimi et al., 2002) between the UAS and NOS terminator sequences

using a Gateway (Invitrogen) cloning approach. Transgenic plants were generated by a standard floral dip method (Clough and Bent, 1998).

To generate the *SCR::abi1-1:RFP* and *COR::abi1-1:RFP* constructs, the primers 'caccATGGAGGAAGTATCTCCGGCG' and 'GTTCAAGGGTTTGCTCTTGAG' were used to PCR amplify the mutated *abi1-1* coding sequence without its stop codon from cDNA synthesized using *abi1-1* root RNA, and cloned into the Gateway compatible D-TOPO vector. Sequencing was performed using M13F 'GTAAAACGACGGCCAG' and M13R 'CAGGAAACAGCTATGAC' to verify the cloning fidelity and the insertion orientation. Free form of RFP was cloned in pDONRP2R-P3 gateway vector, which is a gift from Frederic Berger. Primers 'GGGGACAACTTTGTATAGAAAAGTTGgttgacaatgtgggctaactc' and 'GGGGACTGCTTTTTTGTACAAACTTGggttttggctaatgtgattgtg' were used to clone around 1.2kb of CORTEX (AT1G09750) promoter into the pDONRP4-P1R gateway vector. M13F and M13R primers (as mentioned above) were used to examine the fidelity of cloning. 2kb of *SCARECROW* promoter was also cloned in pDONRP4-P1R. Multisite Gateway (Invitrogen) recombination was use to introduce *SCR::abi1-1:RFP* and *COR::abi1-1:RFP* into a dpGreen-based binary vector, which contains a Kanamycin resistant gene for plant selection.

To generate the *SCR::abi1-1* construct, the primers 'caccATGGAGGAAGTATCTCCGGCG' and 'TCAGTTCAAGGGTTTGCTCTTG' were used to PCR amplify the mutated full length *abi1-1* coding sequence from cDNA synthesized using *abi1-1* root RNA, and cloned into the Gateway compatible

D-TOPO vector. Multisite Gateway (Invitrogen) recombination (Karimi et al., 2005) was use to introduce a *SCR::abi1-1* minigene into a modified dpGreen-based binary vector, which contained a *35S::PM-mCherry* selection marker in place of the Kanamycin resistance gene (pCherry-pickerT).

One Shot® TOP10 Chemically Competent E. coli cells (Invitrogen, C4040-03) was used for propagate plasmids during each cloning steps. And the DNA from BP reaction or LR reaction was added into the cells followed by incubating on ice for 5 to 30 minutes. Then the cells were heat-shocked for 30 seconds at 42°C without shaking before transferred to ice immediately. Add 500µl S.O.C medium (Tryptone (pancreatic digest of casein) 2% (w/v), Yeast extract 0.5% (w/v), NaCl 8.6 mM, KCl 2.5 mM, MgSO4 20 mM, Glucose 20 mM). Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour. Spread the adequate amount from each transformation on a selective plate and incubate overnight at 37°C. Single colonies can be inoculated in LB liquid medium (Tryptone, 10.0 g, Yeast Extract, 5.0 g, NaCl, 10.0 g) with the selection antibiotics for 12-16 hours before used for plasmid extraction (Promega, Wizard® Plus SV Minipreps DNA Purification System).

2.3.2 Agrobacteria-mediated plant transformation

Agrobacteria strain, GV3101 cells, was used for carrying the transgenes and infecting plants. To transform agrobacteria, the binary vectors with transgenes were added to electric competent GV3101 cells on ice. Then the mixture was added into the 1 mm electroporation cuvette (Bio-rad) and shocked with 1.8 kv electricity. LB liquid medium was added to the cells and incubate at 28°C for 2 hrs before spreading on

selective LB plates. After growing under 28°C for 2-3 days, colony PCR was performed on the single colonies in order to verify the transgenes.

Single colonies of GV3101 cells were than cultured in large petri dishes (150 mm diameter) on selective LB agar media for 2 days at 28°C. 50 ml of infiltration media (1/2X MS salts, 0.03% Silwet L77 and 5% sucrose adjusted to pH 5.7 with KOH) was poured on top of the LB agar media and cells were scraped off into the solution. The cell suspension was homogenized by gentle shaking for 5 seconds in a 50 ml conical tube. The cell suspension was then diluted with an additional 150 ml of infiltration media and the combined solution used for floral-dip mediated plant transformation.

Arabidopsis plants were grown at 5-8 seedlings per pot. The primary shoot of the flowering plants was cut off and we waited for around 7 days for secondary shoots to emerge. This method provided considerable more flowers on the plants. Then the plants were dipped in the prepared agrobacteria infiltration media for 30 seconds. Plant pots were then put horizontally under dark for 24 hrs before taken out. Seeds were harvested from treated plants and selected based on Kanamycin resistance or visually based on mCherry fluorescence using an M165 FC fluorescence microscope (Leica).

2.4 Microscopic analysis

For quantitation of lateral root developmental stages, roots were mounted in a modified Hoyer's solution (chloral hydrate:water:glycerol in proportions 8:2:1,

g/ml/ml), then imaged using a Leica DMI6000 inverted compound microscope. Scoring for LR stages was done according to previously described definition (Malamy and Benfey, 1997). The GUS staining protocol was performed as previously described (Swarup et al., 2008). For confocal microscope imaging, roots were mounted in an FM4-64 solution (Invitrogen) (Levesque et al., 2006), and imaged using a Leica SP5 point-scanning confocal microscope. The imaging settings are 488 nm excitation and 505-550 nm emission for GFP, 514 nm excitation and 520-560 nm emission for YFP and 488 nm excitation and >585 nm emission for FM4-64. A Leica fluorescence dissection microscope was used to monitor RAB18 expression level changes in the LRs over time. LR images for RAB18 expression were taken through petri dishes and MS media. RFP images for the transgenic plants *SCR::abi1-1:RFP* and *COR::abi1-1:RFP* were taken under the Leica DMI6000 inverted fluorescence compound microscope under the DSRed filter.

2.5 Phenotypic analysis

Images of 10 days old seedlings were captured using a CanonScan 9000F flatbed scanner (Canon). The sample size for all the analyses was 15-20 roots. LR and PR length was quantified using ImageJ (Abramoff et al., 2004).

For live-imaging analysis of lateral root growth, a custom live-imaging system was developed and consisted of the following: samples were manipulated using a circular platform with six tissue-culture plate holders, which is controlled by an automated Theta/360 degree rotary stage and MFC-2000 controller (Applied Scientific

Instrumentation), samples were backlit using an infra-red LED panel, images were captured using a digital monochrome camera (CoolSnap) fitted with an NF Micro-Nikor 60mm lens (Nikon) and Infra-red filter. Micro-Manager Software (Vale Lab, UCSF) was used to control the stage and automate image acquisition (Edelstein et al., 2010). Images were taken every 15-20 minutes for up to 7 days. Sequential images were collated as a stack for further analysis using imageJ. The StackReg plug-in was used to align the stack of image slices before root growth quantitation. Quantification of data from the time-lapse movies was conducted using the Manual Tracking plug-in for ImageJ. The growth of the root tip was tracked manually from frame to frame.

To determine the effect of salt stress on cell-cycle activity in LRs the expression of the *CYCLINB1;1:GUS* reporter was quantified in seedlings transferred to standard or 100 mM NaCl conditions for 3 days. The total number of GUS positive and GUS negative LRs was counted for LRs where obvious elongation and maturation zones had developed ("long LR") or where no such zonation had developed ("short LRs").

To monitor dynamic changes in ABA signaling in LR, fluorescence of the *RAB18::GFP* reporter was imaged on a Leica fluorescence dissecting microscope in real time. LR length and GFP fluorescence intensity at each time points were quantified using ImageJ (Abramoff et al., 2004).

2-way ANOVA and Student's t-test were used to test for statistical significance in root length measurements using a p-value threshold of less than 0.05 and a bonferroni correction based on the number of tests performed.

2.6 Genetic analysis

To selectively express *abi1-1* in different tissue types, various enhancer trap lines were crossed to plants harboring the *UAS::abi1-1* transgene. Wild-type plants of the C24 ecotype were crossed with *UAS::abi1-1* plants to generate the control genotype. Phenotypic and gene expression analysis were performed using the F1 seeds. To evaluate whether salt-dependent changes in RGA protein stability are ABA-signaling dependent, *RGA::GFP-RGA* transgenic plants were crossed with the *abi1-1* mutant or to the *Ler* ecotype to generate the control genotype. F1 seeds from these crosses were used for confocal image analysis of GFP-RGA fluorescence under standard or salt stress conditions.

2.7 Gene expression

For quantitative RT-PCR analysis of gene expression in different root types, root RNA was extracted from root region A or region B separately using RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions. cDNA was prepared using the iScript advanced cDNA synthesis kit (Bio-Rad) from 300 ng of total RNA. Q-PCR was performed on a Fluidigm BioMark 96.96 Expression Chip using EvaGreen (Bio-Rad) as the fluorescence probe according to the Fluidigm Advanced Development Protocol #37. AT3G07480 was used as a control gene. Genes accession numbers and primers used in these experiments are listed in Table 1.

Table 1. Accession numbers of analyzed genes and primers used in the Real-time quantitative PCR analysis.

Gene name	AGI number	Forward primer	Reverse primer
Internal control	AT3G07480	CTCTTCAGAAACTCTCCTCTCAA	ATTCCTCTGCGATCTGAACCTC
AtWRKY46	AT2G46400	GAGGAAAGTATCGGAGAAGAACA	GTTACAAGTGTGGTTTCCGAG
ABI1	AT4G26080	TCTCAGGTAGCGAACTATTGTAG	TGGTCAACGGATAATGGAAGTG
RD29A	AT5G52310	GCACCCAGAAGAAGTTGAACAT	GAATAATTTCCTCCGATGCTGG
DREB2A	AT5G05410	AAGGAGGACCAGAGAATAGCCG	GAACACAACCAGGAGTCTCAAC
ABI2	AT5G57050	GATCACAAACCGGATAGGGA	CCATCGCGTTCTTCTTATGC
ATNCED3	AT3G14440	AGTGTCCTGTCTGAAATCCG	ATGTATCCTTCGTCTTCCTCTCC
ATNCED5	AT1G30100	GCCATAGCTGAACCGTGG	GAAAGTGCCGTGGAAACCATAC
FUS3	AT3G26790	ACAGGCGATTTTGTGAAT	TGTGGTGACATCATCTATGACTG
MIZ1	AT2G41660	CGCGTTGGAGTGCGAGAA	GCCTCTCATATACAACAACTCAC
DREB1B	AT4G25490	GCTCGGGACTTTCCAAACC	TAAATAGCTTCCACCATCGTCTC
ABF3	AT4G34000	CCTGCAACACAGTGCCAG	TTTGCCTTCTCTCAATCACTTTC
AFP1	AT1G69260	GGAAACTAAAGCGTCCAGTGAC	ATGGCAAACACACATGATCC
GA2OX6	AT1G02400	CAAGTTCAGCTCGGCGAC	GAGCAAATCTCAAGTCCATCTAC
ACH1	AT1G08090	CGCCGAGTACTTCTTTGACAGGT	ATAGAGAAGAGCACCATAGCCAC
SOS1	AT2G01980	ACCAAATTAAGAGGTGTCTGGG	AAACAACAATCGCCGTCCCATC
ABI3	AT3G24650	CAGCTTCTGCTATGCCACGTC	CACAAAATCGCCGGTGTTC
ABI4	AT2G40220	CTTCCTTAATGGTGGGACCTC	ACCAGCTAGAGAGTTCAAATCC
ABI5	AT2G36270	ACAGCAAATGGGAATGGTTGG	AACTCCGCCAATGCATGTTT

ABA1	AT5G67030	TCGATGCTTGACTGGGTCCT	TCGAAACCTGAGACGAAGG
ERA1	AT5G40280	ACATTTTGGCAGGCAGCC	CCACCGTCAGGGATCTTAGA
ATHB12	AT3G61890	AGCAATCTCTGGTCTCTGAGC	TCAAGCAACTATCATCAGCTTTC
SNRK2.2	AT3G50500	AATATGCTGCTGGTGGAGAAC	CCTGTCGAAGAAGAATCTCTGG
SNRK2.3	AT5G66880	GAACTTTACGAGCGGATTTGC	TCTTGCCATCATATTCCTGACG
SNRK2.6	AT4G33950	GGTTGCAGATGTTTGGTCTTG	ACTGAGTGGTCATCGTGTTATC
SNRK2.7	AT4G40010	TGGAAAGATTGCAGATGTGTGG	CCATTATCGCATTTCTCTTCCTCC
SNRK2.9	AT2G23030	TTCTGTTCTGCACTCCAACC	GCGTACTTCTATGTAACGGATTG
MYC2	AT1G32640	CGAGTGCTAGTGGAGGAGATATG	TTGCTCTGAGCTGTTCTTGC
NRT1.1	AT1G12110	AAAGGTATGAAAGGGATGAGCAC	CCATTGGAATACTCGGCTCATC
NAC2	AT5G04410	ACTCCATCTTCATTTGCTTCACAG	GGAGAACCCATAAACACATGAAGAC
WRKY65	AT1G29280	ACAAGAAACGGGCCTAAACC	AGACTCATCCTCTTCTCCCATC
XERICO	AT2G04240	CAGAGTCATTTGATTTCCGGGTC	CTGATGGTCTTCTGGCACAAC
GA3OX1	AT1G15550	ACTCCACCCTCCTAACCATTC	GGCGATTCAACGGGACTAAC
GAMT2	AT5G56300	TCGAGCCAAACATCCTTTCAC	CCATAGGACGAATAGCAGCTTG
GID1A	AT3G05120	CGCGATTGGTACTGGAAAGC	TACAAACGCCGAAATCTCATCC
GID1B	AT3G63010	TGTAATCCCTTTGGCCCGAG	AGGAGTAAGAAGCACAGGACTTG
GID1C	AT5G27320	TAACCCTATGTTTGGAGGGACC	CCTCTTGACCCGCTTTCTTG
RAB18	AT5G66400	AAGATCAAGGAGAAGTTGCCAGG	GTAAACAACACACATCGCAGGACG
RGA1	AT2G01570	GGAAGGAGTTCCGAATAGTCAAG	TAATGAGTGGACGAGTGTGC
RGA2	AT1G14920	TCGACGTTGTTTGACTCGTTG	CCAACCCAACATGAGACAGC
SKP2A	AT1G21410	TCTAGCAGACTGGTGTGTCC	GTTAAGTTCAGACAGCCGCTC

SLY1	AT4G24210	GTTTCACTAAGAGACCTC TTCCAG	CCAACTTAACTTCACGACAG AACC
Peroxidase	AT4G16270	ACCACGGAGACAACCTTG AG	GCTATTACTACCACCGGGTA TCC
Peroxidase	AT4G11290	TCCTCCTTCTCAAACCGTC TCTTC	GCAAAGAACTCTTGCTCCGA TCC
Peroxidase	AT1G05260	CCTAGCCTCAACGATAAC AAGACC	AACGGAACATTGCCTCCTAA CC
Peroxidase	AT1G44970	AACGGTAATAACCAACCA GACG	ACCATCGACTTTGCGAATTG
Peroxidase	AT5G42180	GCTGGATCGAACATGGAT GG	GTGATGTTGGAAGCGTAGA TTGAG
Peroxidase	AT5G66390	ACTATTGCGTCAACGATG TCC	TACGCCTGATCTCTCCCTTG

Chapter 3 RESULTS AND DISCUSSION 1

3.1 ABSTRACT

The endodermal tissue layer is found in the roots of all vascular plants, and functions as a semi-permeable barrier, regulating the transport of solutes from the soil into the vascular stream. As a gateway for solutes, the endodermis may also serve as an important site for sensing and responding to useful or toxic substances encountered in the environment. Here we show that high salinity, an environmental stress widely impacting agricultural land, regulates growth of the root system through a signaling network operating primarily in the endodermis. We report that salt stress induces an extended quiescence phase in post-emergence lateral roots (LRs) whereby the rate of growth is suppressed for several days before recovery begins. Quiescence is correlated with sustained Abscisic Acid (ABA) response in LRs and is dependent upon genes necessary for ABA biosynthesis, signaling and transcriptional regulation. We utilize a tissue-specific strategy to identify the key cell layers where ABA signaling acts to regulate growth. Mis-expressing the abi1-1 mutant protein, which dominantly inhibits ABA signaling in the endodermis, leads to a near complete recovery in LR growth under salt-stress conditions. Gibberellic Acid (GA) signaling, which antagonizes the ABA pathway, also acts primarily in the endodermis and we define the cross talk between these two hormones. Our results define the endodermis as a gateway with an ABA-dependent guard, which prevents root growth into saline environments.

3.2 INTRODUCTION

Root system architecture (RSA) is a complex emergent property of the root that arises due to the growth attributes of the primary root (PR) and individual lateral roots (LRs). In Eudicot species, the PR is specified during embryogenesis and establishes the majority of the root system through the production of LRs along its length. In Arabidopsis, the patterning of LR founder cell populations occurs at regular temporal intervals and leads to the specification of founder cell populations that later develop into LR primordia (De Smet et al., 2007; Moreno-Risueno et al., 2010). Subsequent to primordia formation, the process of LR emergence requires that the primordium communicate with the outer tissue layers of the root to coordinate changes in tissue-integrity with the destructive process of outgrowth (Swarup et al., 2008). Much attention has focused on these initial stages of LR development, and auxin signaling and transport are critical components at every stage of this process (Benkova et al., 2003; De Smet et al., 2007; Dubrovsky et al., 2008; Laskowski et al., 2008; Moreno-Risueno et al., 2010; Overvoorde et al., 2010).

Post-emergence LR development is tightly controlled by environmental stimuli such as nutrients and water stress (Lopez-Bucio et al., 2003; Malamy, 2005). Studies have demonstrated that spatial variation in nutrient conditions of the surrounding media can have important effects on the resultant RSA. Nitrate, for example, can inhibit LR elongation through competition with auxin transportation, and by affecting mitotic activity (Little et al., 2005; Okushima et al., 2011). Localized iron, on the other hand, promotes LR growth by triggering auxin accumulation in root apices through the regulation of gene expression of the auxin influx carrier, *AUX1* (Giehl et al., 2012). Intrinsic differences in the environmental response programs of PRs and LRs have

also been observed. Phosphate promotes PR growth and inhibits LR growth (Zhang and Forde, 1998; Linkohr et al., 2002), though the mechanistic basis for these differences are unknown. The water stress-associated hormone, ABA, is a known inhibitor of LR development, and has been found to act at the post-emergence stage (Signora et al., 2001; De Smet et al., 2003). ABA has a much stronger effect on LR than PR growth, suggesting that differences in environmental responsiveness between these two root types may be due to divergent hormone signaling networks.

Recent work using cell-type specific profiling methods has demonstrated that tissue identity is critical for providing a context in which environmental responses are interpreted at the cellular level (Dinneny, 2010; Wee and Dinneny, 2010). Changes in salinity, nutrient content, pH and oxygen concentration all elicit a large number of transcriptional and post-transcriptional changes that substantially differ between each cell type (Dinneny et al., 2008; Gifford et al., 2008; Mustroph et al., 2009; Iyer-Pascuzzi et al., 2011). While these data have led to a sophisticated understanding of the biological pathways targeted by environmental regulation, it is still unclear what the tissue-specific regulatory mechanisms are that control these changes.

Plant hormones are important secondary-signaling molecules that mediate the response to many environmental stimuli, and recent work has shown that several of these hormones promote growth primarily by acting in specific tissue layers. Gibberellic Acid (GA) signaling leads to the degradation of DELLA-proteins, which negatively regulate growth primarily in the endodermal tissue layer (Ubeda-Tomás et al., 2008; Ubeda-Tomas et al., 2009) while brassinosteroid signaling acts in the

epidermis to promote shoot growth (Savaldi-Goldstein et al., 2007) and regulate root meristem size (Gonzalez-Garcia et al., 2011; Hacham et al., 2011). These results suggest a possible mechanism by which environmental stimuli elicit localized transcriptional changes through the action of hormones that trigger tissue-specific signaling cascades. Hormones associated with stress responses also regulate growth of the plant (Spollen et al., 2000; Sharp and LeNoble, 2002; Achard et al., 2006), but it is not known whether the growth-suppressing activities of these hormones also act in specific cell layers.

Here we dissect the tissue-specific function of ABA signaling in regulating root growth to reveal the mechanism by which salt controls RSA. High salinity is an important and prevalent agricultural contaminant that affects yield (Flowers, 1997), and previous studies have shown that elevated salt levels can inhibit both PR and LR growth (Burssens et al., 2000; He et al., 2005). We reveal that PR and post-emergence-stage LR growth show divergent temporal dynamics during salt stress, which is due to LR-specific ABA signaling. We identify the endodermis as the key tissue layer required for ABA-mediated growth repression in the LR, and dissect points of cross talk with the GA pathway in this process. These data provide insight into a previously uncharacterized function of the endodermis as a sentinel cell layer, which guards against growth into saline environments.

3.3 RESULTS

3.3.1 The growth of primary and lateral roots is differentially affected by salt stress

RSA is determined by three fundamental parameters: 1) The number of LR branch points specified along the PR, 2) The initiation and emergence rates for these LR primordia and 3) The growth rate of each root type, PRs and post-emergence LRs. To quantify the effect of salt stress on these three parameters, Arabidopsis seedlings were grown on standard media for 6 days then transferred to media supplemented with NaCl and grown for an additional 4 days. This treatment established two regions along the PR: region A, in which the PR had grown before the transfer to salt conditions, and region B, in which the PR had grown after the transfer (Fig. 3A). Based on recent work, the patterning of LRs is determined close to the root tip and initiation of LR primordia from founder cells occurs later in development (De Smet et al., 2007; Moreno-Risueno et al., 2010; Overvoorde et al., 2010). Thus, the LR patterning process in region A is expected to have occurred before treatment of roots with high salinity. Consistent with this hypothesis, we found that high salinity has little effect on the total number of LRs initiated in region A (Fig. 3B). Furthermore, we did not observe any strong effects on the distribution of developmental stages for pre-emergent LR primordia (Fig. 3B).

