ABSTRACT

Title of Dissertation: DISSECTION OF MOLECULAR MECHANISMS OF ABIOTIC STRESS TOLERANCE IN *ARABIDOPSIS THALIANA*

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Plants, due to their sessility nature, must cope with different environmental stresses during their lifetime. Elucidation of the molecular mechanisms of abiotic stress responses in plants is important for agricultural production, food security, and global sustainability. For my dissertation research, we employed forward genetic analysis to

study genes essential for different abiotic stresses in *Arabidopsis thaliana*. Here we report the identification and characterization of two genes *ROA1* (REGULATOR OF ABA RESPONSE 1) and *RSA4* (SHORT ROOT IN SALT MEDIUM 4), that are critical for cold stress and salt stress responses, respectively. ROA1 is a splicing factor, belonging to U1 snRNPs (small nuclear ribonucleoproteins). A previous study in our laboratory revealed its regulatory roles in ABA responses. Here, we characterized its role in plant cold stress responses, especially its role in chilling stress tolerance. The *roa1* mutants are hypersensitive to chilling stress and moderate sensitive to freezing stress. Overexpressing *ROA1* led to improved chilling tolerance. These results suggest that ROA1 positively regulates plant cold tolerance. RNA sequencing data revealed that ROA1 controls the proper splicing of many genes with diverse functions. IP-Mass analysis together with BiFC analysis showed that ROA1 interacts with other U1 snRNPs such as U1-70K and PRP40A. Loss-of-function of one of the ROA1 splicing targets, AtDi19-4 (Drought-responsive family protein 19-4), is hypersensitive to chilling stress. Ectopic expression of *AtDi19-4* can partially restore the *roa1* mutant phenotype, suggesting it is critical for ROA1 function under cold stress. Together these results indicate that ROA1 is critical for alternative splicing of genes under cold and cold stress tolerance in plants.

We identified the *rsa4* mutant in a forward genetic screening. *RSA4* encodes a cellulose synthase for primary cell wall synthesis. *rsa4* mutants are sensitive to salt stress and drought stress. The *rsa4* mutants over accumulate reactive oxygen species (ROS). We also observed that the integrity of the cuticle in the *rsa4* mutant plants is

compromised. These results can at least partially explain why mutations in *RSA4* result in increased sensitivity to salt and drought stress conditions.

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by

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Preface

The molecular mechanisms of abiotic stress responses are very complex. Dissecting the signaling pathways, from signal perception to signal transduction and regulations on gene expression, is crucial for understanding plant stress responses. In my dissertation, I summarized my work on a U1 splicing factor, ROA1 (REGULATOR OF ABA RESPONSE1), in response to cold stress, and a cellulose synthase, RSA4, in response to salt stress. Together, the studies will help people understand how plants cope with environmental challenges, and the knowledge will guide agricultural production and contribute to food security and agricultural sustainability.

Dedication

This work is dedicated to my family and friends. Your words become my courage, and this courage walked me through my Ph.D. study.

I dedicate this dissertation to my parents, for their support, trust, and unconditional love. They encouraged me to pursue my Ph.D. in the United States, 7000 miles away from home. I am truly grateful for this precious experience, not only becoming a plant scientist but also being able to see the world differently.

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Special thanks to Dr. Steve Mount. His comprehensive knowledge and expertise in splicing giving me a big picture and helped me understand my project more in-depth.