While the total number of LRs initiated and the developmental progression of pre-emergent LR primordia is not affected in region A, measurement of the average post-

emergence LR length or the total root length for all post-emergence LRs revealed that salt has a strong inhibitory effect on growth at this stage of development (Fig 4). Interestingly, PR growth was much less sensitive to salt treatment compared with LR growth (30.7% compared to 87.5% reduction, respectively) (Fig. 4C). These differences were observed in multiple accessions of Arabidopsis, despite differences in overall salt sensitivity (Fig. 5). Thus, PRs and LRs have unique salt-stress response programs, which likely indicate different priorities for these two organ types in the natural environment.

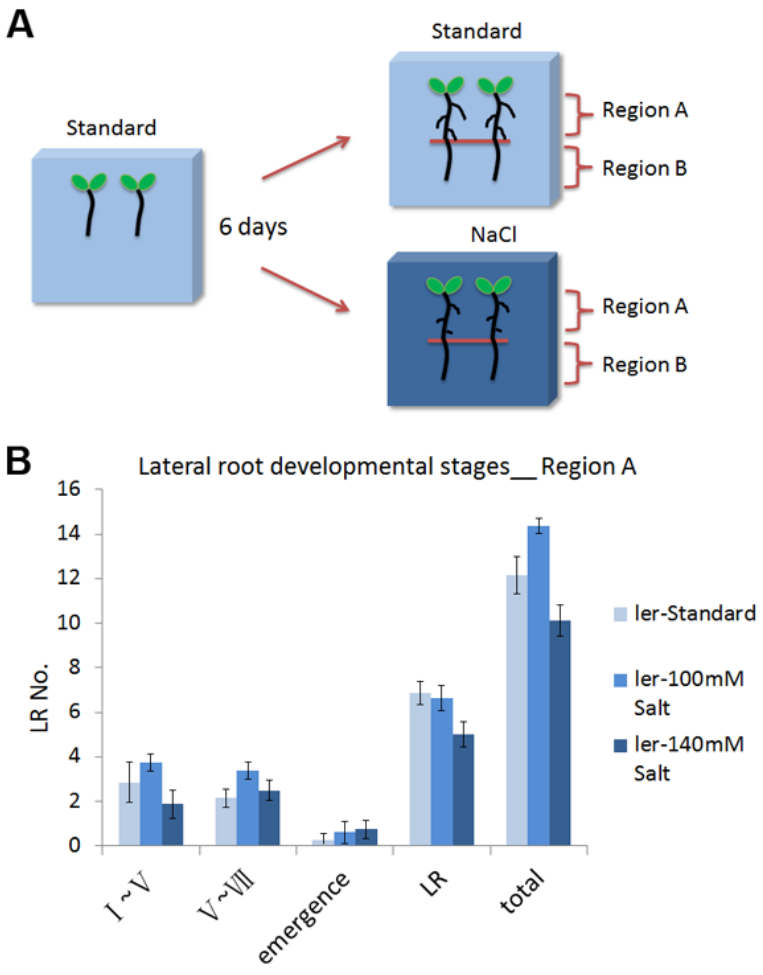

Figure 3. High salinity has minor effect on LR specification on region above the transfer point.

(A) Diagram of the transfer experiment, which divides roots into two distinct regions.

(B) Quantification of pre-emergent LR stages as well as mature LRs in root region A after transfer to standard or salt-stress conditions. Error bars indicate standard error of the mean (SEM).

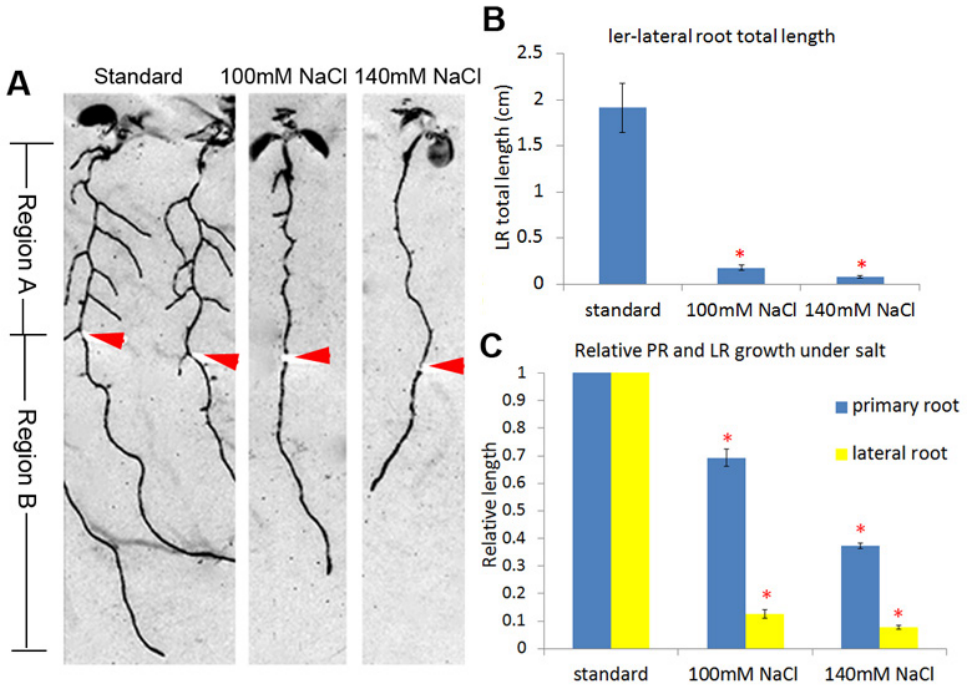

Figure 4. High salinity causes more suppression on LR growth than PR growth.

(A) Morphology of the Ler root system under standard, 100 mM NaCl and 140 mM NaCl media conditions. Red arrowheads mark the position of the root tip at the time of transfer from standard to salt-stress conditions.

(B) Total LR length of Ler under two different concentrations of NaCl.

(C) Average PR and LR length at 100 mM and 140 mM NaCl relative to their length under standard conditions.

Error bars represent standard error of the mean. Red asterisks mark significant changes in salt response, Student's t-test.

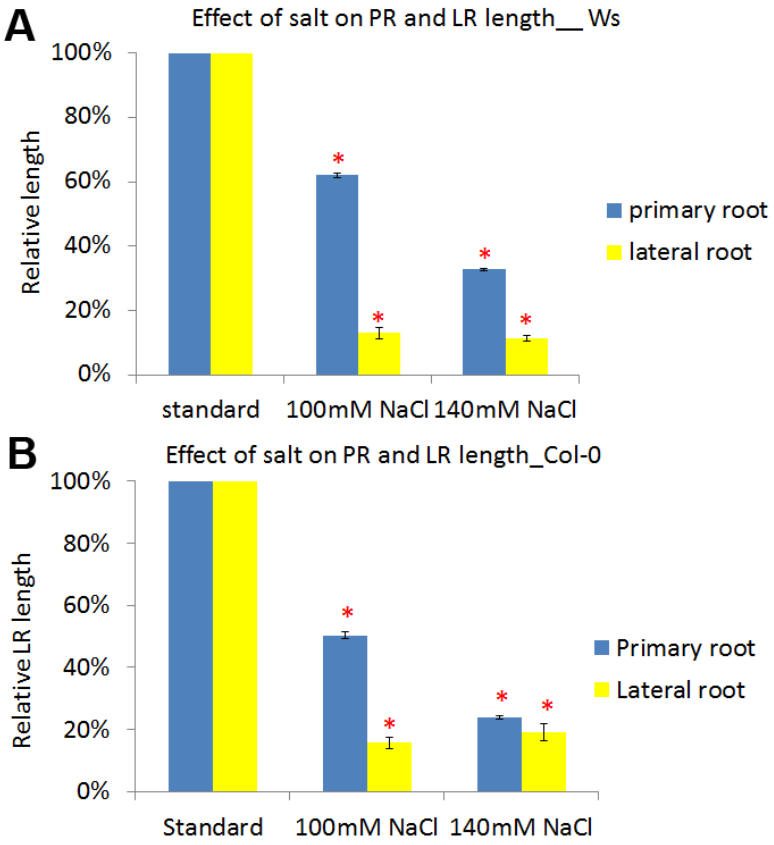

Figure 5. Similar effect of salt stress can be observed in multiple ecotypes.

(A) Relative PR and LR length of Ws under two different concentrations of NaCl.
(B) Relative PR and LR length of Col-0 under two different concentrations of NaCl.

Error bars: SEM; Red asterisks represent significant differences based on Student's t-test for percentage difference.

3.3.2 ABA signaling is necessary for LR growth suppression during salt stress

ABA acts as an important secondary-signaling molecule during abiotic stress (Sharp and LeNoble, 2002; Zhu, 2002; De Smet et al., 2003; Hubbard et al., 2010). Under high salinity, the production of ABA is induced and its signaling pathways are necessary for salt tolerance (Achard et al., 2006). We first treated roots with varying concentrations of ABA to determine if this hormone treatment has a similar effect as high salinity. As previously shown (De Smet et al., 2003), ABA treatment affected LR growth more severely than PR growth (68% compared to 24% reduction, respectively) (Fig. 6A, B). These effects were observed in several different ecotypes (Fig. 6C, D). To determine whether LRs are generally more sensitive to the effects of growth-suppressing hormones, we treated seedlings with ACC (1-aminocyclopropane-1-carboxylic-acid), a biosynthetic precursor to ethylene, which is also induced under salt stress (Achard et al., 2006). Interestingly, ACC treatment caused the opposite trend in growth, having a much stronger effect on PR than LR growth (78% compared to 42% reduction, respectively) (Fig. 7A, B). Other ecotypes also showed similar responses (Fig. 7C, D).

Together, these results indicate that LRs and PRs are distinguished based on their hormone and stress-response profiles. The similarity in the response of the root system to NaCl and ABA suggests that this hormone may be most critical for eliciting the suppression of growth in LRs.

To test whether endogenous hormone signaling is responsible for RSA changes during salt stress, we measured the effect of salt on average LR length for various

mutants affecting the ABA pathway. Multiple mutants tested caused reduced sensitivity of LR growth to NaCl. This analysis identified mutations affecting ABA biosynthesis (*aba2-1*, *aba2-sail*), signal transduction (*abi1-1, abi2-1*) and transcriptional regulation (*abi4-1 fus3-3*) that mediate salt-dependent LR growth suppression (Fig. 8A). Importantly, none of these mutations significantly affect PR growth under these conditions (Fig. 8B).

Based on the differential response of LRs and PRs to exogenous ABA treatment, which eliminates the possibility of different ABA biosynthesis in different organs, we hypothesize that differences in signaling, and not biosynthesis, are likely to be the basis by which these two root types are distinguished.

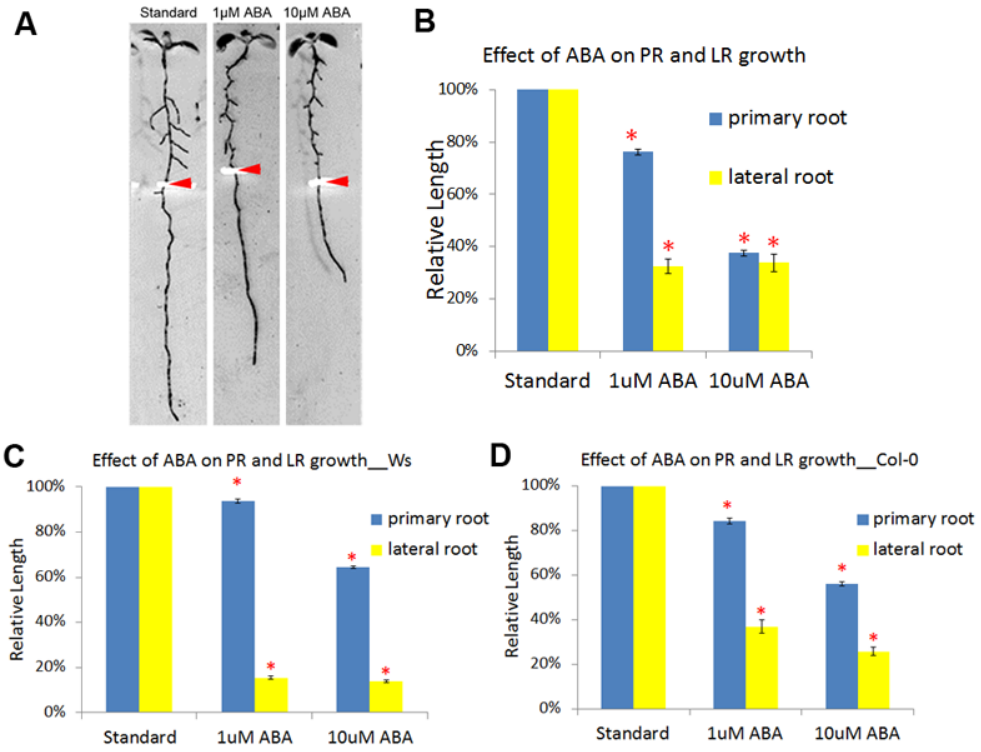

Figure 6. Effect of ABA on LR and PR growth in various ecotypes.

(A) Ler root system at 1 μM or 10 μM ABA. Red arrowheads mark the position of the root tip at the time of transfer from standard to hormone-supplemented conditions.

(B) Relative PR and LR length after treatment with 1 μM or 10 μM ABA.

(C) Relative length of PR and LR of Col-0 under two concentrations of ABA.

(D) Relative length of PR and LR of Ws under two concentrations of ABA.

Error bars: SEM; Red asterisks represent significant differences based on Student's t-test for percentage difference.

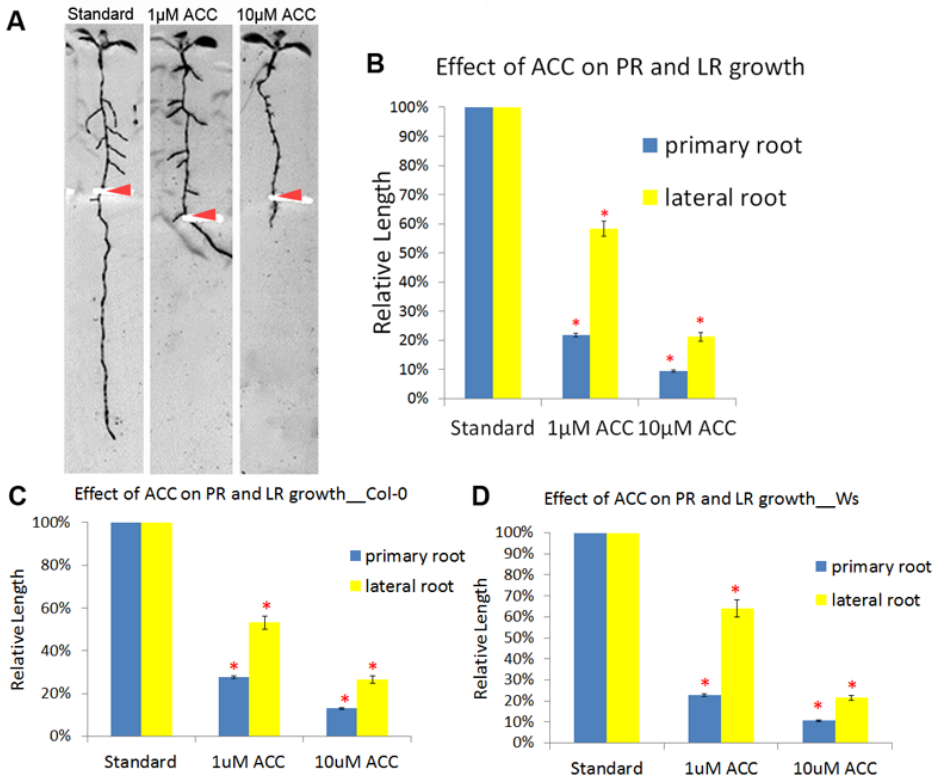

Figure 7. Effect of ACC on LR and PR growth in various ecotypes.

(A) Morphology of the Ler root system under ACC treatment.

(B) Relative length of PR and LR of Ler under two concentrations of ACC.

(C) Relative length of PR and LR of Col-0 under two concentrations of ACC.

(D) Relative length of PR and LR of Ws under two concentrations of ACC.

Error bars: SEM; Red asterisks represent significant differences based on Student's t-test for percentage difference.

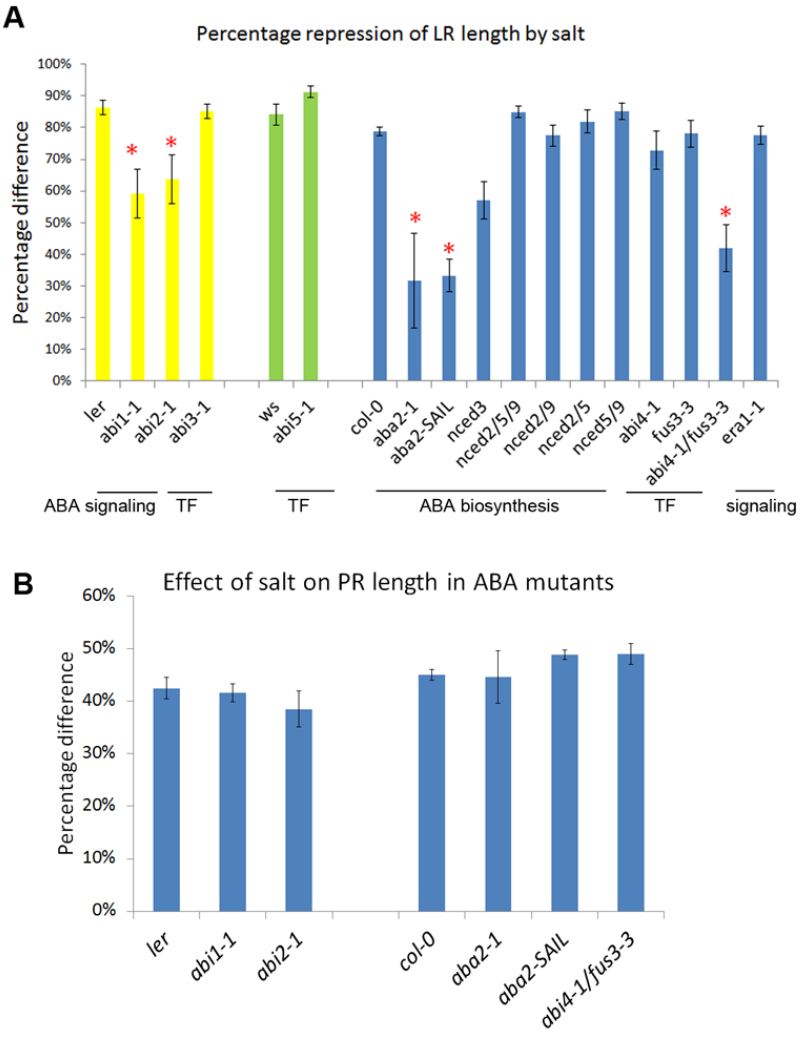

Figure 8. ABA mutants show reduced sensitivity to salt in LR growth

(A) Suppression of LR growth under 100 mM NaCl conditions measured as a percent difference relative to standard conditions for the various genotypes. Mutations in the Ler background are shown in yellow bars, Ws background in green bars and Col-0 background in blue bars.

(B) Percentage difference in PR length in different ABA mutants which have reduced sensitivity in LR length upon 100 mM NaCl treatment.

Error bars: SEM; Red asterisks represent significant differences based on Student's t-test for percentage difference.

3.3.3 The endodermis is the target cell layer for ABA-dependent salt-stress regulation of LR growth

Several plant hormones have been shown to regulate growth through tissue-specific signaling (Swarup et al., 2005; Savaldi-Goldstein et al., 2007; Ubeda-Tomás et al., 2008; Ubeda-Tomas et al., 2009). To determine if ABA operates by a similar principle, we utilized the GAL4-VP16/UAS enhancer trap system (Fig. 9) to drive tissue-specific expression of abi1-1, which dominantly suppresses ABA signaling (Kiegle et al., 2000; Ubeda-Tomas et al., 2009). ABI1 encodes a PP2C-type phosphatase and ABA co-receptor, which dephosphorylates SnRK-type kinases to inhibit ABA-dependent signal transduction (Meyer et al., 1994; Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). The *abi1-1* mutation renders the protein constitutively active. We verified that the enhancer-trap lines used in our study drive strong expression in the various cell layers of post-emergence LRs using the associated *UAS::erGFP* reporter (Fig. 9).

We first determined in which cell layers ABA acts to regulate root growth. F1 seedlings generated by a cross between the *UAS::abi1-1* transgenic plant and the various enhancer trap lines conferred insensitivity of LR growth to ABA treatment when expression was driven in the ground tissue (J0571), endodermis and pericycle (Q2500) or stele (Q0990) (Fig. 10A, C). This pattern of activity partially overlaps with the cell layers observed to be important for ABA signaling in the PR, where enhancer traps driving *abi1-1* expression in the epidermis and cortex (J2812), ground tissue layers (J0571) and endodermis and pericycle (Q2500) had a significant effect

while stele expression (Q0990) did not (Fig. 10D). These results indicate that *abi1-1* dependent ABA signaling acts to regulate growth in a spatially restricted fashion and that the ground tissue layers are common sites for such regulation in the PR and LR.

We next asked whether salt stress-dependent ABA signaling acts through similar tissue layers as exogenous ABA treatment. Interestingly, *abi1-1* was most effective at rescuing LR growth when expressed in the endodermal and pericycle tissue layers of LRs (Q2500) and less so in the ground tissue as a whole (J0571) or when expressed in the epidermis and cortex (J2812) (Fig. 10B, C). Use of a pericycle specific enhancer trap (J0121) to drive *abi1-1* expression had a very mild effect on LR length during ABA and salt treatment (Fig. 13A, B), which suggests that endodermal expression of *abi1-1* in the Q2500 line, is the primary cause of salt and ABA insensitivity. Expression of *abi1-1* in the various tissue layers had little effect on PR growth (Fig. 10D), consistent with the LR specificity of the ABA response we inferred from our genetic analysis. Importantly, while our media contains 1% sucrose, we did not find that this significantly affected the ABA-dependent regulation of LR growth during salt stress treatment (Fig. 14).

To confirm the function of ABA signaling in ground tissue during salt stress, an endodermis specific gene promoter, SCARECROW (SCR) (Di Laurenzio et al., 1996), and a cortex specific promoter, COR (Lee et al., 2006), were used to drive the expression of an *abi1-1:RFP* translational fusion. Two independent *SCR::abi1-1:RFP* transgenic lines showed a rescue in LR growth under both ABA and salt treatment (Fig. 11A). Fluorescence imaging of these lines revealed endodermal-specific

localization (Fig. 11C). These results were further confirmed by transforming plants with a *SCR::abi1-1* transgene lacking any fluorescence tag (Fig. 12A, B). The *COR::abi1-1:RFP* lines also showed a mild rescue in LR growth with ABA or salt treatment (Fig. 11B). However, we found that the *abi1-1:RFP* fusion showed cortex and endodermal localization (Fig. 11D). Together, our data indicate that ABA signaling acts primarily in the endodermis to limit LR growth during salt-stress conditions, though the cortex may also have some importance in this process, as well. These data also show that the pattern of activity for the ABA pathway is environment dependent, with salt acting through a more restricted domain of the root than with exogenous ABA treatment.

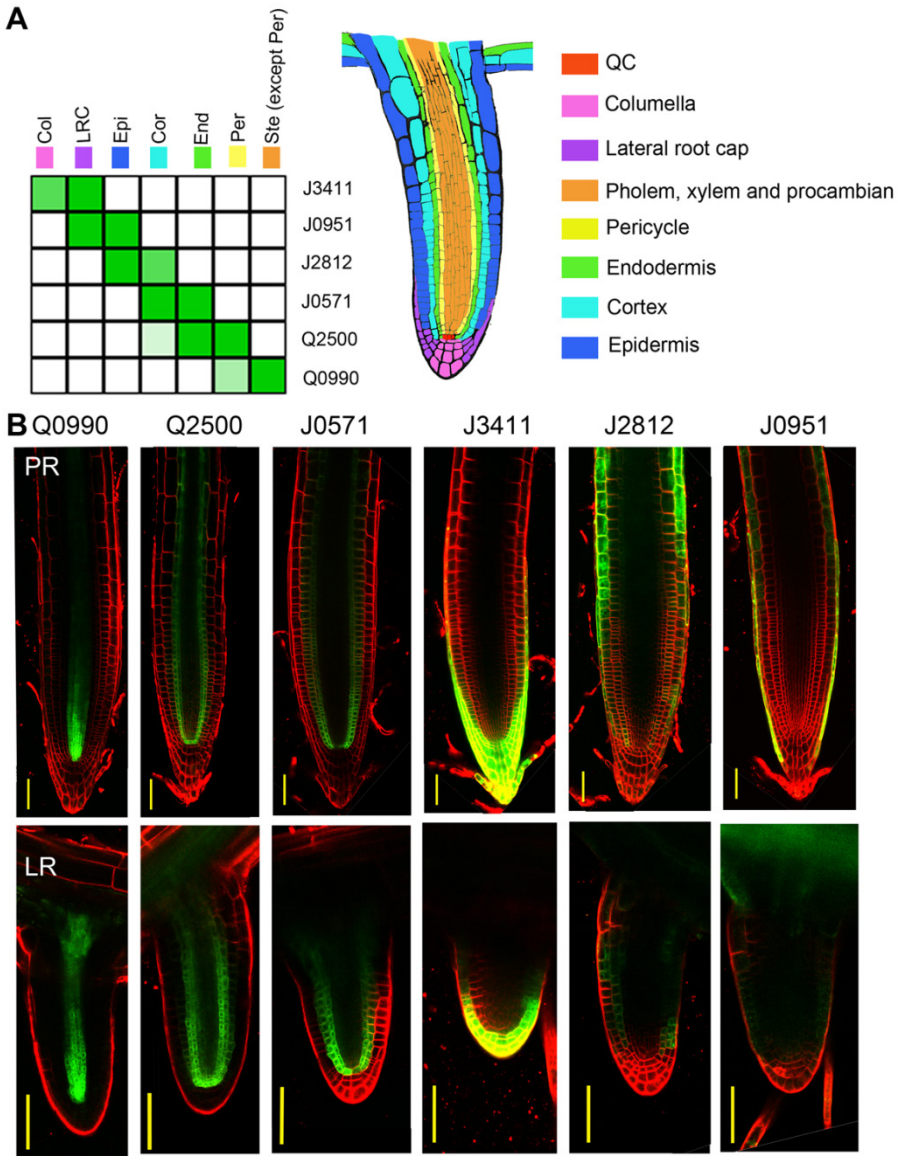

Figure 9. Six enhancer-trap lines show distinct expression patterns in Arabidopsis roots.

(A) Heat map illustrating the expression pattern of the various GAL4-VP16/UAS enhancer trap lines used in this study (Left panel). The intensity of green represents GFP intensity of the associated *UAS:erGFP* reporter and a false-colored confocal image of an LR illustrating the different cell types of the root.

(B) Confocal images of both the PR and LR of various enhancer-trap lines. Scale bar represents 50 microns.

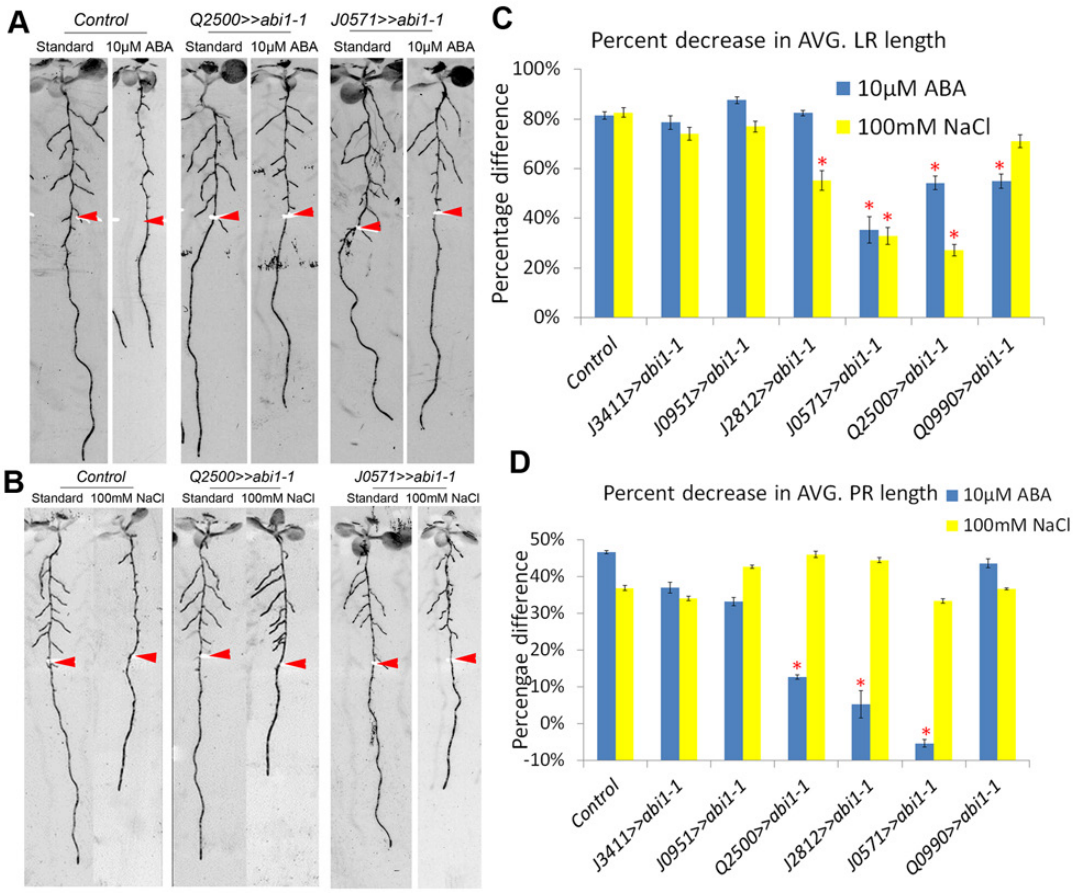

Figure 10. Effect of tissue-specific *abi1-1* expression on ABA and NaCl mediated LR and PR growth suppression.

(A) Scanned images of 10 days post germination roots. Roots of the control, *Q2500>>abi1-1,* and *J0571>>abi1-1* transactivation lines under standard or 10 μM ABA treatment.

(B) Scanned images of 10 days post germination roots. Roots of the control, *Q2500>>abi1-1,* and *J0571>>abi1-1* transactivation lines under standard or 100 mM NaCl conditions.

(C) Quantification LR growth suppression under 100 mM NaCl or 10 μM ABA conditions shown as the percentage difference in average root length between standard and treatment conditions. Red asterisks mark signaficant differences based on a Student's t test and 2-way anova analysis.

(D) Percentage difference in PR length in various *abi1-1* transactivation lines grown under ABA and 100 mM NaCl treatment.

Red arrowheads mark the position of the root tip at the time of transfer. Error bars indicate standard error of the mean (SEM).

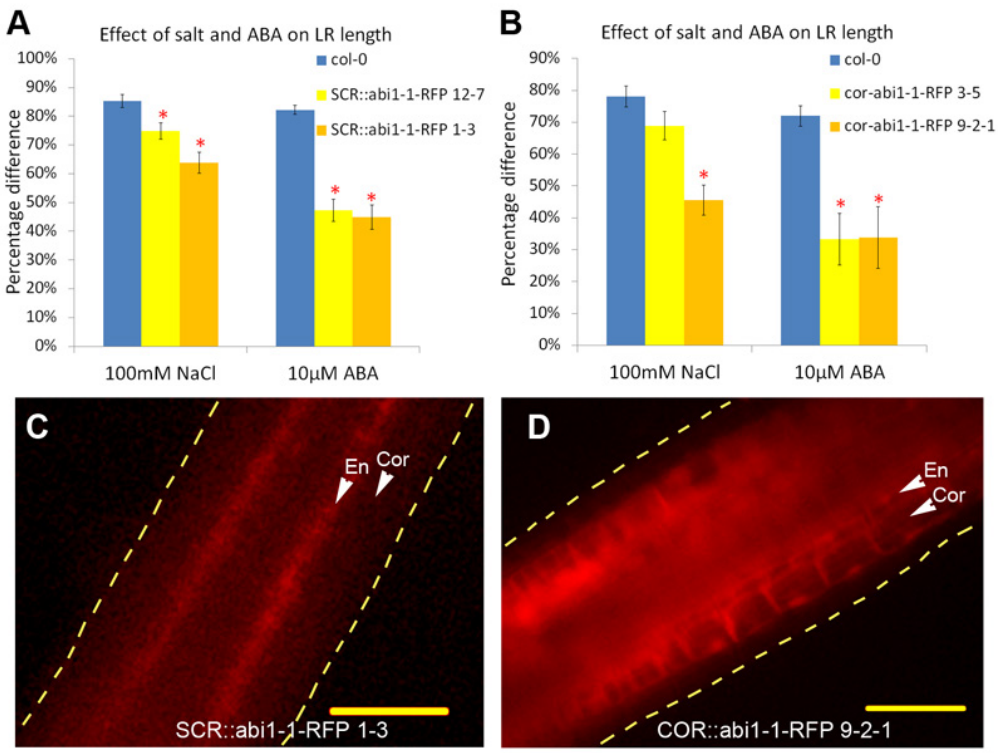

Figure 11. Endodermis-specific expression of *abi1-1* rescues LR length during ABA and salt treatment.

(A) Percentage suppression in LR length for two independent *SCR::abi1-1:RFP* lines after ABA and 100 mM NaCl treatment.

(B) Percentage suppression in LR length for two independent *COR::abi1-1:RFP* lines after ABA and 100 mM NaCl treatment.

(C) Fluorescence image for *SCR::abi1-1:RFP* 1-3 line in PR.

(D) Fluorescence image for *COR::abi1-1:RFP* 9-2-1 line in PR. Mis-localization of red protein in the endodermis is observed.

Scale bar represents 50 microns. Error bars: SEM; Red asterisks represent significant differences based on Student's t test for percentage difference.

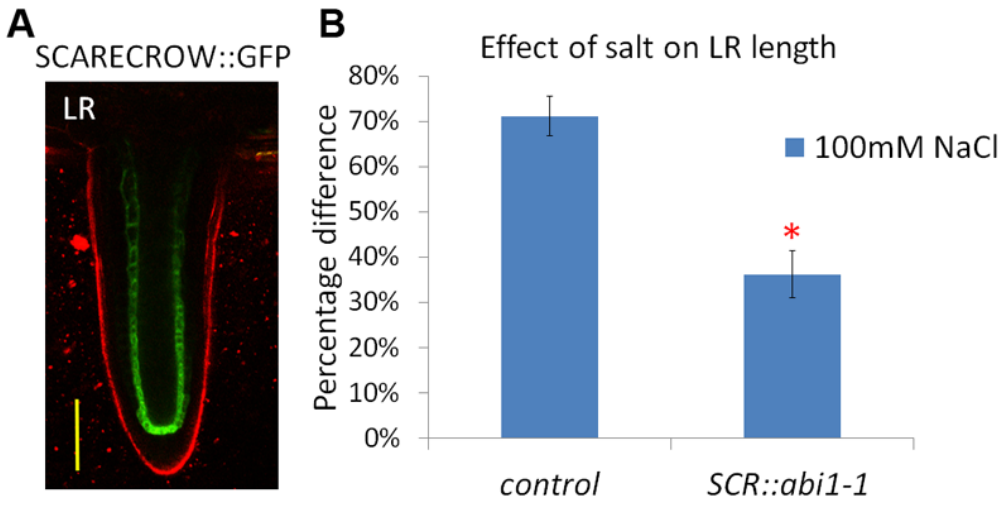

Figure 12. Expression of *abi1-1* from the Scarecrow promoter rescues LR length during salt stress

(A) Confocal image of *SCARECROW::GFP* in LR.

(B) Percentage suppression of LR length of *SCR::abi1-1* transgenic line after 100mM NaCl treatment.

Scale bar: 50 microns. Error bars: SEM; Red asterisks represent significant differences based on Student's t-test for percentage difference, P-value<0.05.

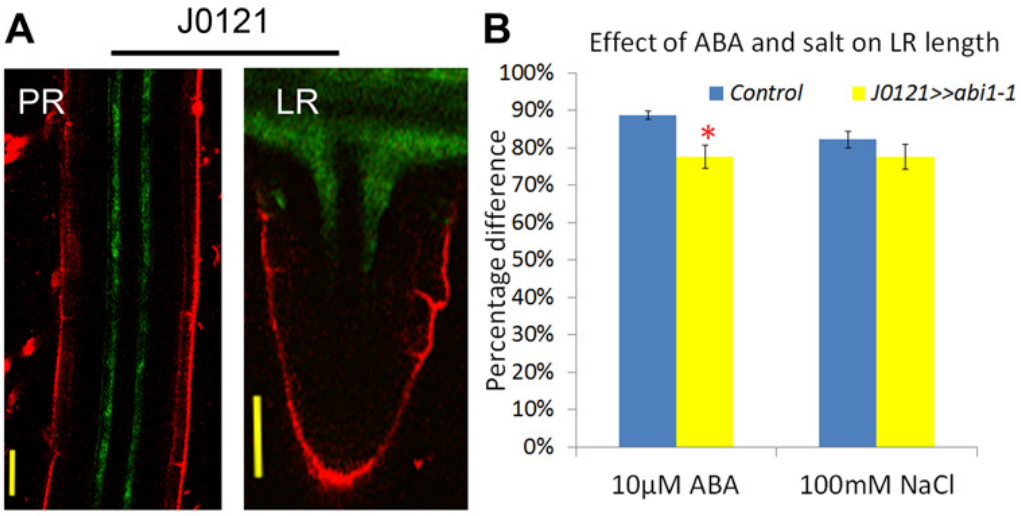

Figure 13. ABA signaling in the pericycle does not significantly regulate LR growth during salt stress.

(A) Confocal images of the J0121 enhancer trap line in both the PR and young LR. Scale bar: 50 microns.

(B) Percentage suppression in LR length for the *J0121>>abi1-1* transactivation line under either ABA or 100 mM NaCl conditions.

Error bars: SEM; Red asterisks represent significant differences based on Student's t-test for percentage difference, P-value<0.05.

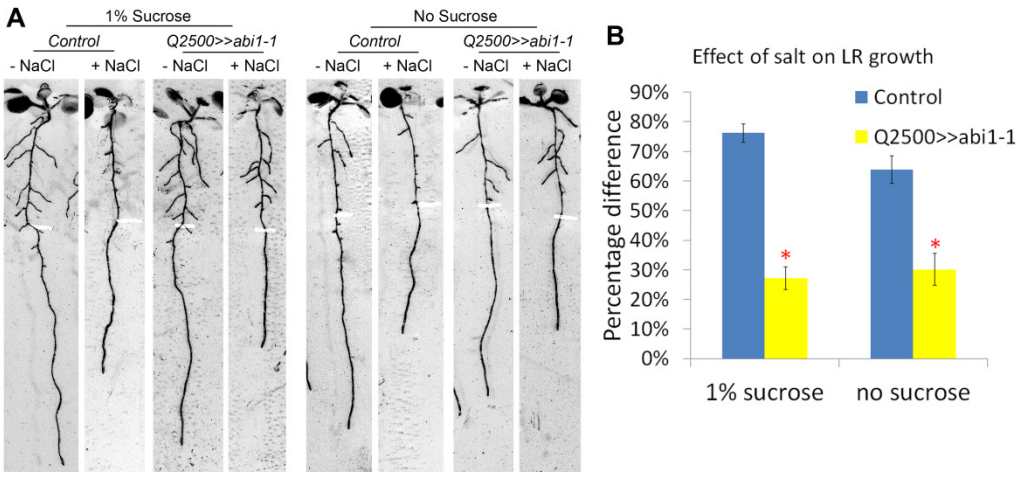

Figure 14. Sucrose supplement in the growth media does not affect the LR growth rescue phenotype in *Q2500>>abi1-1* trans-activation line.

(A) Root system morphology of control plants and *Q2500>>abi1-1* during salt treatment, with 1% sucrose in the media (left panel) or without sucrose in the media (right panel).

(B) Percentage suppression in LR length in the *Q2500>>abi1-1* transactivation line grown under 100 mM NaCl with 1% sucrose or no sucrose in the medium.

Error bars: SEM; Red asterisks represent significant differences based on Student's t-test for percentage difference, P-value<0.05.

3.3.4 Sustained ABA signaling is associated with LR quiescence during salt stress

To understand how salt stress and ABA signaling affect LR growth, live imaging was used to monitor the temporal dynamics of growth regulation. Growth rates of post-emergence LR were quantified for 5-7 days after transferring roots to standard or saline media. Interestingly, growth was not stably suppressed throughout the salt response, but instead, LRs were maintained in a quiescent stage for several days (Fig. 15A, B). Similar quiescent LRs were observed under standard conditions, however much less frequently. After several days of dormancy, LRs resumed growth at rates similar to roots grown under standard conditions (Fig. 15B). This response dynamic differed substantially for the PR, which showed an immediate recovery of growth after being transferred to salt-stress conditions (Fig. 17). We examined the expression of the cell-cycle marker, *CYCB1;1:GUS* and found that expression was greatly reduced in quiescence-stage LR primordia (Fig. 15C) (de Almeida Engler et al., 1999). No changes in the structure of the stem-cell niche or radial organization that could affect growth rate were observed in quiescent-stage LRs (Fig. 16). Together, these results indicate that salt induces a period of quiescence in newly emerged LRs, which may be, in part, a consequence of suppressed cell-cycle activity.

To determine if the quiescence of LRs is correlated with the timing of ABA signaling, we examined the activity of an ABA-sensitive reporter, *RAB18::GFP* (Kim et al., 2011), in the root system after salt treatment. Under standard conditions, *RAB18::GFP* was expressed at very low levels in both the PR and LRs (Fig. 18A). During salt stress, *RAB18::GFP* was weakly and transiently induced in the PR.