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List of Abbreviations

ROA1/RBM25: Regulator of ABA response 1/ RNA binding motif protein 25

OSCA1: Reduced hyperosmolality induced $[Ca^{2+}]_i$ increase 1

COLD1: Chilling tolerance divergence 1

ER: Endoplasmic reticulum

ROS: Reactive oxygen species

ABA: Abscisic acid

PYR: Pyrabactin resistance

PYL: PYR1-like

RCAR: Regulatory components of ABA receptor

PP2C: type 2C Protein phosphatase

SnRK2s: Snf1-related protein kinases

ABRE: ABA-responsive elements

CBF: C-repeat binding factor

DREB1B: Dehydration-responsive element binding factor 1B

CRT/DRE: C-repeat/Dehydration-responsive element

CAMTA3: Calmodulin-binding transcription activator 3

ICE1: Inducer of *CBF* expression 1

bHLH: Basic helix-loop-helix transcription factor

HOS1: High expression of osmotically responsive gene 1

SIZ1: SAP and Miz 1

SUMO: Small ubiquitin-related modifier

OST1: Open stomata 1

pre-mRNAs: Precursor messenger RNAs

snRNPs: Small nuclear ribonucleoproteins

AS: Alternative splicing

PTC: Premature termination codon

NMD: Nonsense mediated decay

STA1: Stabilized1

RCF1: Regulator of *CBF* gene expression1

CIR1/RVE2: Circadian1/Reveille2

PRR5: Pseudo-response regulator5

LHY: Late elongated hypocotyl

CCA1: Circadian clock associated1

TOC1: Timing of cab expression1

PRR: Pseudo-response regulator

PIF7: Phytochrome-interacting factor7

phyB: Phytochrome B

SD: Short-day

SNRF1: a small nuclear ribonucleoprotein family protein encoded by *At2g14285*

RBM7: RNA-binding RRM/RBD/RNP motifs family protein

WD40RL: transducin/WD40 repeat-like superfamily protein

ATOSA1: *Arabidopsis thaliana* oxidative stress-related ABC1-like protein1

UPI: Unusual serine protease inhibitor

DSP4: Plant and fungi atypical dual-specificity phosphatase4

TRAF1B: TRAF-like superfamily protein1b

AtDi19-4: Drought-responsive family protein19-4

LHCB7: Light-harvesting complexb7

NCED: 9-cis epoxycarotenoid dioxygenase

CED1: 9-cis epoxycarotenoid dioxygenase defective1

RSA1: Short root in salt medium1

RITF1: RSA1 interacting transcription factor1

EMS: Ethyl methanesulfonate

Overview

Abstract

Plants, with the sessile property, must cope with the environmental stresses during their growth and development. These stress conditions caused by non-living environmental factors are known as abiotic stresses, including drought, salinity, cold, and heat. Changes in the environments in the past few decades adversely affect plant growth and crop production, which has become a great challenge to global food security (Mickelbart et al., 2015). Understanding the mechanisms of abiotic stress responses in plants is crucial for agricultural production and sustainability. In this chapter, I reviewed and summarized some well-studied molecular mechanisms of plant abiotic stress responses. These mechanisms are highly complex and overlapped in stress responses.

Plant abiotic stresses, signal perception and transduction

Abiotic stresses adversely affect cellular functions and activities in plants. Researchers categorized these stress conditions according to different physical and chemical stimuli, such as cold, heat, drought, salinity, etc. In general, researchers are working on dissecting the signal transduction pathways and networks to understand abiotic stress responses.

Plants evolved different strategies to face abiotic stress, for instance, stress avoidance and stress tolerance. In our lab, we are focusing on stress tolerance, understanding how plants adapt to unfavorable growth conditions. Mechanisms of plant abiotic stress responses include stress signal perception, signal transduction (the process of cellular signal transmission), and downstream responses via gene expressions. In general, plants perceive environmental stimuli, and these signals are transmitted into the cell to trigger gene expression.

Many research questions need answers to understand abiotic stress tolerance mechanisms. For example, how plants sense the stress signals, and what are the putative plant stress sensors? However, characterizing plant stress sensors remains very hard. Limitations on technology impede the accurate detection of stress signals (physical signals) in plants, and the challenges remain on utilizing the genetic approach to identify the gene(s) that either is essential for plants or has functional redundancy (Zhu, 2016). Researchers hypothesized that changes in the membrane fluidity may involve in the cold signal perception in plants (Murata and Los, 1997; Martinière et al., 2011). Over the past few decades, several stress sensors were identified, such as OSCA1, COLD1, etc. *OSCA1* (*REDUCED HYPEROSMOLALITYINDUCED* $[Ca^{2+}]_i$ *INCREASE 1*) encodes a plasma membrane protein, and the *osca1* mutant plants showed impaired $[Ca^{2+}]_i$ induction in response to osmotic stress, indicated that OSCA1 has a putative role as an osmosensor in plants (Yuan et al., 2014). Ma et al. identified COLD1 (CHILLING-TOLERANCE DIVERGENCE 1) in rice, a transmembrane protein that localizes on