During LR development, reporter expression was also weak just after emergence but became highly induced 2 days later. Reporter expression in post-emergence LR was maintained at very high levels for several days until the time at which growth resumed (Fig. 18B, C). The substantially higher level of ABA signaling in the LR, compared to the PR, explains why the suppression of ABA signaling by our various perturbations had an LR-specific effect. In response to ABA treatment, *ProRAB18:GFP* was also induced after LR emergence as it entered into a 'quiescent phase'; however expression tended to be maintained for a longer time period (Fig. 19B, C). To confirm and extend our results, we utilized high-throughput Q-PCR to analyze salt-regulated changes in gene expression for a collection of known ABA-responsive genes. RNA was isolated from either region A or B of 9-day-old root systems of the *aba2-sail* mutant background or *Q2500>>abi1-1* transaction line and controls. As shown, known ABA-inducible genes (*RD29A* and *ABI2*) and others showed strong transcriptional activation by salt stress predominantly in region A, which contains post-emergent LRs, while expression was much reduced in region B (Fig. 21). Induction of RAB18 expression by salt was alleviated in the *aba2-sail* mutant and also strongly reduced in the *Q2500>>abi1-1* transactivated line (Fig. 21). The tissue specific expression pattern of the *ProRAB18:GFP* reporter is not informative since it uses a non-localized GFP reporter protein, which may translocate between cell layers. However, based on cell-type specific transcriptional profiling data, RAB18 is most strongly activated in the endodermis (Fig. 19A). Analysis of other salt-responsive genes identified several that were strongly dependent upon ABA signaling in the endodermis including *AtHB12*, an ABA-induced gene necessary for

growth suppression during water stress (Fig. 21) (Olsson et al., 2004; Son et al., 2010).

Live-imaging analysis of the *Q2500>>abi1-1* transactivated line revealed that LRs showed an immediate activation of growth under salt conditions (Fig. 20), which confirms that ABA signaling in this subdomain of the LR is critical for maintaining quiescence. However, greatly reduced elongation of most LRs was observed in *Q2500>>abi1-1* after a few days rapid growth during salt stress (Fig. 20B), while in control plants, LRs were healthier after growth activation though going through a long 'quiescent phase' after emergence (Fig. 20A). This result suggests that endodermis ABA signaling could be necessary to prime emergent LR and enter 'quiescence' during salt stress in order to protect plants from further damage from high salinity.

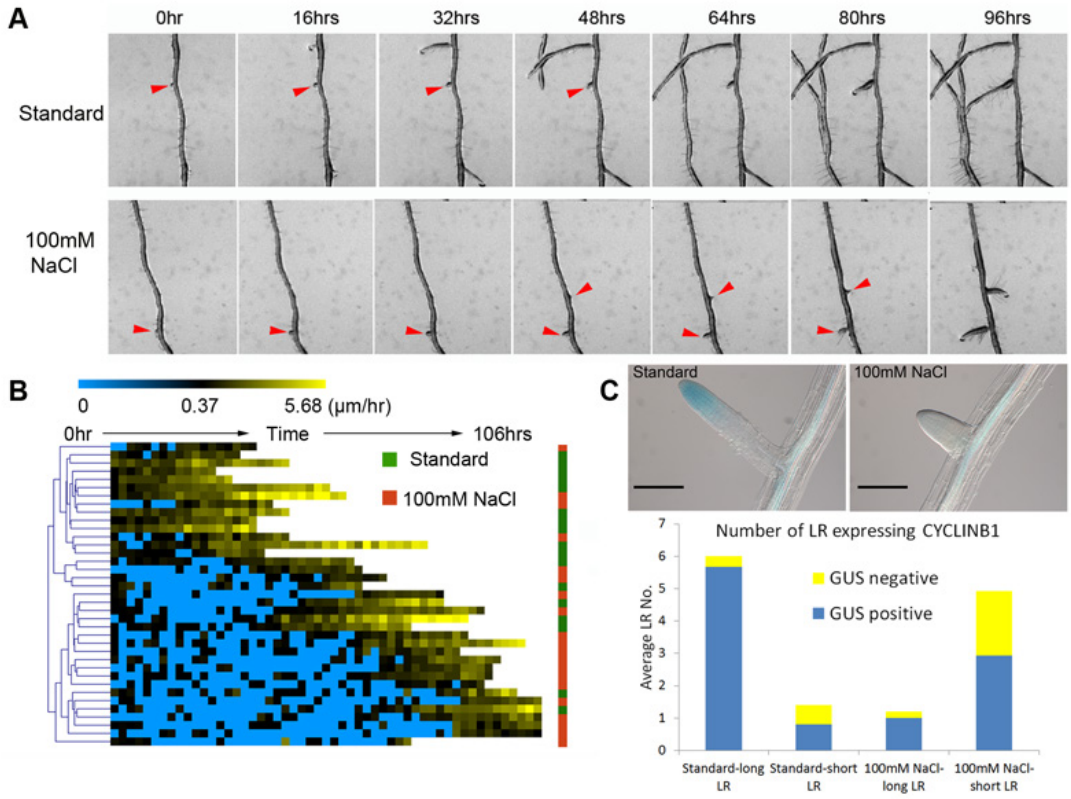

Figure 15. A prolonged quiescent phase in post-emergent LR growth is induced during salt stress.

(A) Time-lapse images of LR growth under standard conditions (upper panel) and 100 mM NaCl (lower panel) conditions. Red arrows mark quiescent LRs.

(B) Quantification of LR growth rates from time-lapse imaging data. Growth represented as a heat-map profile for each root. Data from roots grown under standard or salt-stress conditions organized by heiarchical clustering. Note, most LR under salt stress conditions show a distinct growth profile with an extended quiescence phase followed by growth recovery.

(C) Staining pattern of the *CYCB1:GUS* repoter in LR. Upper panels: standard conditions and 100 mM NaCl conditions. Lower panel: quantification of GUS staining activity. Scale bar represents 100 μm.

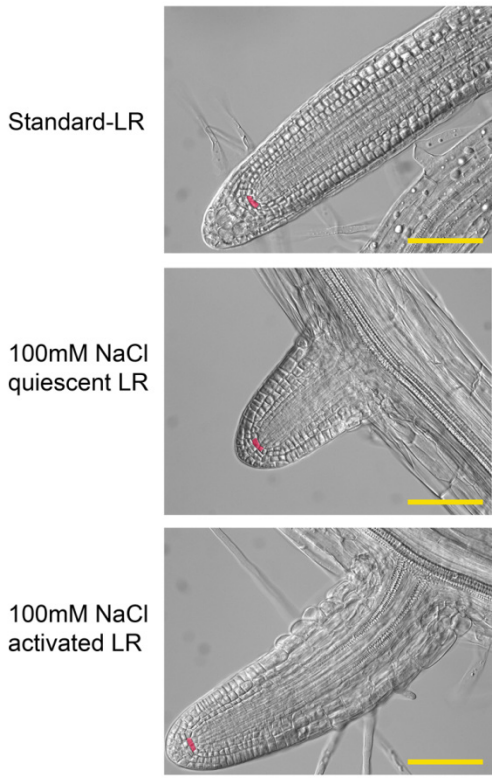

Figure 16. Cellular organization is not changed by the quiescence in LR growth

DIC images of LR grown under either standard or 100 mM NaCl conditions. Red color marks the QC cells in the meristem. Scale bar: 50 microns.

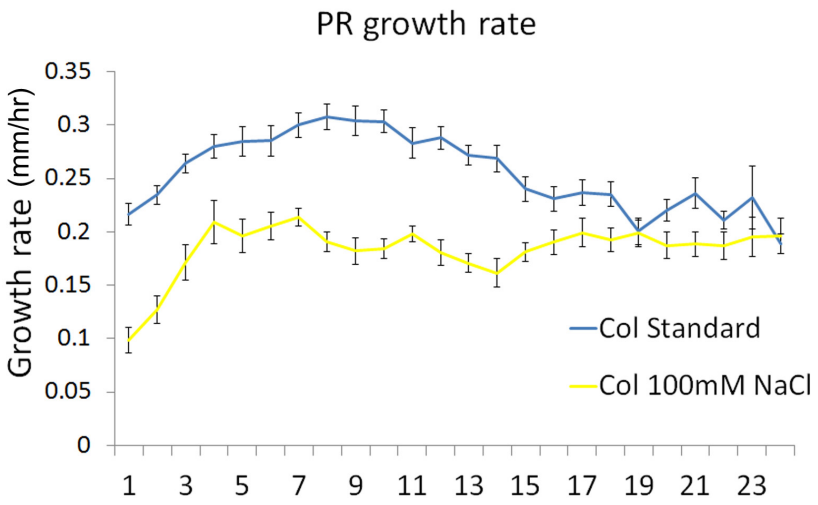

Figure 17. The growth dynamics of primary root under 100 mM NaCl.

Growth rate quantification from time-series imaging of PR grown under standard or 100 mM NaCl conditions.

Error bars: SEM.

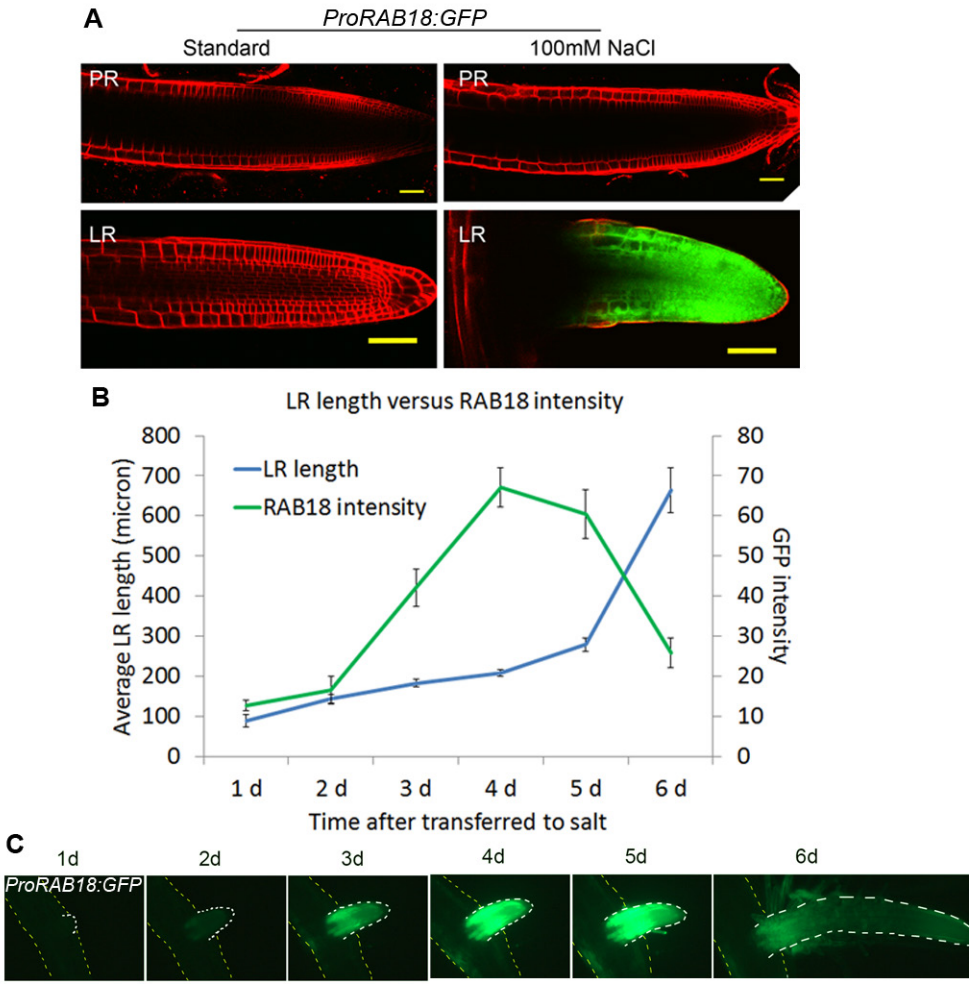

Figure 18. ABA signaling is induced and sustained in the 'quiescent LR'.

(A) Confocal images of plants expressing the *ProRAB18:GFP* reporter in either PR or LR under standard or 100 mM NaCl conditions. Scale bar: 50 microns.

(B) Quantification of LR growth and *ProRAB18:GFP* intensity in the LR during 100 mM NaCl treatment from 1day to 6days. Error bars indicate standard error of the mean (SEM)

(C) *ProRAB18:GFP* expression under 100 mM NaCl conditions at different time points post-treatment.

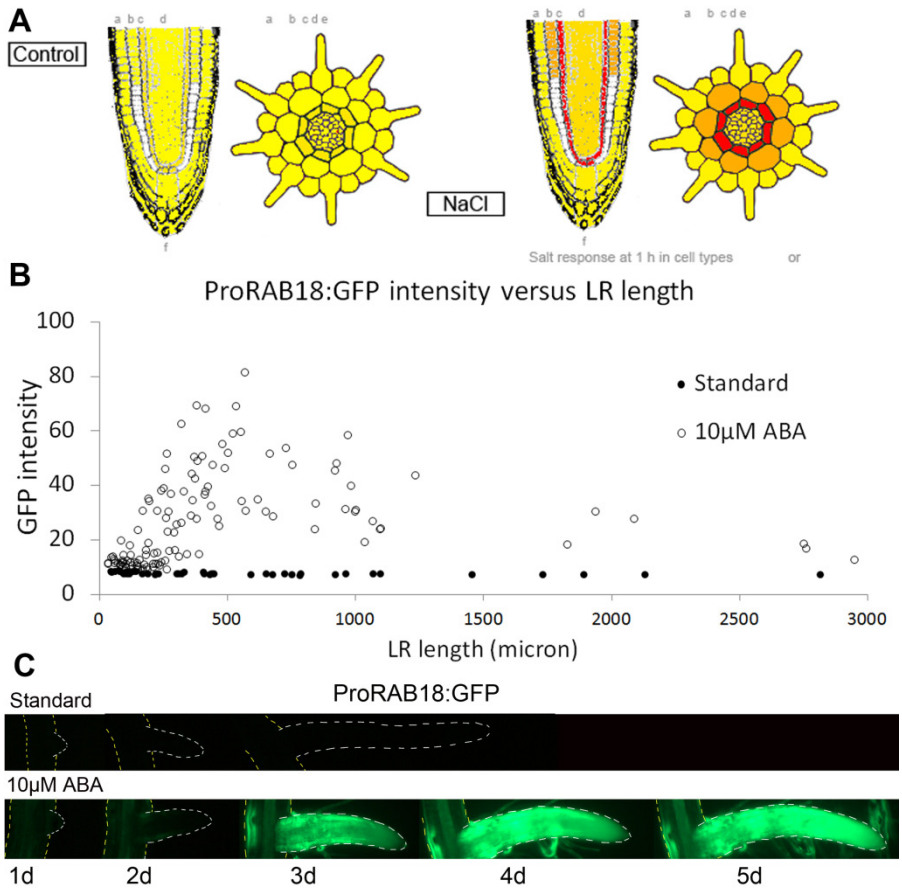

Figure 19. ABA signaling is induced by salt stress in particular developmental stages of LR growth

(A) RAB18 expression level in various root tissue types. Left panel: standard condition; right panel: 1hr 100 mM NaCl treatment. Images are from eFP browser.

(B) Scatter plot showing *ProRAB18:GFP* fluorescence intensity and LR length for roots grown under standard or ABA treatment conditions.

(C) *ProRAB18:GFP* expression under standard or ABA treatment conditions at different time points post-treatment.

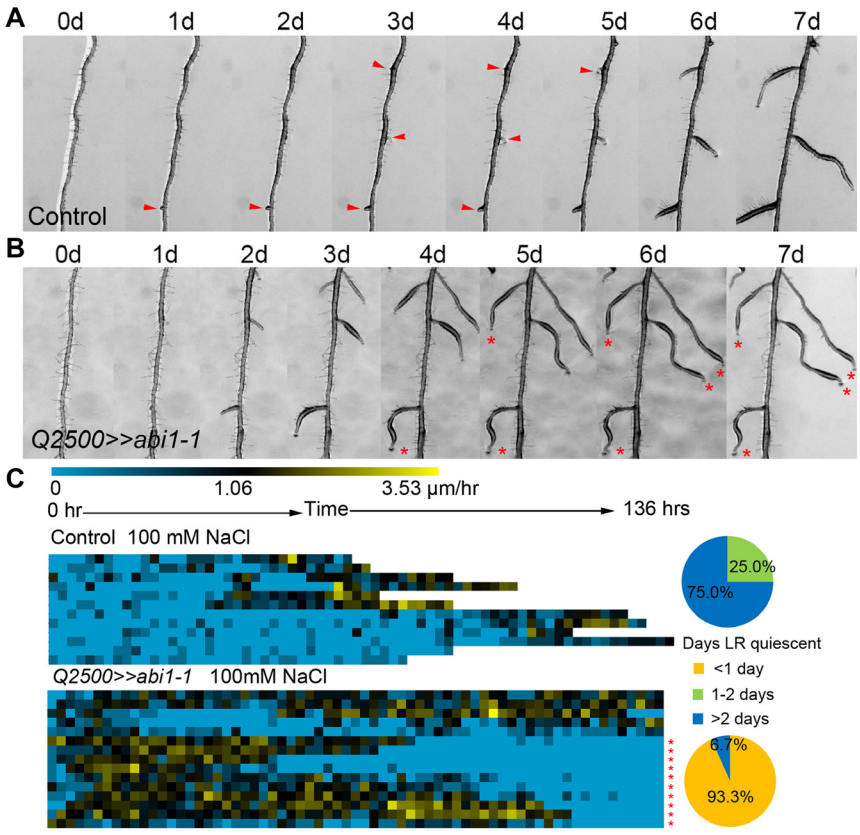

Figure 20. ABA signaling in the endodermis rescues the quiescent phase in post-emergent LR growth during salt stress.

(A) Time-lapse images of LR growth under 100 mM NaCl (lower panel) conditions in control root. Red arrows mark quiescent LRs.

(B) Time-lapse images of LR growth under 100 mM NaCl (lower panel) conditions in *Q2500>>abi1-1* root. Red asterisks mark the LRs that have stoped or slow down growth.

(C) Heatmap showing the growth profiles of individual LRs quantified from time-lapse imaging data. Each pixel in the heatmap represents the total growth over 2 hours. Data quantified from enhancer-trap control seedlings or *Q2500>>abi1-1* transactivation seedlings transferred to 100 mM NaCl conditions and imaged for 7 days. Pie charts to the right of the heatmaps quantify the proportion of LRs that are quiescent for different lengths of time. Red asterisks mark LRs that show unsustainable growth recovery, 10 out of 15.

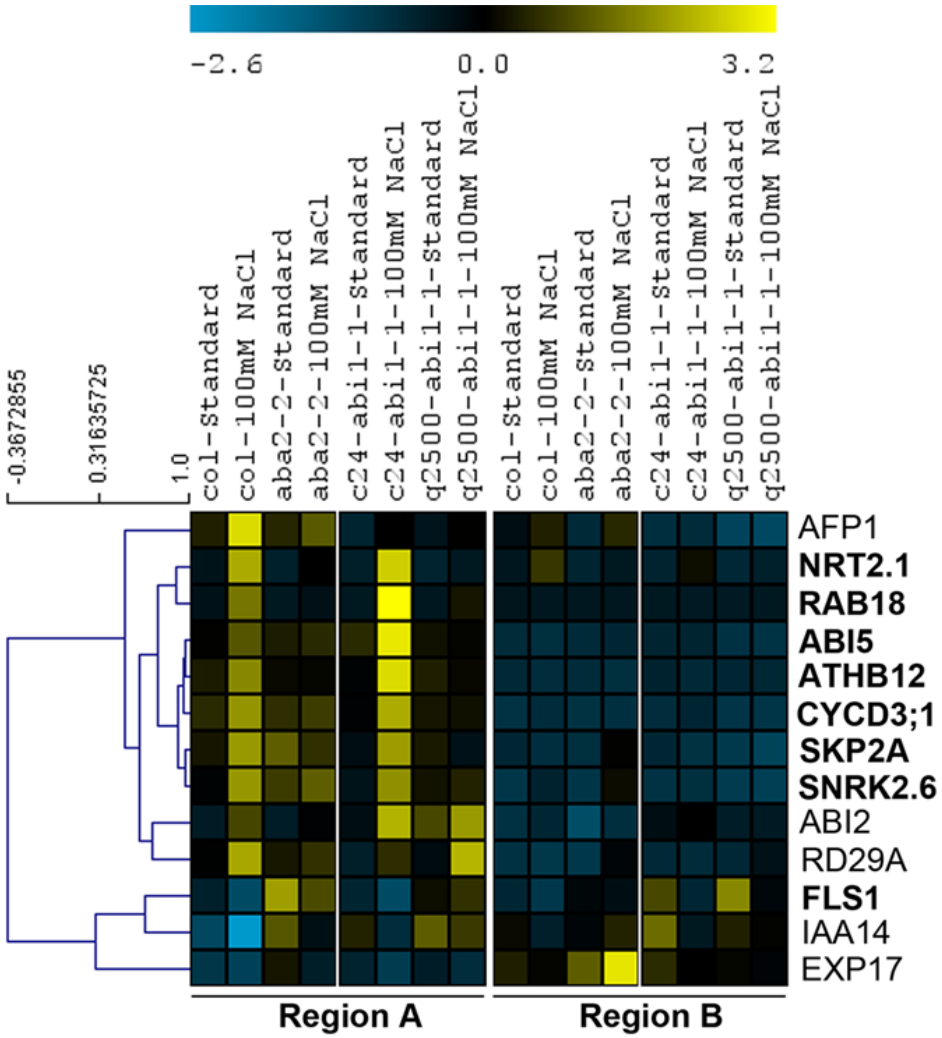

Figure 21. Regulation of many salt responsive genes is dependent upon endodermis ABA signaling.

Gene expression levels of salt regulated ABA-dependent genes were examined in Region A and Region B. Expression in control, *aba2-SAIL* and *Q2500>>abi1-1* genotypes determined. Heat-map shows normalized gene expression values. Bold highlighted genes are strongly affected in the *Q2500>>abi1-1* genotype.