the plasma membrane and endoplasmic reticulum (ER) membrane. It interacts with RGA1, the G-protein subunit α in rice, and further regulates Ca^{2+} signals in response to cold stress (Ma et al., 2015). Currently, researchers are still working to obtain more direct experimental evidence to understand how these putative sensors perceive the physical signals and respond to different stress stimuli.

During signal transduction, many molecules are either produced or recruited to respond to different stimuli. For instance, the concentration of calcium ion was increased when plants response to environmental stresses. Reactive oxygen species (ROS) can be accumulated in response to different stress conditions including cold, heat, salinity, and drought, etc. Over-production of ROS can cause oxidative stress, which leads to damage to functional cellular components including nucleic acids and proteins (Río, 2015). Also, the induction of ROS can serve as a second messenger in signal transduction, which triggers downstream stress responses in plants (Vanderauwera et al., 2009).

After the plants perceive the stress signals and transmit the signals via second messengers in the cell, signaling cascades occur to promote gene expression to respond to different stress conditions. Here, I mainly discuss plant responses to cold stress, salt stress, and drought stress.

Plant cold stress responses

CBF-mediated regulation pathway

Cold stress, one of the abiotic stresses, limits the geographical distribution of the plants, and severely affects worldwide crop production. Defining by the temperatures that plants are exposed to, cold stress can be classified as chilling stress and freezing stress. These two stresses represent injuries resulted from a low temperature above or below zero degrees Celsius, respectively. Moreover, the causes of these injuries are different: cellular malfunction is the main cause of chilling injury, displaying symptoms such as wilting, yellowing, and even death; ice formation, which leads to membrane damage and cellular dehydration, is the predominant cause of freezing damage (Knight and Knight, 2012). During cold stress, plant organelles showed damages directly from low temperatures. For instance, chloroplast, as the photosynthetic machinery in plants, showed rapid damage (chloroplast and thylakoid swelling) after exposure to extreme temperatures (Kratsch and Wise, 2000).

In the 1990s, extensive studies were conducted on freezing tolerance and the role of cold acclimation in plants. Cold acclimation is a process of exposing the plants under non-freezing low temperatures for a short period (Thomashow, 1999). cold acclimation, plants experience changes at the molecular, biochemical, and metabolic levels. Previous studies discovered several signaling pathways for cold stress. CBF (C-REPEAT BINDING FACTOR) cascade is one of the most well-established pathways in plant cold stress response. The CBF-mediated cold response pathway is

conserved in many plant species (Jaglo et al., 2001). *CBF1*, *CBF2*, and *CBF3*, also known as *DREB1B* (*Dehydration-Responsive Element Binding Factor 1B*), *DREB1C*, and *DREB1A*, are rapidly induced after low-temperature exposure (Gilmour et al., 2004). CBFs, acting as transcription factors, can bind to the *cis*-elements named C-repeat (CRT)/dehydration-responsive element (DRE) located in the promoters of many cold-responsive genes (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). These genes, also known as the CBF regulon, are then differentially expressed to contribute to freezing tolerance in plants.

Transcriptional regulation on CBF pathway

CBFs act as one of the central regulators in freezing stress tolerance. The upstream regulations on CBFs are very complex. Several components are shown to regulate the expression of *CBF* genes directly or indirectly. CAMTA3 (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3) activates *CBF2* expression, indicating a possible connection between calmodulin regulation on plant cold stress response via calcium signaling (Doherty et al., 2009). CBFs are either activated or inhibited by several components. ICE1 (INDUCER OF *CBF* EXPRESSION 1) is a plant MYC-like bHLH transcription factor, which activates the expression of *CBF3* by directly binding to its promoter (Chinnusamy, 2003). The activation of CBF via ICE1 can be inhibited by HOS1 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1). HOS1 is an E3 ubiquitin ligase that negatively affects CBF function by degrading ICE1 via ubiquitination (Dong et al., 2006). On the other hand,

SIZ1, a SUMO (small ubiquitin-related modifier) E3 ligase, can stabilize ICE1 via sumoylation that reduces ICE1 ubiquitination (Miura et al., 2007). Recently, Ding et al. (2015) showed that a protein kinase OST1 (OPEN STOMATA 1), also known as SnRK2.6, is an important upstream regulator of ICE1 that act positively to achieve cold stress tolerance by inhibiting HOS1-mediated ICE1 degradation under cold stress in *Arabidopsis* (Ding et al., 2015).

Roles of alternative splicing in plant cold stress responses

The regulation of gene expression is very intricate. In eukaryotes, including plants, one of the critical steps of gene expression is pre-mRNA splicing. During splicing, noncoding region (introns) are removed, and exons are spliced to form the spliced mRNA. This process is precisely controlled by a protein complex called the spliceosome. Alternative splicing (AS) is the process of choosing alternative splice sites to produce different transcript isoforms other than the constitutive splicing product. AS plays a critical role in the regulation of gene expression since AS can generate different protein isoforms from a single gene, or degrade the mRNA isoforms caused by the occurrence of the premature stop codon, etc.

Alternative splicing appeared to have a universal effect on abiotic stress responses (Filichkin et al., 2010). Staiger and Brown (2013) suggested a working model of AS regulation. This regulation can lead to signaling cascades in response to environmental factors or developmental stimuli. Such cascades caused by AS include the regulation of some regulatory components (e.g. AS of transcription factors) and

the regulation of other splicing factors or itself (Staiger and Brown, 2013). Lee et al. (2006) identified a mutant named *sta1* (*stabilized1*) from the screening of *RD29A*:*LUC* (luciferase) EMS mutant population. The mutant plants showed enhanced stability of luminescence signal after 72 h of cold treatment, suggesting the critical role of STA1 in mRNA stability and turnover. *STA1* expression can be upregulated by cold stress. It encodes a splicing factor in plants. Splicing defect of *COR15A*, a cold-responsive gene, was only found in *sta1-1* plants under cold stress but not under ABA or NaCl treatments. Overall, this study suggested that STA1 is necessary for pre-mRNA splicing and mRNA turnover under cold stress (Lee et al., 2006). The previous work done by Dr. Jianhua Zhu's laboratory characterized the function of a cold-inducible DEAD-box RNA helicase, called RCF1 (REGULATOR OF *CBF* GENE EXPRESSION1). *rcf1* mutants showed chilling sensitive and freezing sensitive phenotypes. Over 200 genes are mis-spliced in the *rcf1* mutant under cold stress. Several downstream target genes of RCF1 are cold-responsive, including circadian clock regulators *CIR1* (*CIRCADIAN1*), *PRR5* (*PSEUDO-RESPONSE REGULATOR5*), etc. Overall, RCF1 functions as a positive regulator in plant cold stress tolerance by maintaining the proper splicing on the pre-mRNAs of cold-responsive genes (Guan et al., 2013b).

Effects of circadian and light signals on plant cold stress responses

Circadian rhythm and light signaling also regulate plant cold stress responses. The expression levels of *CBF* genes are regulated by circadian rhythm (Fowler et al.,

2005). Two key components of the circadian clock, LHY (Late Elongated Hypocotyl) and CCA1 (Circadian Clock Associated 1) can directly bind to *CBF* promoters and contribute to circadian regulation of *CBF* gene expression in the morning (Dong et al., 2011). The expression levels of *CBF* genes are generally higher in *prr9/prr7/prr5* triple mutant (*d975*) compared with the wild type, indicating those circadian-related PRRs (PSEUDO-RESPONSE REGULATOR) can repress the expression of *CBFs* (Nakamichi et al., 2009).