3.3.5 GA signaling acts in parallel and antagonistically with ABA in LRs

Our data show that post-emergence LRs undergo a period of quiescence during salt stress, which is induced by ABA. Another phase of plant development characterized by an ABA-dependent period of growth arrest is seed dormancy (Finkelstein et al., 2008). Here, GA acts antagonistically to promote radical germination. To test whether GA may also suppress LR quiescence, we treated roots with varying concentrations of GA during salt stress. Under GA treatment, the suppression of LR growth by salt was significantly reduced (Fig. 22A). PR growth was less sensitive, again highlighting important differences in hormone signaling between the two root types (Fig. 23A). To understand what function endogenous GA levels might have in regulating growth during salt stress, we studied the effects of the GA biosynthesis inhibitor, paclobutrazol (PAC). Under standard conditions, PAC treatment suppressed LR growth indicating that GA biosynthesis is important under this condition (Fig. 24B). The *abi1-1* mutation partially suppressed this effect, indicating PAC treatment affects LR growth, in part, by elevating ABA signaling. PAC was not able to fully suppress the *abi1-1* phenotype under salt stress conditions, which suggests that *abi1-1* also acts to regulate growth independent of changes in GA biosynthesis (Fig. 24B). Together, these data show that ABA and GA signaling act in opposing ways to regulate LR growth and that this regulation occurs through mutual antagonism as well as through independent pathways. Furthermore, we found that a gene encoding a GA-biosynthetic enzyme (*GA3OX1*), GA-receptors (*GID1A/B/C*) and signaling components (*RGA1/RGA2/SLY1*) showed an increase in expression

when ABA signaling was suppressed in the endodermis (Fig. 24A), which indicates that this tissue layer is an important site for GA-ABA signal integration.

GA signal transduction leads to the degradation of the DELLA-class of growth repressors (Achard et al., 2006; Harberd et al., 2009; Skirycz and Inze, 2010). Consistently, the *della* quadruple mutant combination caused a similar rescue in LR growth as GA treatment under salt-stress conditions (Fig. 22B), while PR growth was not affected (Fig. 23B). Previous work has shown that ABA or salt treatment can stabilize DELLA proteins in PRs (Achard et al., 2006). The effect of salt stress on DELLA protein levels was examined in both LRs and PRs using the *RGA::GFP:RGA* reporter. In salt-treated quiescence-stage LRs, we observed relatively high levels of GFP:RGA in the ground tissue layers, including the endodermis (Fig. 22C) whereas PRs maintained much lower levels (Fig. 23C). Previous studies have identified the endodermis as the key site for DELLA-dependent growth regulation of the PR (Ubeda-Tomás et al., 2008; Ubeda-Tomas et al., 2009). We observed that DELLA activity in the endodermis also suppressed the growth of LRs (Fig. 26A). We asked whether the increase in RGA protein levels is dependent upon ABA signaling by visualizing GFP:RGA fluorescence in an *abi1-1* mutant background. Interestingly, we did not see a change in fluorescence levels even though LR growth was clearly rescued (Fig. 25A-C). These data suggest that ABA acts largely by non-DELLA dependent mechanisms to inhibit LR growth. Consistent with this model, we found that the *della* quadruple mutant only showed a slight reduction in ABA sensitivity (Fig. 22B) and had little effect on ABA-dependent changes in gene expression during the salt-stress response (Fig 26B).

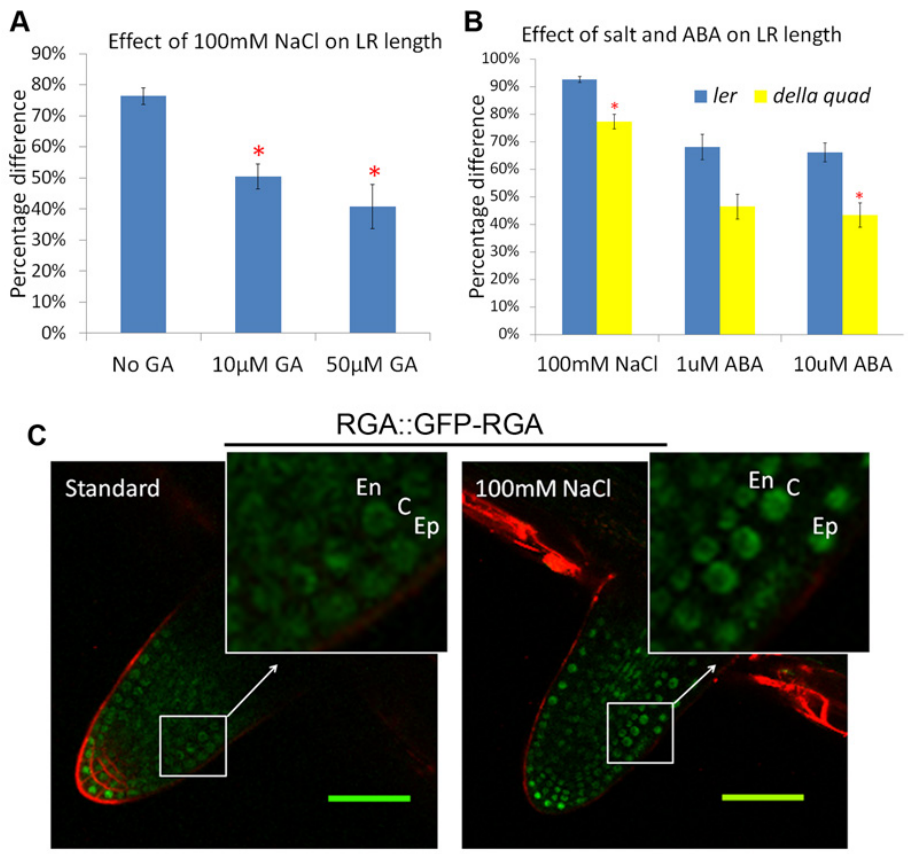

Figure 22. GA signaling is involved in alleviating the growth suppression of LR during salt stress.

(A) Suppression of LR growth by 100 mM NaCl with or without supplementing media with GA.

(B) Suppression of LR growth by 100 mM NaCl or two different concentrations of ABA in wild type or the *della* quadruple mutant.

(C) Confocal images of post-emergence stage LRs grown under standard or 100 mM NaCl conditions and expressing the *RGA::RGA-GFP* reporter. Scale bar represents 50 μm.

Error bars indicate standard error of the mean (SEM). Red asterisks represent significant differences as determined by the Student's t-test, P-value < 0.05.

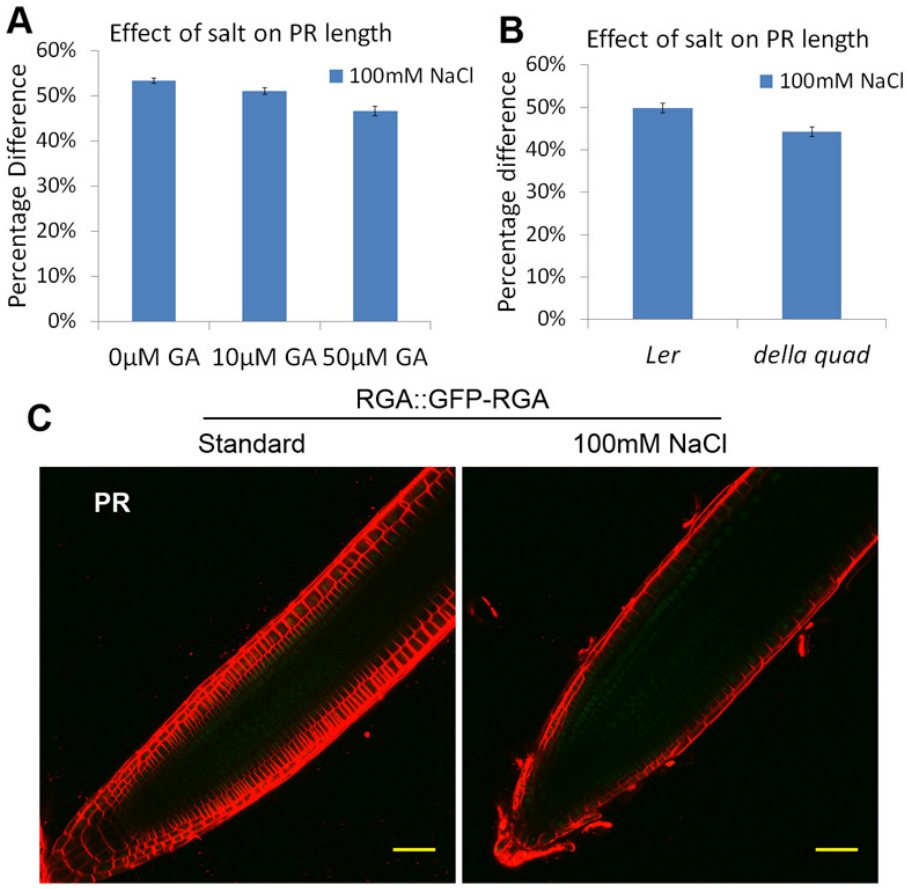

Figure 23. PR length is not significantly affected by exogenous GA or endogenous signaling.

(A) Percentage suppression in PR length during salt treatment with or without the presence of exogenous GA.

(B) Percentage suppression in PR length of *della* quadruple mutants under 100 mM NaCl.

(C) Confocal images of PR of *RGA::GFP:RGA* under either standard or 100 mM NaCl conditions.

Error bars: SEM. Scale bar: 50 microns.

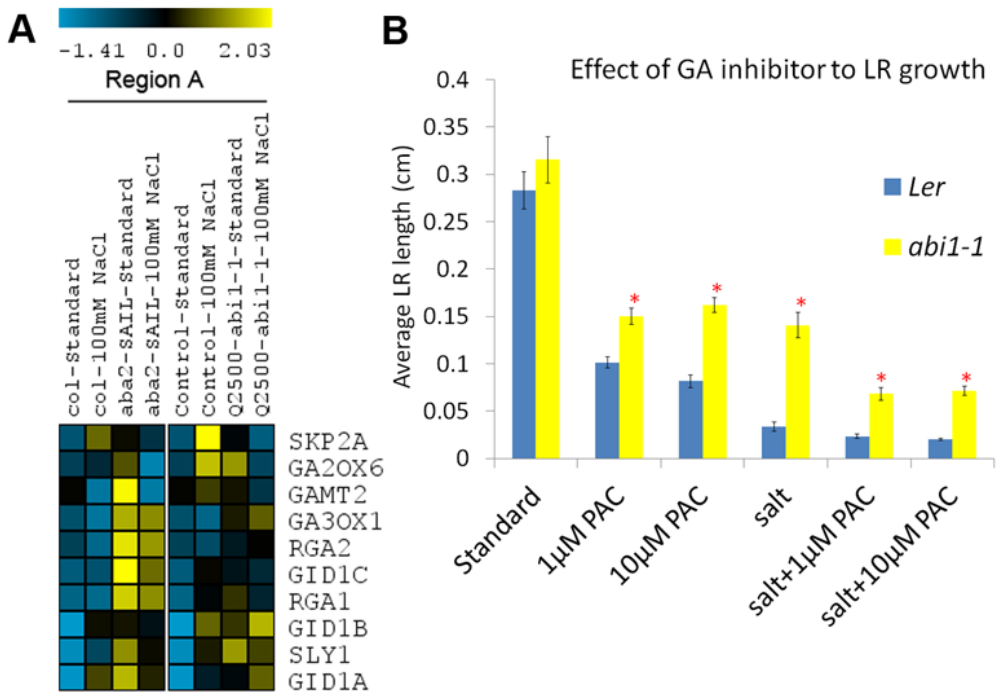

Figure 24. Integration of GA signaling in ABA regulated LR growth

(A) Heatmap of normalized gene expression data for GA-pathway assocaited genes in the *aba2-SAIL* or *Q2500>>abi1-1* genotypes. Expression is examined in tissue collected from regions A under standard or salt stress conditions.

(B) LR length of wild type and *abi1-1* mutants after treatment with standard or 100 mM NaCl media supplemented with varing concentrations of the GA biosynthesis inhibitor, PAC.

Error bars indicate standard error of the mean (SEM). Red asterisks represent significant differences as determined by the Student's t-test, P-value < 0.05.

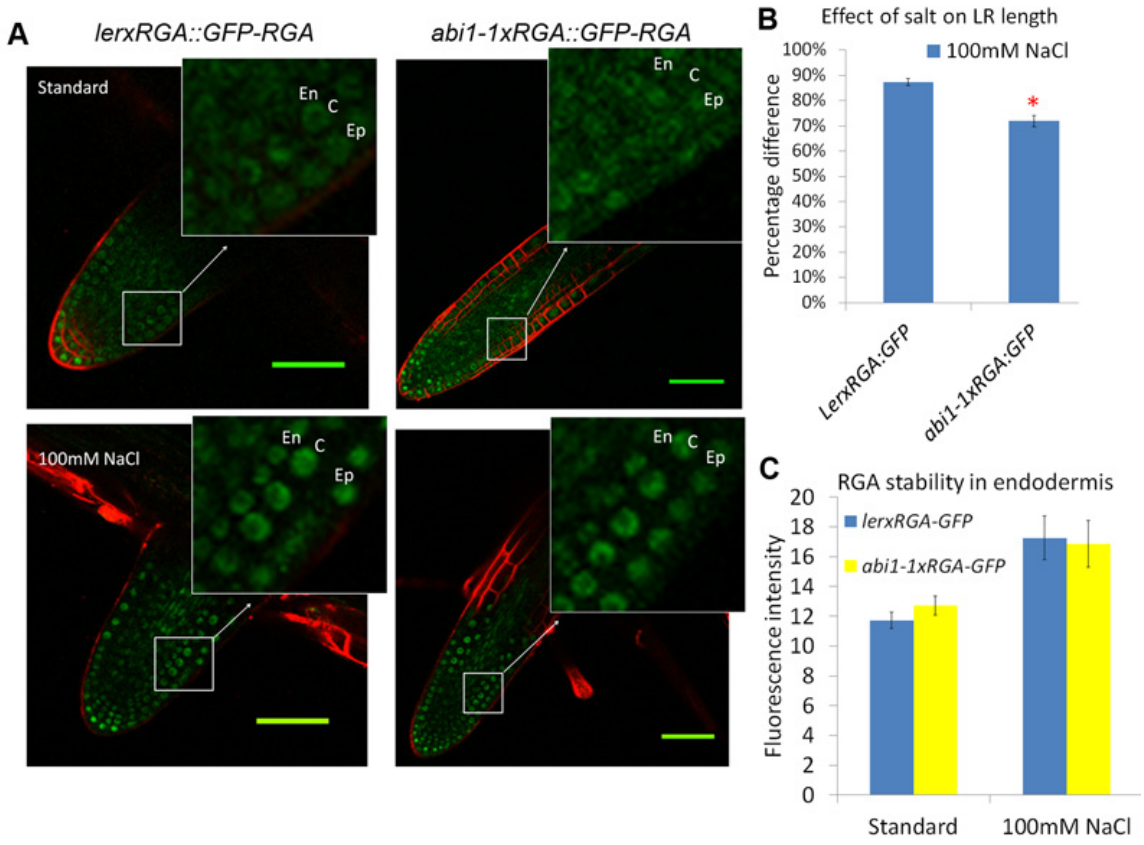

Figure 25. RGA protein in endodermis inhibits LR growth, but its stability in the LR is not affected by *abi1-1* (+/-).

(A) Confocal images of LR from an *Ler x RGA::GFP:RGA* (left panel) F1 hybrid and an *abi1-1 x RGA::GFP:RGA* F1 hybrid (right panel). Upper panels are standard conditions, and lower panels are 100mM NaCl conditions.

(B) Percentage suppression in LR length of *abi1-1 x RGA::GFP:RGA* F1 hybrid under 100mM NaCl compare to standard conditions.

(C) Quantification of GFP intensity in F1 hybrid plants described above grown under standard or 100 mM NaCl conditions.

Error bars indicate standard error of the mean (SEM). Scale bar: 50microns. Red asterisks represent significant differences as determined by the Student's t-test, P-value < 0.05.

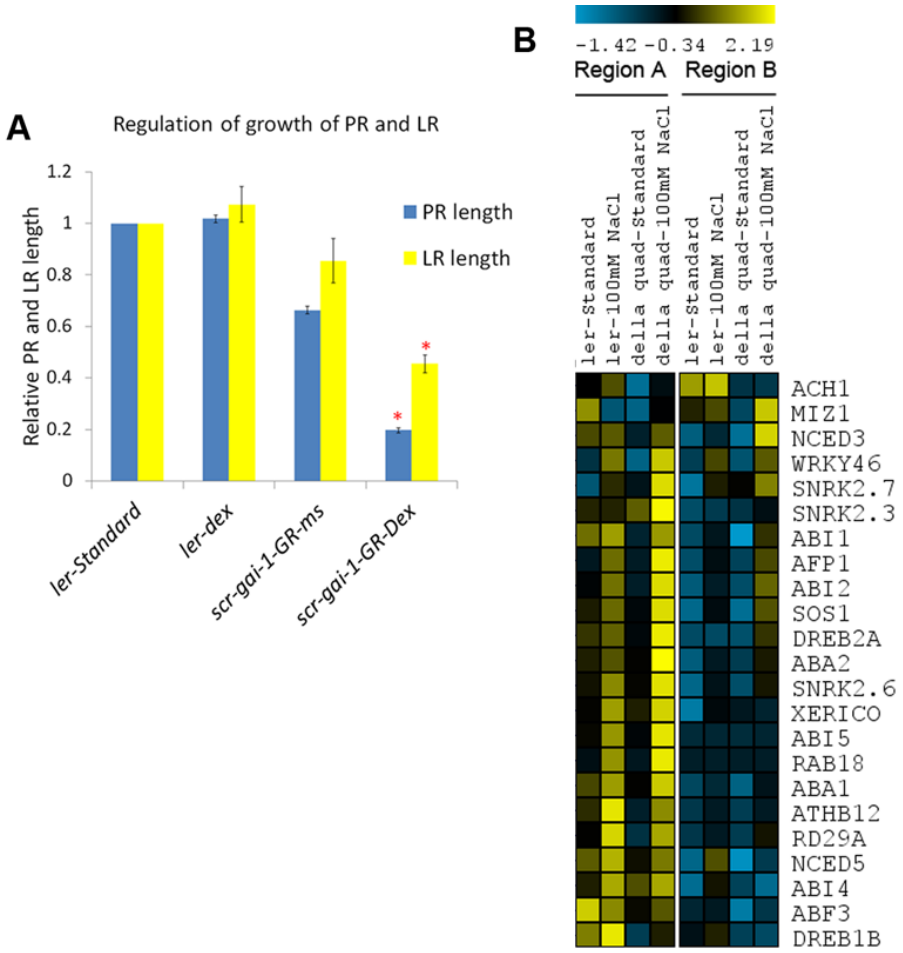

Figure 26. Stabilized DELLA in endodermis suppresses LR growth while gene expression regulation is not affected in *della* quadruple mutant

(A) Relative PR and LR length of *SCR::gai-1:GR* after treatment with 1μM DEX for 4 days.

(B) Expression data in *della* quadruple mutant backgound is presented in heat-map with normalized expression values. Blue represents low expression level while yellow represents high expression level.

Error bars: SEM. Red asterisks represent significant differences based on Student's t-test for percentage difference, P-value<0.05.

3.3.6 ROS production in the endodermis is involved in LR growth regulation

Reactive Oxygen Species (ROS) is chemically reactive molecules which can be involved in regulating many processes of plant development. Enzymes produced in plants can function to remove ROS. For example, superoxide dismutase and peroxidase can remove superoxide and hydrogen peroxide respectively, while some enzymes like glutathione reductase, maintain the antioxidant in reduced status (Pandhair and Sekhon, 2006). Recent study has shown that UPB1, a transcriptional factor, targets peroxidase expression and regulates the balance between cell division and differentiation in Arabidopsis root (Tsukagoshi et al., 2010). Interestingly, in the study of cell type specific gene expression profiling, a group of peroxidase genes are found to be expressed specifically in the endodermis (Dinneny et al., 2008), where we expect the regulatory signaling for LR growth occurs. Example of the expression pattern of peroxidase is shown in fig. 27A. To understand whether the endodermis expressed peroxidases are targeted by ABA signaling in the same cell type and involved in LR regulation, their expression level was examined in both *aba2-sail* and *Q2500>>abi1-1* background. Firstly, some peroxidases were found to be preferentially expressed in the LRs while others are highly enriched in the PR (Fig. 27B). In region A, peroxidases expression are mostly down-regulated by salt, suggesting an enhanced ROS accumulation in the LRs under salt treatment. Interestingly, in ABA deficient mutant, peroxidases genes are expressed with higher level and in endodermis expressed *abi1-1* lines, peroxidases genes are mostly up-regulated (Fig. 27B), suggesting that ABA signaling could be involved in suppressing

peroxidases during salt stress, and this regulation is likely to be endodermis dependent.

In previous sections, we have shown that GA signaling partially rescues LR growth during salt stress, while stabilization of the negative GA signaling regulator, DELLA protein, in the endodermis suppresses LR growth. To understand whether GA signaling regulates LR growth through targeting endodermis peroxidases and modulating ROS accumulation, the expression level of various endodermis expressed peroxidases were examined in both DELLA loss of function (*della* quadruple) and gain of function (*SCR::gai-1-GR*) lines. Intriguingly, in della quadruple mutant, the expression of most peroxidases are enhanced, especially under salt treatment (Fig. 28A), while after the stabilized protein, gai-1, is induced driven by SCR promoter, the expression of peroxidases are largely suppressed (Fig. 28B). These results indicate that DELLA protein in the endodermis could target the peroxidases genes and by suppressing their expression levels, ROS will be highly accumulated, which can result in the reduced LR growth during salt stress.