In addition, photoperiod regulation reflects freezing tolerance in response to seasonal changes. Kidokoro et al. (2009) found that phytochrome-interacting factor7 (PIF7), a bHLH transcription factor, was shown to repress the accumulation of *CBF2* transcript by binding to the G-box motif in the *CBF2* promoter. PIF7 activity is regulated by a photoreceptor phytochrome B (phyB) and a circadian oscillator TOC1 (Kidokoro et al., 2009). CBF-mediated freezing tolerance is repressed under long-day (LD) conditions, whereas short-day (SD) conditions remove such repression and lead to an increase in freezing tolerance. This photoperiodic regulation of *CBF* genes is correlated to higher transcript levels of *PIF4* and *PIF7* and increased PIF4 and PIF7 protein stability under LD. Genetic analysis indicated that phyB may also negatively affect the CBF pathway and freezing tolerance under LD by its interaction with PIF4 and PIF7 (Lee and Thomashow, 2012).

Plant drought stress and salt stress responses

Plant drought and salt stress shared overlapping mechanisms related to loss of water in plants. Osmotic stress is the primary stress signal of drought stress. In salt stress, osmotic stress and/or ionic stress (or ion toxicity), are usually the primary stress signals.

ABA-dependent stress responses

Abscisic acid (ABA) biosynthesis is closely related to the abiotic stress response. ABA accumulates under osmotic stress (Zhu, 2002). NCED (9-cis epoxycarotenoid dioxygenase) catalyzes a rate-limiting step in ABA biosynthesis. *NCED3* is strongly induced after drought exposure in *Arabidopsis*, and it contributes to drought tolerance by elevating endogenous ABA levels (Iuchi et al., 2001). In *Arabidopsis*, *ced1* (*9-cis epoxycarotenoid dioxygenase defective 1*) mutant is hypersensitive to polyethylene glycol treatment, and it showed a reduced expression level of *NCED3* and *ABI1* (Wang et al., 2011). ABA-INSENSITIVE 1 (ABI1) is a PP2C, which negatively regulate ABA signaling (Gosti et al., 1999). Therefore, CED1 regulates ABA biosynthesis and downstream ABA signaling. CED1 encodes a protein containing alpha/beta hydrolase fold domain, and it is found to be important for cuticle biosynthesis in *Arabidopsis* (Wang et al., 2011).

Chemical genetics identified the ABA receptors called PYR/PYL/RCAR family (Ma et al., 2009; Park et al., 2009b). Park et al. (2016) characterized the association between PYL and type 2C protein phosphatase (PP2C) via protein-protein interaction

analysis. They found that PP2Cs function as the inhibitor in ABA signaling (Park et al., 2009b). When ABA is absent, PP2Cs can bind to the protein kinases, Snf1-related protein kinases, well known as SnRK2s, and inactivate their kinase activities. When the PYL-ABA-PP2C complex is formed, SnRK2s are released from such inhibition and are able to phosphorylate transcription factors like ABRE (ABA-responsive elements) binding proteins, and trigger ABA response in plants (Park et al., 2009b; Nakashima and Yamaguchi-Shinozaki, 2013).

SOS pathway plays a key role in plant salt stress tolerance

Salt stress tolerance consists of ionic stress tolerance and osmotic stress tolerance in plants (Zhu, 2016). Extensive studies revealed a central SOS3-SOS2-SOS1 (salt overly sensitive) regulatory complex in maintaining ion homeostasis in response to salt stress in plants. The SOS pathway is mediated by calcium-dependent signal transduction, of which mainly the tolerance to Na^+ (Zhu, 2002). *SALT OVERLY SENSITIVE 3* (*SOS3*) encodes an EF-hand calcium-binding protein, which activates SALT OVERLY SENSITIVE 2 (SOS2) by physical interaction. SOS2 is a serine/threonine protein kinase. SOS3-SOS2 complex then activates SALT OVERLY SENSITIVE 1 (SOS1), a Na^+/H^+ antiporter to extrude an excessive level of Na^+ from the cell (Zhu, 2002).