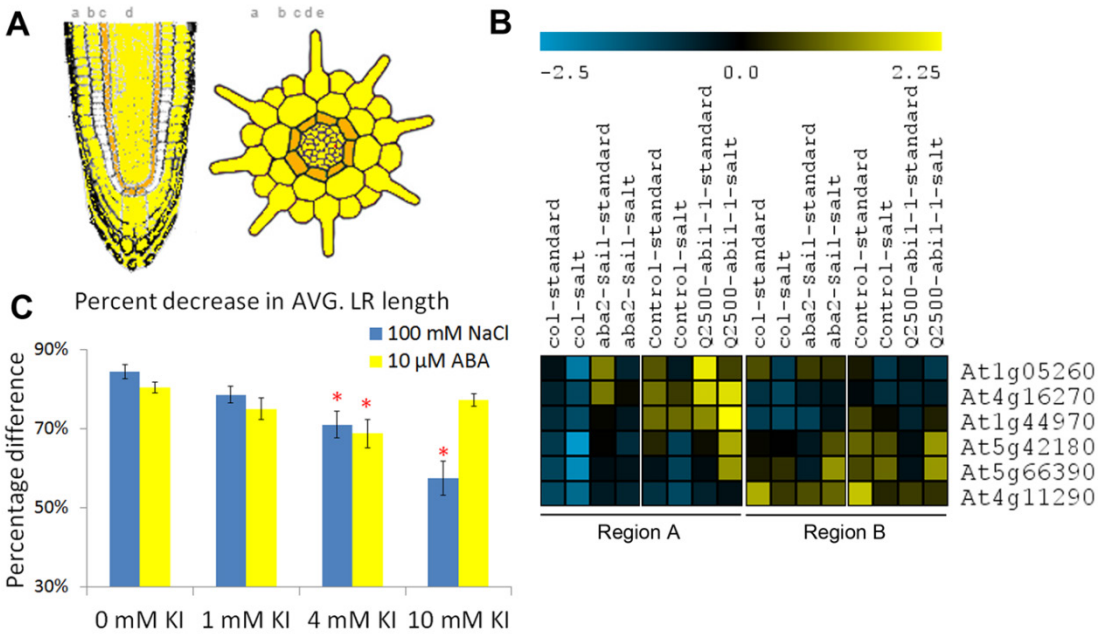

Figure 27. Endodermis ROS accumulation can be induced by ABA signaling and suppresses LR growth.

(A) The expression pattern of a representative peroxidase, AT4G16270, in various root cell types (Dinneny et al., 2008).

(B) Heat-map of the expression level of various endodermis expressed peroxidases in *aba2-sail* and *Q2500>>abi1-1* transactivation line in either root Region A or Region B under standard or salt condition.

(C) Quantification of the percentage decrease in LR length by either salt or ABA treatment with or without various concentrations of potassium iodide supplementation.

Error bars: SEM. Red asterisks represent significant differences based on Student's t-test for percentage difference.

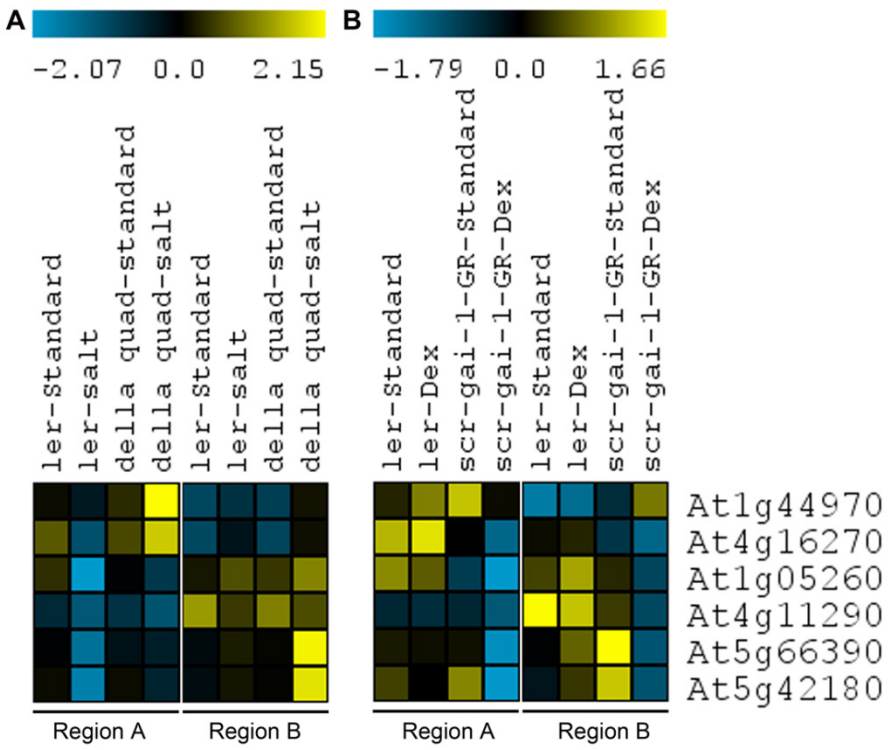

Figure 28. The expression levels of endodermis peroxidases are regulated by endodermis GA signaling.

(A) Heat-map of the expression level of various endodermis expressed peroxidases in *della* quadruple in either root Region A or Region B under standard or salt condition.

(B) Heat-map of the expression level of various endodermis expressed peroxidases in *SCR::gai-1-GR* in either root Region A or Region B under standard or 1 μM DEX condition.

3.4 DISCUSSION

Our study reveals an important interplay between developmental and environmental pathways that ultimately transforms RSA to suit a new environmental condition. We show that LRs and PRs have intrinsically different response programs to salinity and the associated hormones elicited downstream. ABA is the critical effector in this process and preferentially suppresses the growth of LRs. The site of action for this hormone is localized to the endodermis, thus highlighting how cell identity provides an important signaling context for salt-stress responses. Finally, the ultimate outcome of the stress-signaling network outlined here is to dynamically control the growth of LRs, which will cause a transformation in the emergent properties of the root system due to changes in the ratio between LR and PR growth. We hypothesize that these differences are likely related to the unique functions of each root type in a taproot system; the PR functions to extend the root system into deeper terrain, while LRs exploit the resources identified at specific depths. In a saline environment, LR-specific activation of ABA signaling will shift the balance of root growth towards soil exploration, away from resource acquisition.

3.4.1 The root system is composed of root types with distinct salt stress and hormone signaling properties

The Arabidopsis root system is composed of primary, lateral and adventitious roots (roots emerging from the root/hypocotyl junction). Lateral roots are differentiated from the primary root due to their post-embryonic origin from the pericycle cell layer

of the PR (Malamy and Benfey, 1997). Other differences in growth have been described; however the mechanistic basis controlling such distinctions is unclear. The response to gravity, for example, differs between the PR and LR (Kiss et al., 2002; Guyomarc'h et al., 2012). The PR is able to immediately respond to the gravity vector and in flax has been shown to be responsive even before germination (Ma and Hasenstein, 2006). On the other hand, newly emerged LRs in Arabidopsis show little sign of gravitropism and become more gravitropic over time. These differences have important effects on RSA and enable larger domains of the soil environment to be explored and increase anchorage. Work by Guyomarc'h et al. revealed that the expression pattern of PIN transporters changes extensively through post-emergence LR development, which may explain some of the differences in gravitropism (Guyomarc'h et al., 2012).

In our study, we show that hormone and salt-stress signaling differ significantly between LRs and PRs, as well. Salt stress activates high levels of ABA signaling exclusively in LRs, as demonstrated by the LR-specific expression of the *ProRAB18:GFP* reporter. These data suggest that ABA signaling may have little activity in the PR at the concentrations of salt used here (100 mM NaCl). Indeed, all mutants affecting ABA signaling primarily rescue growth of the LR, while the PR is unaffected or grows slower. These data suggest that salt may induce LR-specific ABA biosynthesis or that the LR may be hypersensitive to ABA. Exogenous application of ABA clearly shows that the LR exhibits significantly higher sensitivity to ABA than the PR. Thus, we propose that LRs and PRs diverge in their salt response due to differences downstream of ABA biosynthesis. Root-type specific

expression of the various components of the core ABA signaling pathway may be involved; however, no exhaustive profiling experiments have yet compared the transcriptomes of post-emergence LRs with PRs to uncover such differences.

In the shoot, well-characterized sets of transcription factors are known to determine the identities of different meristem types (Bowman et al., 1993; Wellmer and Riechmann, 2010). The vegetative meristem transitions to an inflorescence meristem, while lateral branch meristems are specified as floral meristems. While currently uncharacterized, the various meristem types in the shoot may also have unique hormone and stress signaling properties that allow the plant to regulate context appropriate environmental responses. We speculate that post-emergent LRs may have a unique identity, in the developmental sense, that controls the specific suite of genes controlling hormone, stress signaling and gravity response. In species such as Maize, where additional root types exist like seminal, crown and brace roots, mechanism to determine the identity of each root type may exist (Hochholdinger and Tuberosa, 2009). To understand how plants tailor stress signaling for particular environmental and field conditions, it will be important to understand the mechanisms that regulate the identity of each root type and the associated stress-signaling networks.

3.4.2 Tissue-specific ABA signaling regulates root growth

In our study, we examined the function of tissue-specific ABA signaling in the context of exogenous ABA treatment as well as an environmental stress that acts through the ABA pathway to regulate growth. These studies focused on LR growth,

but also examined the PR. This comparative approach enables us to distinguish where ABA is able to regulate growth from where ABA actually acts during a physiologically relevant execution of the pathway. Interestingly, we find that the endodermis is not the only cell type where ABA signaling can regulate growth. With exogenous ABA treatment, the stele is also important. In other cell types, ABA signaling may control the same downstream pathways to affect growth. Alternatively, ABA signaling may regulate growth by distinct developmental mechanisms in each cell type. We find that the salt-induced expression of some ABA-regulated genes (e.g. *RD29A* and *ABI2*) is not strictly dependent on endodermal ABA signaling. Thus, we propose that ABA signaling may be active in multiple tissue layers of the root during salt stress, but that certain functions, such as growth regulation, are regulated by tissue-specific ABA signaling pathways.

Several recent studies have also highlighted the role of developmental pathways regulating formative divisions, which generate the endodermis and ground tissue, in the control of stress response. Iyer-Passcuzi et al. has shown that the *scr* mutant exhibits defects in germination upon ABA treatment (Iyer-Pascuzzi et al., 2011; Cui et al., 2012). This work and another study by Cui et al. have shown that SCR can directly regulate the expression of several transcription factors controlling ABA-response (Cui et al., 2012), including *AtHB12*, which we find here to be regulated by ABA signaling in the endodermis. This work and others have now clearly demonstrated that developmental regulation and tissue-specific signaling are important for generating a stress response program and for salt tolerance , a model we

proposed in our original work studying tissue-specific responses to abiotic stress (Dinneny et al., 2008).

3.4.3 The endodermis as a gateway with an ABA-dependent guard

The endodermis is an evolutionarily conserved cell type found in all flowering plants and acts as a semi-permeable barrier due to the presence of a specialized cell-wall modification termed the Casparian strip. Recent work has shown that lignin deposition in the Casparian strip primarily contributes to its function as a barrier to the apoplastic diffusion of solutes into the vascular stream (Roppolo et al., 2011; Naseer et al., 2012). Consequently, transport of nutrients must occur by entering the symplast of the outer tissue layers or the endodermis itself. Indeed, several transporters are expressed in this tissue layer and are localized to a specific side of the cell, consistent with their function in nutrient uptake (Ma et al., 2006a; Ma et al., 2007; Alassimone et al., 2010; Takano et al., 2010; Sasaki et al., 2012).

All solutes in the symplast must pass through the cytoplasm of the endodermis. Thus, this cell type can be considered a gateway for entry into the vascular stream. For salinity stress in particular, the extent to which a plant absorbs sodium into the vascular stream and transports it to the shoot is negatively correlated with overall stress tolerance (Munns, 2002; Møller and Tester, 2007). The endodermis may cause a bottleneck in the diffusion of sodium ions through the root, and as a consequence, ABA signaling would be most strongly activated there and growth arrested (Fig. 29). This hypothesis is consistent with data from Møller et al. showing that sodium ions accumulate in the outer tissue layers of the root while the inner stele has lower levels

(Møller et al., 2009). The endodermis may also be particularly sensitive to the presence of sodium ions and activate ABA signaling at a lower threshold than other cell types. As a consequence of either mechanism, ABA signaling acts as a "guard" at the endodermal "gate" ensuring that growth of the LR does not proceed into high saline environments. The quiescent phase of growth activated by ABA appears to be necessary for acclimation and ultimate growth recovery, as plants suppressed in endodermal ABA signaling exhibit unsustainable growth. Thus, other processes besides growth regulation may be downstream of endodermal ABA signaling, and enable long-term growth and acclimation.

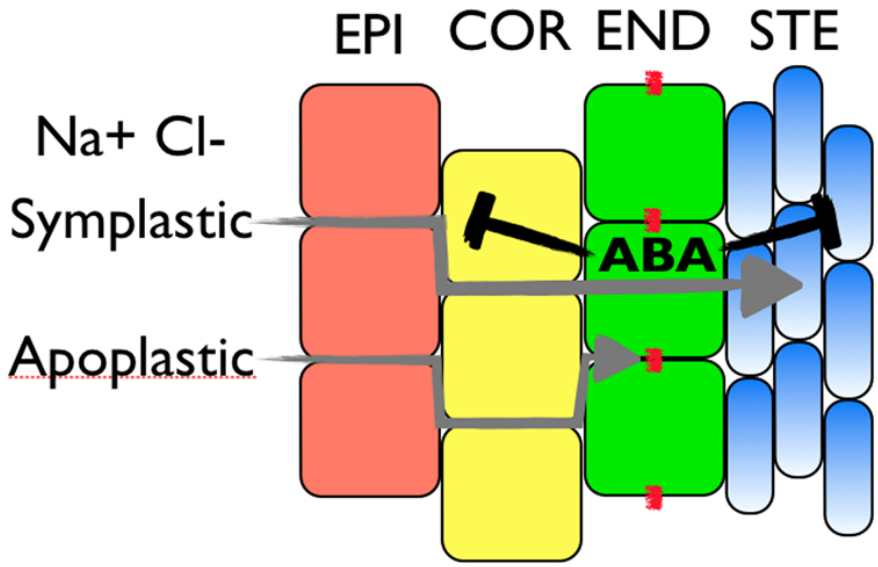

Figure 29. Model of ABA signaling in regulating growth in the endodermis

Chapter 4 RESULTS AND DISCUSSION 2

4.1 ABSTRACT

Among the different developmental programs in determining root system architecture (RSA), the patterning of lateral roots (LRs) along the primary root (PR) is the most early and essential factor. Auxin has been found to be an important plant hormone in specifying LR founder cells. Here we show that as an important agriculture stress, high salinity does not only suppress the outgrowth of LRs, but also disrupts the early patterning process of LRs, which is conferred by reduced LR number and disrupted spacing. This phenotype is largely due to the loss of maintenance of auxin maxima during salt stress indicated by an auxin responsive reporter, *DR5::Luciferase*, which can be used to monitor the changes of auxin response and the auxin determined LR pre-branch sites along the PR in a more dynamic fashion (Moreno-Risueno et al., 2010). Rather than auxin biosynthesis, the repressed transportation strength of auxin from root tip upwards during salt stress is likely to be the major reason of the disrupted LR spacing. This study provides very detailed phenotypic characterization of the various stages of pre-activated LR development during salt stress. It also elicits a model for LR specification based on auxin circulation and distribution under high salinity environment.

4.2 INTRODUCTION

In plants, the root system architecture (RSA) does not only physically support the whole organism, but also determines the ability of plants to absorb water and nutrients from soil. In Arabidopsis, the embryonic primary root is not the only

component of RSA. Instead, the production and patterning of the post-embryonic lateral roots largely constitute and determine the shaping of RSA. Different from the radical emergence, LR initiates from differentiated pericycle cells (Dubrovsky et al., 2000), and then destructive process takes place in order to facilitate LRP breaking through the PR. The various developmental stages during this process has been defined previously (Malamy and Benfey, 1997). Auxin has been well studied to trigger the organogenesis of LR. The spacing of LRs along the PR is highly correlated with a regular oscillation of auxin response in the root elongation region at a time interval of about 6 hours (Moreno-Risueno et al., 2010), indicating the importance of auxin response in LR early determination process. A transcriptional factor, GATA23, has been shown recently to be regulated through IAA/ARFs module and it functions to specify LR founder cells and trigger first anticlinal division (De Rybel et al., 2010). Interestingly, 'fused LR' was found in pin2/3/7 mutant (Laskowski et al., 2008), suggesting the regular LR spacing can be disrupted by abnormal auxin transportation.

As an important agronomic feature, RSA is highly plastic in response to environmental factors. For instance, excessive nitrate treatment leads to reduced LR growth while localized nitrate patch induces proliferation of LR (Zhang and Forde, 1998). This modulation enables plants to adapt extreme environmental changes and maximize the usage of favorable factors. However, the regulations of environmental stresses on early LR determination as well as the details on specific LR developmental stages before emergence have largely not been studied. In this study, we use high salinity as a model and perform detailed analysis on the regulation of salt on various phases of LR development before meristem activation. It has been shown

that lateral root development can be suppressed by high salt stress (Deak and Malamy, 2005). However, the details are still lacking. We reveal that salt stress strongly suppresses both the early patterning and emergence of LRP. We also show that the auxin maxima determined LR branching sites are less well maintained during salt stress. The resulting disruption of normal LR spacing cannot be rescued by excessive amount of free auxin. Thus, we propose a distorted auxin circulation system under salt stress results in the disrupted LR patterning.

4.3 RESULTS

4.3.1 High salinity results in repression of LR initiation as well as outgrowth on newly developed root.

To understand the basic effect of salt stress on lateral root development, seedlings were grown on standard conditions for 5-6 days before transferred to medium supplemented with various concentrations of NaCl, which created two distinct zones of the root—the region before transfer (Region A) and the region after transfer (Region B) (Fig. 30A). Two different GFP marker lines, *SCARECROW::GFP* and *MIR390a::GFP*, which mark the lateral root initiation, were used to evaluate the effect of salt (Fig. 30B) (Malamy and Benfey, 1997; Marin et al., 2010). In a time-course study, the number of visible lateral roots, as well as the GFP expression dots, was counted for both regions every day. It was shown from the results that as the concentration of NaCl increases, only modest effect was found on the region before transfer, while distinct repression could be observed on the region after transfer (Fig.

30C-F). It suggested that only the newly developed region could be affected by salt stress in lateral root initiation.

Sophisticated studies have been done to describe the whole Lateral root developmental stages in detail (Malamy and Benfey, 1997). Two-stage-theory was also raised to emphasize the two key steps, initiation and meristem organization of lateral root (Laskowski et al., 1995). To investigate the effect of salt stress on the different stages of LR development, 100 mM NaCl was used to treat *Arabidopsis* root for different days, and the newly developed region was fixed for stage-specific characterization (Fig. 31A). A general retardation on every stage of LR development was observed under salt stress (Fig. 31B, C). To investigate the effect of salt on different stages of LR development, we calculated the total number of LRPs that have been passing through each stage every day. Figure 32A illustrates the calculation method for the growing trend of each stage. Calculation of the percentage suppression induced by salt stress was performed in order to visualize the effect of salt stress, and a two-group-pattern could be found (Fig. 32B). The constant 30% or higher suppression on the LR initiation stage suggested that salt stress highly inhibits LR initiation process. The large gap between emergent LRP and LRP before emergence indicates that the outgrowth process is also a key step that salt stress has the repression effect on. Importantly, the overall decreasing sensitivity to salt stress (Fig. 32B) suggested that an acclimation process was occurring in the root.

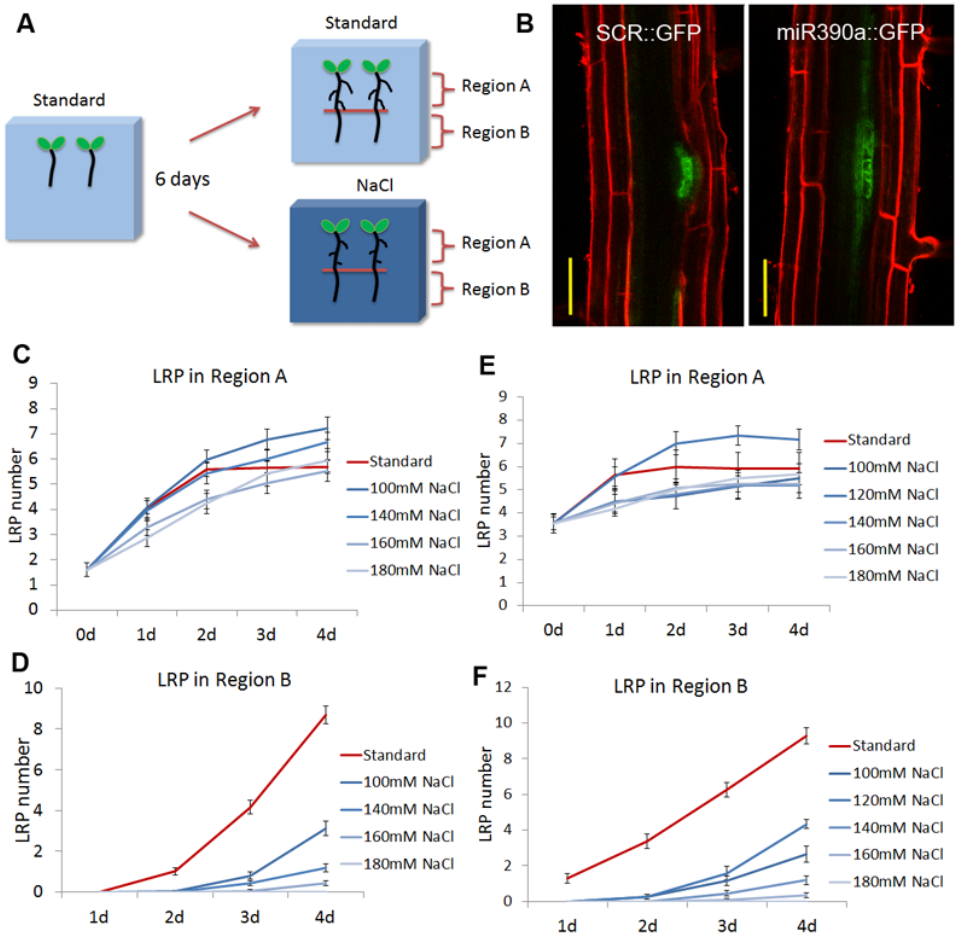

Figure 30. Salt stress suppresses LR initiation through signaling to the primary root tip.

(A) Diagram illustrates the transfer experiment to salt. Two distinct regions, region A and region B was created during this experiment.

(B) Confocal images of early LR stages GFP marker lines: *SCARECROW::GFP* (left panel) and *miR390a::GFP* (right panel). Scale bar: 50 microns.

(C-D) Quantification of LRP marked by the *SCR::GFP* under various NaCl concentrations in root Region A (C) and Region B (D).

(E-F) Quantification of LRP marked by the *miR390a::GFP* under various NaCl concentrations in root Region A (C) and Region B (D).

Error bars: SEM.

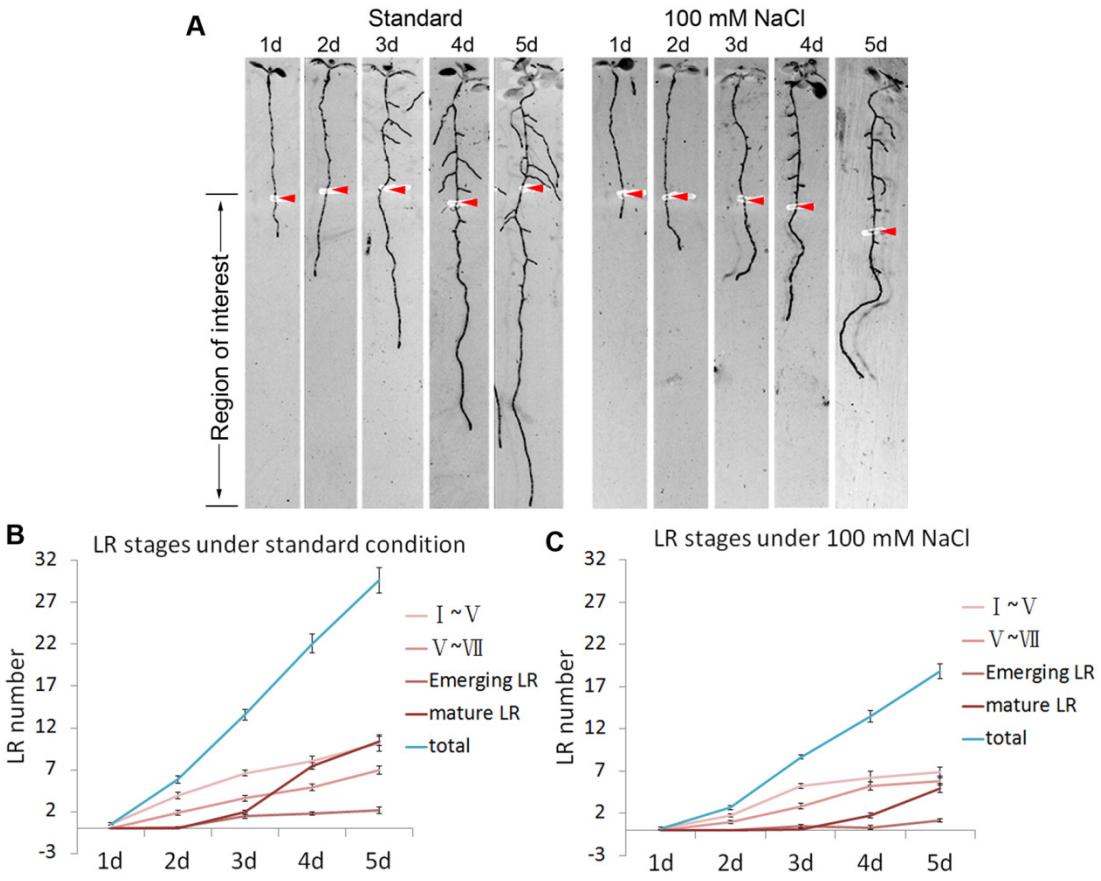

Figure 31. LR developmental stages during a time-course treatment of 100 mM NaCl.

(A) Morphology of Col-0 Root system during either standard or 100 mM NaCl treatment. Red arrowheads mark the transfer point. Only the newly developed region was characterized.

(B) Quantification of various stages of LR development from fixed tissue grown under standard condition from 1 day to 5 days after transfer.

(C) Quantification of various stages of LR development from fixed tissue grown under 100 mM NaCl condition from 1 day to 5 days after transfer.

Error bars: SEM.

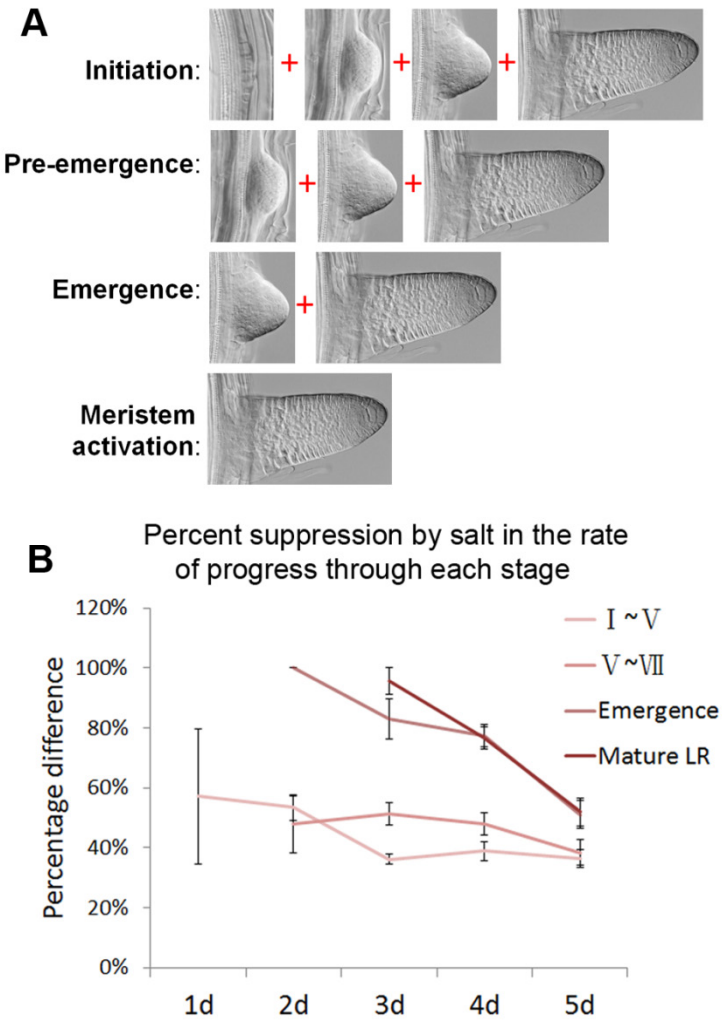

Figure 32. Salt suppresses both LR initiation and outgrowth processes.

(A) Diagram illustrates the calculation method of the total growth of each stage every day. For instance, the total LR initiation events before day 1 equal to the sum of all four different stages at day 1.

(B) Percentage difference of the total events number passing through different stages between standard and 100 mM NaCl.

Error bars: SEM.

4.3.2 Salt stress disrupts LR pre-branching site formation

According to the pre-branching site hypothesis proposed by Moreno-Risueno et al., LRP is pre-determined by auxin maxima, which is environmental independent. However, not all pre-branching sites can be maintained and eventually develop to primodia.

To investigate whether salt stress can affect even earlier stages than LR initiation, *DR5::Luciferase* oscillation was monitored under salt stress. *DR5::Luciferase* seedlings were grown on standard condition for 5 days and then transferred to 100 mM NaCl. Time-series imaging were taken within the subsequent 24 hours. Interestingly, it showed that DR5 could still oscillates in salt treated roots. However, the standard condition grown roots had sustained auxin response maxima, which will eventually stabilize and give rise to PBS (Fig. 33A), while in salt treated roots, the accumulated auxin response failed to be stabilized and form functional PBS (Fig. 33B). Quantification of the time interval between DR5 oscillations showed that during salt treatment, DR5 oscillated slightly faster that during standard condition (Fig. 34A, B). Consistent with this observation, slightly more PBSs were found to be initiated within the 24 hours after transferred to salt, while much less PBSs were remained after 24 hours under salt (Fig. 34C). This result suggests a very early effect of salt stress in LR patterning determination. Figure 33 shows the root Region B of *DR5::Luciferase* grown on either standard or salt media. In standard grown root, well-spaced and evenly bright PBS can be observed; while in salt grown root, lack of luciferase maintenance can be seen on the distal part of the root and patchy luciferase

can be found on the basal part of the root (Fig. 35). Consistent with these observations, the measurements of luciferase brightness under both standard and 100 mM NaCl show that salt grown roots have a wider distribution of brightness, especially having more highly bright PBS (Fig. 35B) but less total number of PBS, which could suggest a lack of auxin circulation. The measurements of the distance between adjacent expression spots show that the peak of normal distribution was shifted by salt treatment (Fig. 35C). These results confirmed that the patterning of LR primordia is disrupted at the earliest stages.

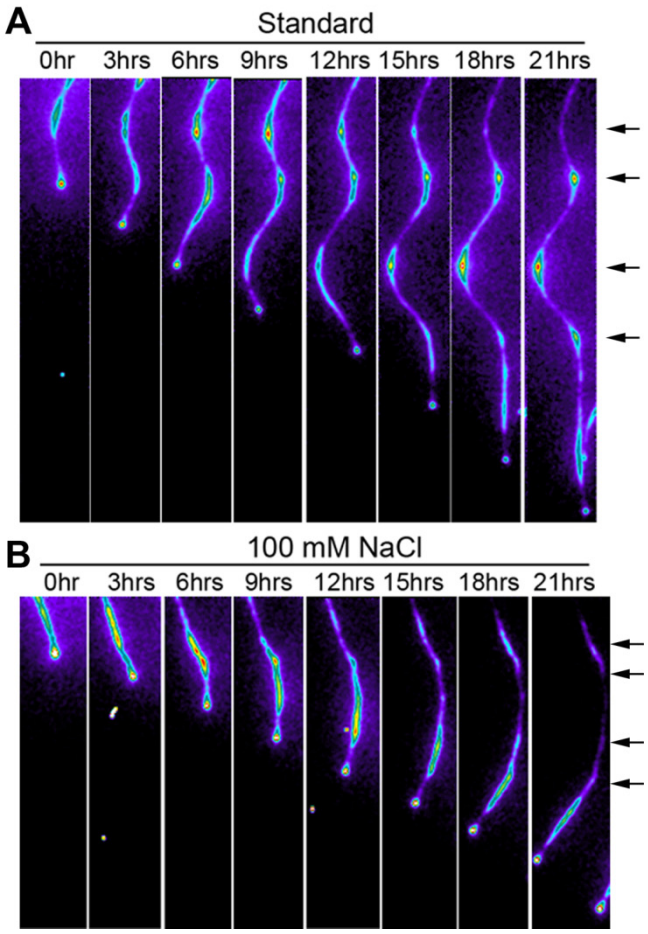

Figure 33. Salt stress affects the maintenance of PBS.

(A-B) Time-lapse images of *DR5::Luciferase* roots grown under standard condition (A) or 100 mM NaCl condition (B). Black arrows point to the positions where auxin response oscillates and supposed to become LR PBS. Note, on salt grown root, the brightness of the hypothetical PBS is very hard to be maintained.

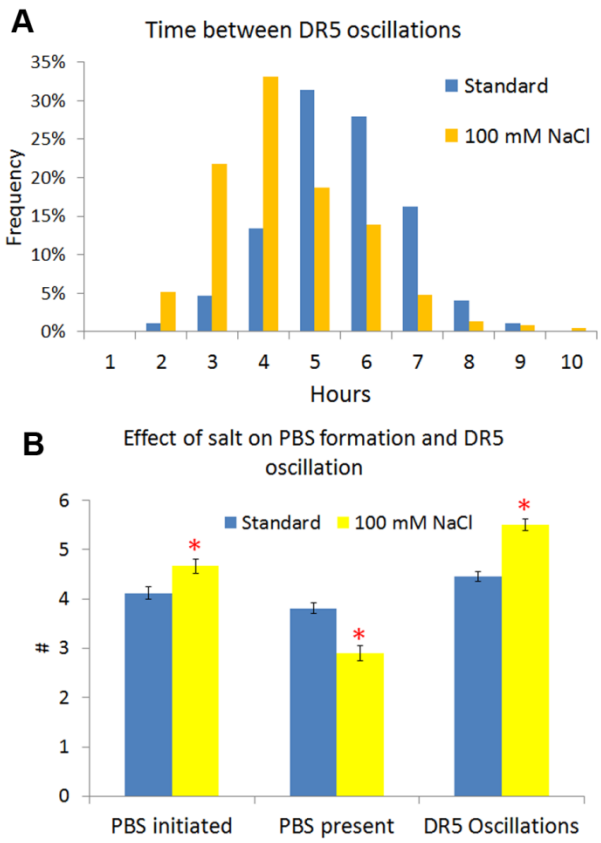

Figure 34. LR pre-branch sites maintenance is disrupted by salt stress.

(A) Distribution of the time interval between DR5 oscillations during standard condition or 100 mM NaCl within 24 hours after transfer.

(B) Quantification of the number of initiated PBSs within the 24 hours after transfer, the number of PBSs present after 24 hours and the number of DR5 oscillation events within the 24 hours.

Error bar: SEM. Red asterisks represent significant differences based on Student's t-test, P-value<0.05.

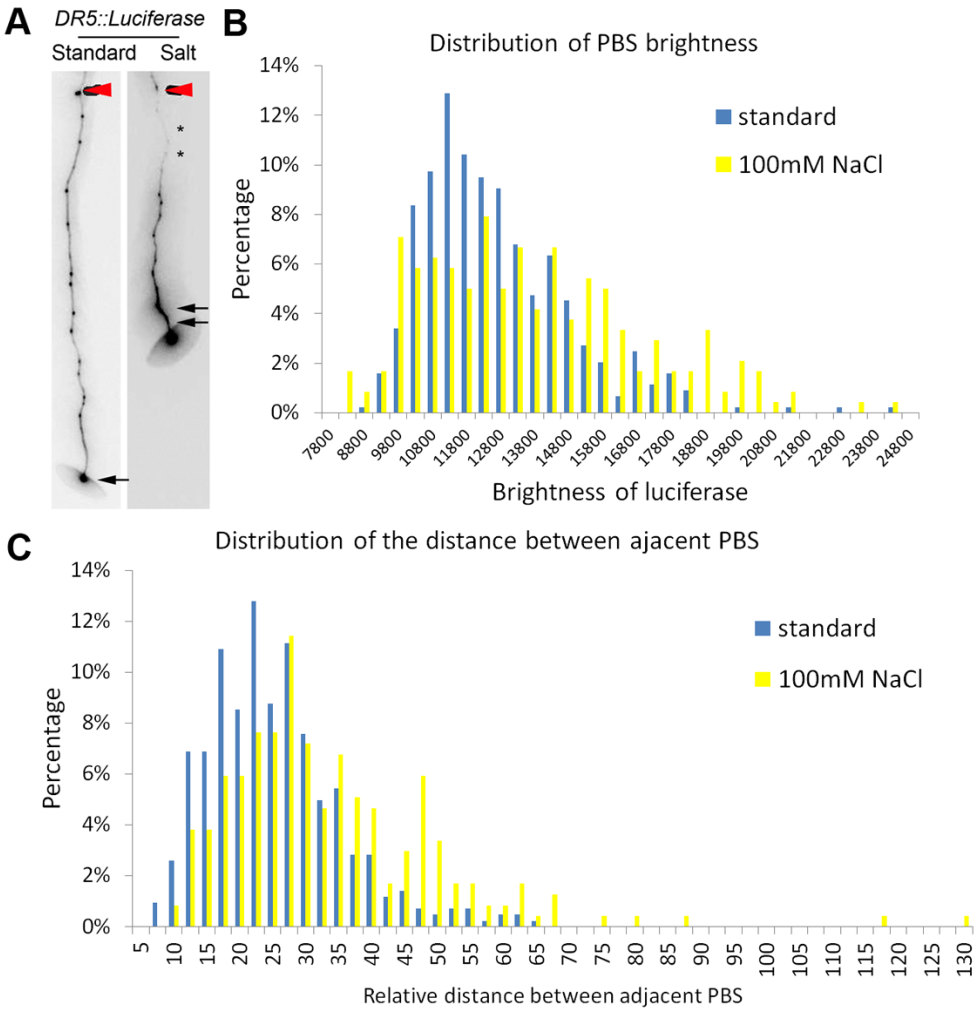

Figure 35. Salt stress disrupts the patterning of LR pre-branch sites.

(A) False colored *DR5::Luciferase* roots grown under either standard condition or 100 mM NaCl. Black dots represent the enrichment of *DR5::Luciferase* expression. Red arrows label the transfer points while black arrows point to the strong accumulation of auxin response in the root tip. Asterisks mark the root region that lack DR5 maintenance.

(B) Quantification of the brightness of PBS in both standard and 100 mM NaCl grown roots. Data is presented as distribution of brightness.

(C) Quantification of the distance of adjacent PBSs in both standard and 100 mM NaCl grown roots. Data is presented as distribution of the distance.

4.3.3 Exogenous auxin could not rescue LR patterning or outgrowth for salt-grown root

Based on the phenomenon we observed in the previous section, that auxin maximum fails to be maintained during salt stress, we want to understand whether exogenous free auxin is able to restore the auxin maxima and facilitate the transition from pre-branching site to a lateral root primordium. Previous research has shown that a non-transportable form of auxin, 2,4-D, could induce ectopic LRP on the established PR (Laplaze et al., 2007), which suggests an excessive amount and mis-location of IAA may also lead to ectopic LRP induction. To test whether the reduced maintenance of pre-branching sites under salt treatment could be rescued by a proper amount of exogenous IAA supply, gradient concentrations of IAA were used in this experiment and a two-step transfer strategy was used.

Col-0 roots were grown on standard media for 5 days and then transferred to 100 mM NaCl media for additional 3 days. Then the seedlings from both standard and salt plates were transferred to media supplemented with various concentrations of IAA for 2 days. The LR stages of the 3-day-grown root region were scored. In this region, primary roots were primed under either standard condition or 100 mM NaCl condition before subjected to IAA treatments. Firstly, it was observed that as the IAA concentration increased, the total number of LRP increased for both roots under standard and salt condition (Fig. 36A). However, under relatively lower IAA (50-250nM IAA), the repression effect of salt was retained (Fig. 36A). As indicated in Fig. 36B, on salt treated roots, IAA was able to induce more LR initiation; especially

1 μM IAA onwards could induce extra primodia, which suggested that salt treated roots could be more sensitive to high auxin in term of inducing LR initiation. However, at high IAA concentration, the ectopic LRP were inhibited from growing out (Fig. 36C, D), which indicated IAA can only rescue the lateral root initiation, but not outgrowth process.

In order to further understand whether the similar total LR number under 1 μM IAA was a result of rescued pre-branching site formation, the distribution of LRP along root was studied through measuring the epidermis cell number between two adjacent primodia. It is shown in Fig. 37A that IAA shortens the distance between adjacent LRPs by shifting the peak of distance distribution left under standard condition. However, for salt treated roots, the normal distribution is disrupted by IAA, and many primordia are almost connecting together near the root tip (Fig. 37B). These results implied that exogenous free form of auxin could not rescue the normal spacing of LRP (Fig. 38), which raised the possibility of impaired auxin circulation system.

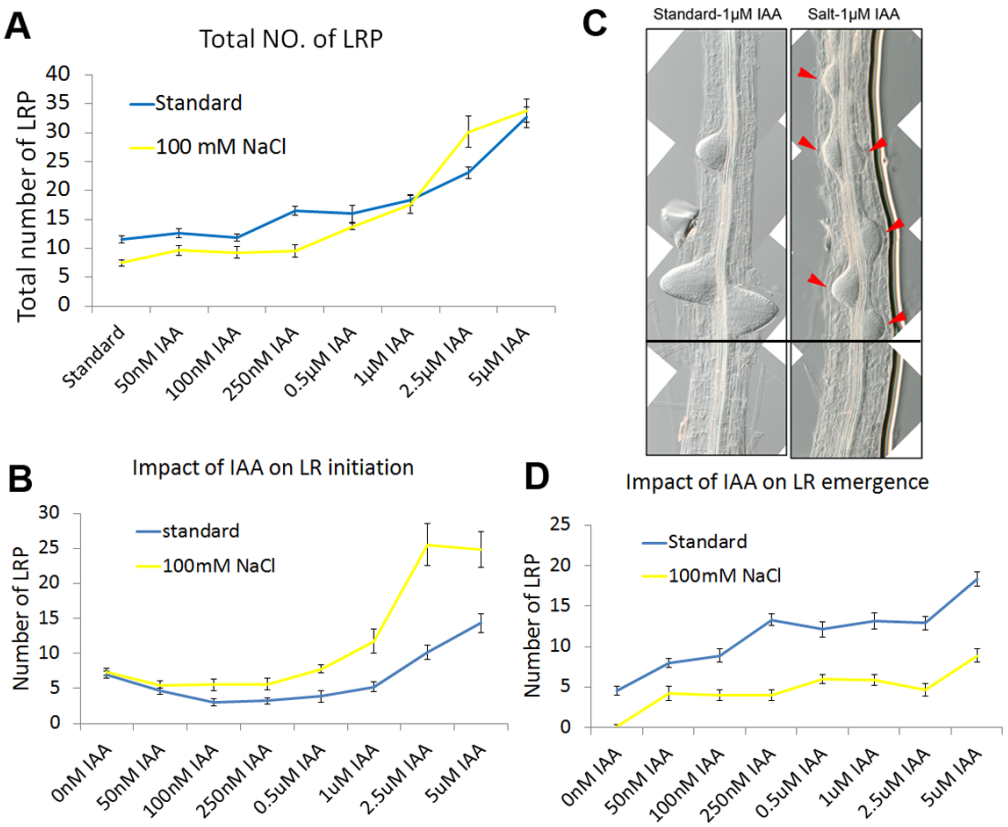

Figure 36. High IAA induces LR initiation but unable to overcome salt inhibition of LR outgrowth.