RSA1 (*SHORT ROOT IN SALT MEDIUM 1*) encodes a calcium-binding protein localized in the nuclear. It interacts with a bHLH transcription factor RITF1 (RSA1 INTERACTING TRANSCRIPTION FACTOR 1). RITF1 further regulates gene

expression of which is important for salt stress tolerance, including *SOS1* (Guan et al., 2013a). *SOS6* (*SALT OVERLY SENSITIVE 6*) encodes a cellulose synthase-like (CSL) protein, as known as CELLULOSE SYNTHASE LIKE D5 (AtCSLD5) in *Arabidopsis*. ROS level is significantly increased in *sos6* mutants under NaCl treatment and mannitol treatment, indicating SOS6 is required for maintaining the ROS level in response to osmotic stress (Zhu et al., 2010).

Chapter 1: ROA1, a U1 splicing factor, is required for plant cold stress responses

Abstract

Cold stress, including chilling and freezing stresses, is one of the major abiotic stresses that adversely affect global agricultural production. Hence, elucidation of the molecular mechanisms of cold stress responses in plants is critical. Extensive studies have been conducted on the cold acclimation-related aspects of cold tolerance in plants; however, the molecular mechanisms of chilling stress-related aspects of plant cold tolerance remain largely unknown. Our lab focuses on the study of abiotic stress tolerance determinants in plants. From a previous forward genetic screen, we had isolated two allelic *Arabidopsis* mutants termed as *regulator of ABA response 1* (*roa1*), which are hypersensitive to ABA treatment at the seedling stage. *ROA1* encodes a splicing factor that is vital for pre-mRNA splicing of genes involved in plant growth and development, including those functioning in ABA signaling pathways. *roa1* mutants have altered responses to cold stress. *roa1* plants are hypersensitive to chilling stress both during germination and post-germination stages and moderately more sensitive to freezing temperatures than the wild type. Overexpressing *ROA1* led to improved chilling tolerance. These results suggest that ROA1 positively regulates plant cold tolerance. RNA sequencing data revealed that

ROA1 controls the proper splicing of 755 genes. IP-Mass analysis together with BiFC analysis showed that ROA1 interacts with other U1 snRNPs such as U1-70K and PRP40A. Loss-of-function of two ROA1 splicing targets, AtDi19-4 (Drought-responsive family protein 19-4) and SNRF1 (a small nuclear ribonucleoprotein family protein), are hypersensitive to chilling stress. Ectopic expression of *AtDi19-4* and *SNRF1* respectively can partially restore the *roa1* mutant phenotype, suggesting they are critical for ROA1 function under cold stress. Together these results indicate that ROA1 is critical for alternative splicing of genes under cold and cold stress tolerance in plants.

Introduction

Pre-mRNA processing and alternative splicing

During transcription, precursor messenger RNAs (pre-mRNAs) with exons and introns are synthesized from the DNA template. mRNA splicing occurs during pre-mRNA processing. At the time, introns are generally removed (partial or entire intron(s) can be retained), and exons are united or re-arranged by a protein complex called spliceosome. Spliceosome contains five core snRNPs (small nuclear ribonucleoproteins), U1, U2, U4, U5, and U6, and numerous non snRNP proteins (Wahl et al., 2009). In general, intron contains main splicing codes, 5' splice site, 3' splice site, and the branch point (an adenine locates at the intron upstream of 3' splice site). Spliceosome recognizes splicing codes and removes introns in the intron-

containing genes. This process includes the recognition of the 5’ splice site, the attachment of the 5’ splice site to the branch point, cutting at the 3’ splice site, and finally releasing the intron as a structure called the lariat. At the same time, the adjacent exons spliced together to form the spliced mRNA.