(A) Quantification of total number of LRP for either standard condition primed or 100 mM NaCl primed roots during various concentrations IAA treatment.

(B) Quantifications of LR stages before emergence for either standard condition primed or 100 mM NaCl primed roots during various concentrations IAA treatment.

(C) DIC images of the root tip region after 3 days growth on either standard or salt media. Horizontal black line represents the transfer point to IAA treatment. Red arrows point to the LRPs which induced by IAA, but failed to emerge on salt stress primed root.

(D) Quantifications of emergent LR stages for either standard condition primed or 100 mM NaCl primed roots during various concentrations IAA treatment.

Error bar: SEM.

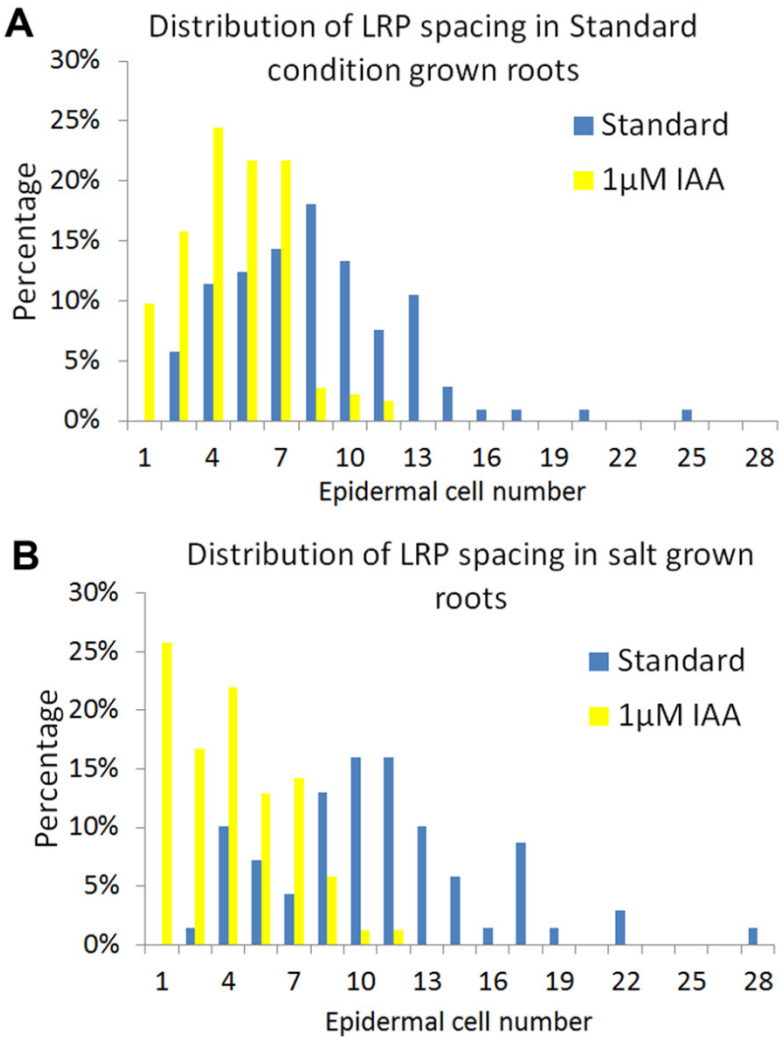

Figure 37. The regular distribution of LRP spacing is disrupted by salt stress and cannot be rescued by IAA.

(A) Distribution of LRP spacing of the standard condition primed roots, which were transferred to either standard or 1 μM IAA media.

(B) Distribution of LRP spacing of the 100 mM NaCl primed roots, which were transferred to either standard or 1 μM IAA media.

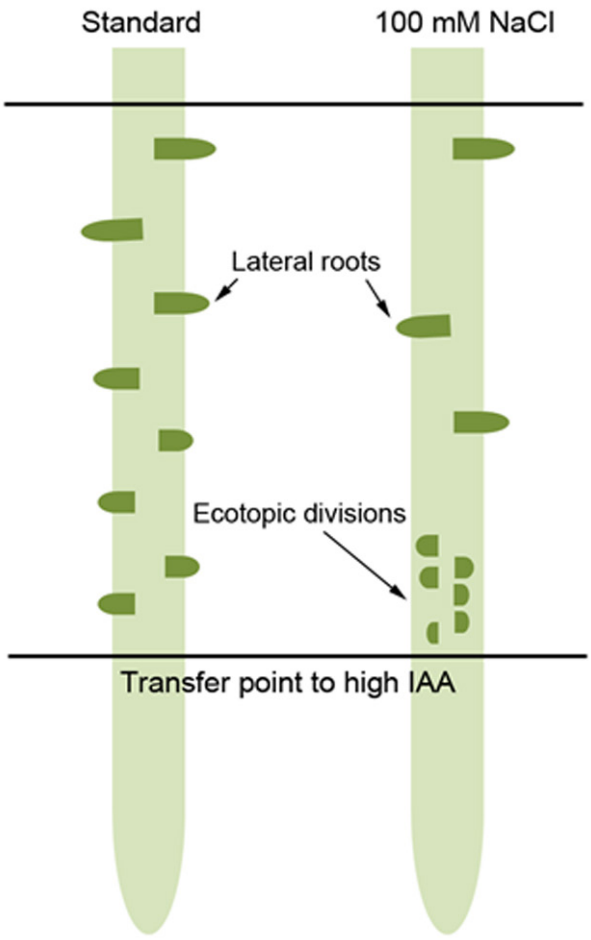

Figure 38. Exogenous IAA cannot rescue the regular spacing of LRP on the root primed under salt stress.

Diagram visualizes the result of the double transfer experiment. IAA induces ectopic divisions in the root region near the transfer point.

4.3.4 High salinity represses auxin transportation and signaling

Various membrane localized transporters have been identified for polarized auxin transportation in the plant. Auxin synthesized in the shoots can be transported down to the root tip, and the various auxin carriers ensure directed auxin flow and distribution in the root. This process is necessary to maintain many developmental programs including the organogenesis of lateral roots (Casimiro et al., 2001; Laskowski et al., 2008). To study the long term effect of salt stress on auxin transportation, protein intensity as well as localization of various auxin transporters was examined after 3 days treatment on salt. Among the various auxin transporters, AUX1 is an auxin influx carrier which is expressed in lateral root cap, epidermis, collumella and stele (Swarup et al., 2004). The plasma membrane localization allows auxin uptake into the cell. PIN2 is an auxin efflux carrier which has polarized membrane localization in both epidermis and cortex in the root meristem, and it can direct auxin flow from collumella shootwards (Blilou et al., 2005). Interestingly, AUX1 (auxin influx carrier) and PIN2 (auxin efflux carrier) are the only auxin transporters that direct auxin flow from the root tip to the maturation zone, and they were found to be repressed under salt stress condition (Fig. 39A-D), suggesting that auxin re-location from the root tip maxima toward the mature region of the root could be suppressed.

Recent work has identified GATA23 as an early regulatory protein on LR founder cells specification and initiation, and it is regulated by IAA/ARF module. The expression of GATA23 is highly correlated with auxin oscillation pattern (De Rybel

et al., 2010). To understand whether the early auxin induced signaling is affected by salt stress, *GATA23::GUS* seedlings were grown on 100 mM or 140 mM NaCl for 3 days before 5 days growth on standard condition, and the expression level and pattern of GATA23 was evaluated on the 3-day-grown region. Three gradual levels were defined for GUS level and spotted expression pattern and patched pattern were separated in the quantification, indicated in the lower panel of Fig. 40. Interestingly, the highly expressed GATA23 spots were greatly reduced by salt treatments, while the number of weak GUS spots was even mildly induced by 140 mM NaCl (Fig. 40), together with the greatly reduced total number of GATA23 expression spots; it suggests that the auxin signaling during early LR initiation process is largely suppressed by salt stress.

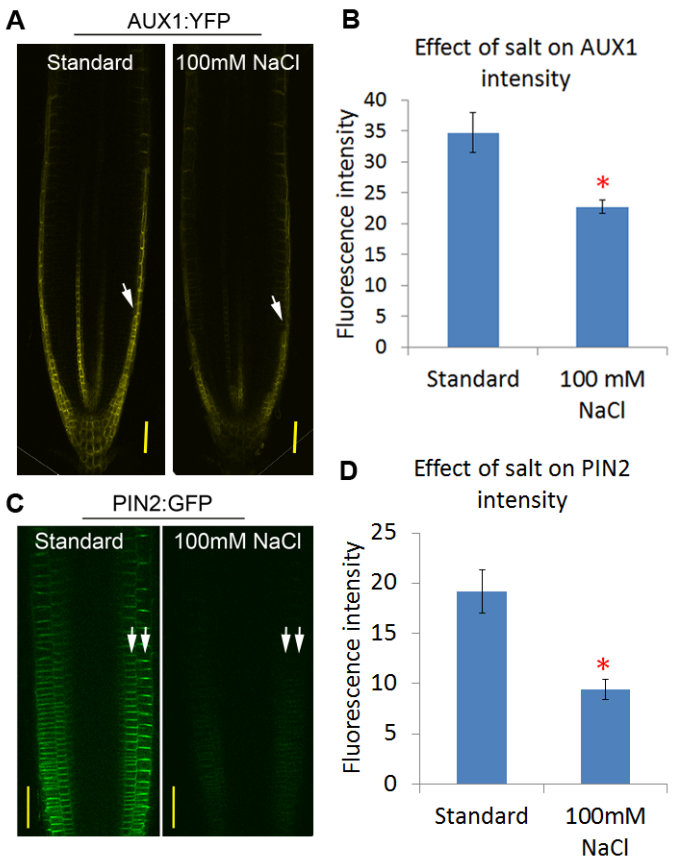

Figure 39. The Auxin transportation from basal to distal is suppressed by salt stress.

(A) Confocal images of *AUX1:YFP* fusion protein under either standard or 100 mM NaCl condition. White arrow point to the lateral root cap localization of AUX1.

(B) Quantification of the fluorescence intensity of AUX1 in the lateral root cap.

(C) Confocal images of *PIN2:GFP* fusion protein under either standard or 100 mM NaCl condition. White arrow point to the epidermis and cortex localization of PIN2.

(D) Quantification of the fluorescence intensity of PIN2 in both cortex and epidermis.

Error bars: SEM. Scale bar is 50 microns. Red asterisks represent significant differences based on Student's t-test for percentage difference, P-value<0.05.

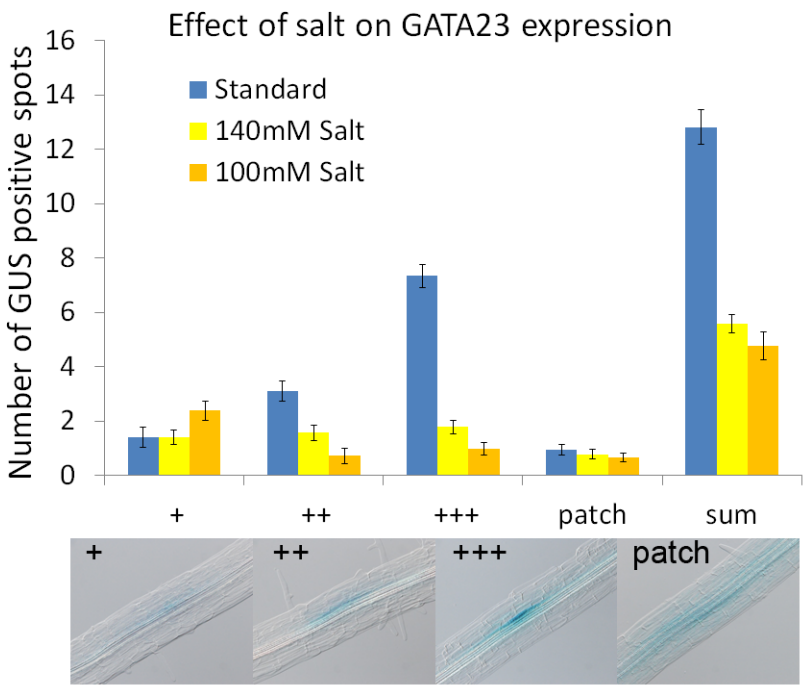

Figure 40. GATA23 expression is affected by salt stress

Quantification of the number and level of GATA23 expression along the primary roots. The expression of GATA23 is scored as 3 gradual levels in spotted form and a patched form of expression. Lower panel is the representative images for the different scoring levels.

4.4 DISCUSSION

This study provides detailed analysis about the effect of salt stress on various stages of LR development. We found that salt stress can only suppress LRP formation on the PR root region which directly grow on salt medium, and also highlight both LR initiation and emergence as two key steps during this process. Importantly, the reduced initiation events and disrupted spacing of LRP is largely due to the less well maintained LR pre-branch sites, which are determined by auxin maxima. Under environmental stresses such as high salinity, root tip functions to sense the rapid changes and subsequently adjust internal growth programs to modulate new organogenesis.

4.4.1 Salt stress suppresses two distinct stages during LRP development

A morphological study has defined the process of LRP development into various distinct stages (Malamy and Benfey, 1997). During this continuous process, a developmental transition needs to take place between the two major steps, LR initiation from the pericycle cells and the meristem formation within the LRP in both structural and functional point of view (Laskowski et al., 1995). Our results show that during the whole LR organogenesis process, salt stress largely suppresses the two key steps, LR initiation and emergence; though a general mild repression can be observed in other LR developmental stages (Fig. 32). It indicates that the signals from environment stresses can be integrated into the decision making of triggering the two key functional steps during LR organogenesis. This mechanism allows a secondary

decision making step in the roots and reduces unnecessary exposure to harmful environmental. Auxin has been found to be play essential roles in the whole developmental process of LR, especially the early initiation step. However, LRP emergence can be regulated through different aspects of auxin signaling. For example, during early seedlings growth, the high concentration of auxin in the root tip controls LR initiation, while the first emergence event of LR is highly correlated with the shoot derived auxin in the root (Bhalerao et al., 2002). In addition, during LR initiation, auxin accumulation triggers pericycle cell to differentiate and promotes cell dividing activities (Dubrovsky et al., 2008) and the spacing of LRP is regulated by various auxin transporters (Laskowski et al., 2008). However, during the LRP emergence process, auxin can be taken up by an auxin influx carrier, LAX3, in the cortical cells surrounding the LRP and it triggers the expression of cell wall remodeling enzymes in order to facilitate the breaking through process (Swarup et al., 2008). In our study, salt stress repressed LR initiation events can be rescued by excessive auxin supplementation to the root, however the emergence of LR is still arrested (Fig. 36), suggesting the different signaling module triggered by salt stress during these two stages. Again, this suppression of LR emergence independent of root derived auxin reduces surface exposure of RSA to the environment. The secondary signaling underlies the suppression of LR emergence by salt stress could be the future direction of this study.

4.4.2 Regulation of RSA by Auxin distribution under the control of environmental factors

RSA appears to be highly plastic in response to environmental changes, and the rapid responses are always correlated with auxin re-distribution. For example, phosphate starvation represses PR length while promotes LR density and the gene *siz1* regulates this process and mediates auxin distribution in the meristem region of both root types (Miura et al., 2011). More interestingly, a nitrate transporter, NRT1.1, is also found to be able to transport auxin. By moving auxin through epidermis in the LRP, the emergence process during different nitrate conditions is regulated by NRT1.1 (Krouk et al., 2010). This mechanism allows roots system to sense environment changes and make responses rapidly. However, none of these studies are focused on the early patterning process of LRPs, which are largely determined by auxin oscillation and distribution in the PR. In our study, the reduction of expression level of both PIN2 and AUX1 during salt stress indicates a suppressed shootward auxin flow, which is highly correlated with the reduced maintenance of LR pre-branch sites and the visualization of *DR5::luciferase* (Fig. 35A). This finding suggests that under a constant salt stress environment, the early LR patterning program could be disrupted by an altered auxin transportation system (Fig. 40). Interestingly, salt stress has been found to induce clathrin to be localized in the plasma membrane (Konig et al., 2008), which may potentially serve as a mechanism to mediate the re-localization of membrane localized auxin transporters through vesicle trafficking.

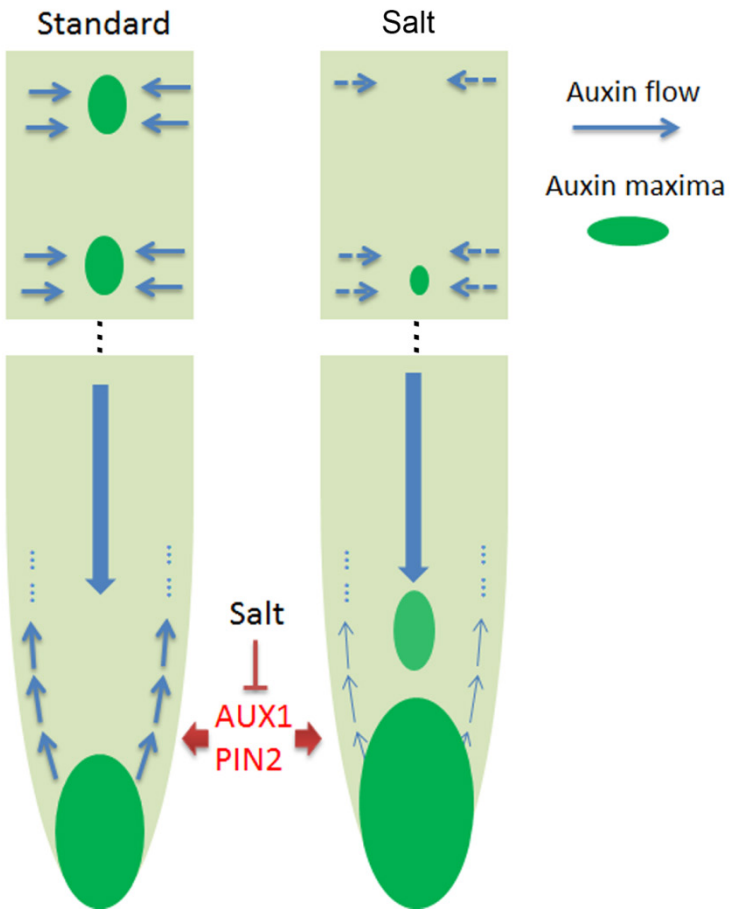

Figure 41. Model for auxin flow and PBS formation during salt stress.

The auxin flow in the root tip is shown by blue arrows. The line thickness represents the strength of auxin transportation. The repression of AUX1 and PIN2 expression under salt results in reduced circulation of auxin from root tip upwards. Consequently, the formed LR pre-branch sites are less able to be maintained under salt stress.

Chapter 5 CONCLUSION

This thesis reveals that the impact of salt stress on various aspects of RSA development is complicated and may function through different signaling pathways. During the morphogenesis process of LRs, three key steps, including patterning, emergence and post-emergence growth activation, are found to be targets of salt stress. Firstly, salt stress needs to be present prior to each decision making steps in order to trigger a defect or retarded growth. For instance, since the LR patterning is determined by an auxin response oscillation occurring at the root tip, the root region grown prior to transferring to salt does not have LR patterning defects, while the emergence process is repressed by salt stress. Secondly, instead of a general suppression on this continuous developmental process, stage specific disruption or quiescence is observed under salt stress. Specifically, from LR initiation to meristem activation, the initiation and emergence are strongly inhibited by salt, while the stages in between them are only mildly affected. Similarly, during the post-emergent LR growth process, salt also induces a specific 'quiescent phase' before growth recovery instead of general growth suppression. Importantly, this growth quiescence is necessary for root growth recovery and better adaptation to salt stress.

In this thesis, we also reveal the importance of spatial and temporal dynamics during the regulation of LR development under salt. First of all, different root types confer distinct sensitivity to salt treatment in growth, which is due to a sustained ABA signaling in newly emerged LRs. The temporal dynamics of LR growth suppression is highly correlated with the induction of ABA signaling. Secondly, a specific cell type, endodermis, is found to be the primary targets of ABA signaling in regulating LR growth, and it may also integrate GA signaling and function as a regulatory center

for root growth during environmental stresses. Lastly, while ABA signaling is mostly induced after LR emergence and induces the 'quiescent phase' in LR growth, auxin signaling plays more important roles in early LR patterning process. Under standard condition, the auxin response oscillates regularly and is stabilized at certain positions in the PR to form the pre-branch sites for LRs. Under salt stress, oscilations are irregular and the highly auxin responsive cells are less able to be maintained and develop to mature pre-branch sites under salt stress.

In summary, this study provides detailed analysis of the impact of salt stress on various developmental stages of lateral roots and it also reveals the temporal dynamics and spatial regulations of different plant hormones during these processes.

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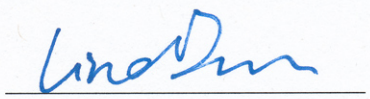
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



Lina Duan

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