Alternative splicing (AS) occurs during pre-mRNAs processing. It produces mRNA variants with different combinations of the splicing sites from a single gene. Splicing events are facilitated by the spliceosome. Alternative splicing may produce different protein isoforms by changing amino acid sequences and further affect protein function. In addition, it may introduce premature termination codon (PTC) or a long 3’-UTR without stop codon in the transcript, leading to either truncated protein products or rapid degradation of mRNA mediated by nonsense-mediated decay (NMD) (Kertész et al., 2006; Kalyna et al., 2012). Therefore, AS can increase the protein diversity from the genome, and contribute to the regulations of gene expression.

Alternative splicing is crucial for eukaryotic gene expression

Alternative splicing (AS) occurs during RNA processing in eukaryotes. In plants, alternative splicing is highly involved in circadian rhythm, abiotic stress, and biotic stress responses (Zhang et al., 2017). In *Arabidopsis*, around 61% of genes with multiple exons are alternatively spliced (Marquez et al., 2012). Alternative splicing events can be classified as intron retention, cassette exon/exon skipping, alternative

acceptor, alternative donor, etc. Intron retention is the most frequent AS events in plants whereas its frequency is relatively low in humans (Chaudhary et al., 2019).

U1 snRNPs are critical to RNA splicing by identifying the 5' splice site at the beginning of RNA splicing. In *Arabidopsis*, U1 snRNPs contain U1-70K, PRP40s (PRE-mRNA-PROCESSING PROTEIN 40), PRP39, LUC7 (LETHAL UNLESS CBC7), etc. (Wang et al., 2007; Kang et al., 2009; Amorim et al., 2018). In *Arabidopsis*, AtPRP40s (encoded by *PRP40A*, *PRP40B*, and *PRP40C*) can physically interact with the carboxyl-terminal domain (CTD) of RNA polymerase II (RNAP II) (Kang et al., 2009), indicating that PRP40s are crucial for pre-mRNA processing. CTD is a key domain of RNAP II that can be phosphorylated to regulate gene transcription (Egloff and Murphy, 2008). Specifically, alternative splicing plays an important role in abiotic stress responses. AtU1A is one of the U1 snRNPs, it was found to be required in salt tolerance in *Arabidopsis*. *atu1a* mutant showed salt-sensitive phenotype and ROS overaccumulation under salt stress. Furthermore, *CSD1* and *ACO1*, two genes related to ROS detoxification, showed different splicing patterns compared with the wild type under salt stress. Ectopic expression of *ACO1* in *atu1a* background can partially restore *atu1a* salt sensitivity, suggesting that other AtU1A target genes, together with *ACO1*, contribute to salt stress response in *Arabidopsis* (Gu et al., 2018).

ROA1 (RBM25) is a splicing factor required in ABA responses in *Arabidopsis*

ABA is crucial for plant growth and development, including the regulation of seed maturation, plant stress responses (drought, salinity, etc.), senescence, etc. A genetic screen with growth defects at seedlings post-germination stage under ABA treatment identified two allelic *roa1* mutants, *roa1-1*, and *roa1-2* (Zhan et al., 2015). Phylogenetic analysis showed that ROA1 is similar to the human RNA-binding protein 25 (RBM25) (Zhan et al., 2015). In yeast, Snu71 is the ortholog of RBM25. A structural biology study revealed the structure of the U1 snRNP complex in yeast (*Saccharomyces cerevisiae*) and suggested that Snu71/RBM25 is one of the U1 snRNPs (Li et al., 2017). U1 snRNPs function in the early recognition of 5' splice sites during RNA splicing (Will and Lührmann, 2011).

In the previous study, we found that ROA1 functions as a splicing factor in *Arabidopsis*. Many genes are aberrantly spliced in the *roa1-1* mutants. *HAB1* (*HYPERSENSITIVE TO ABA1*) is one of the critical genes among ROA1 targets. *HAB1* encodes a type 2C protein phosphatase (PP2C), which is one of the key negative regulators in ABA signaling. RT-PCR and qRT-PCR assays showed that *roa1* mutants failed to produce functional *HAB1.1* isoform. Furthermore, constitutive expression of the functional *HAB1.1* restored the hypersensitive phenotype in *roa1* mutants in response to ABA treatment. These results suggested that *roa1* mutation causes the impairment of *HAB1* functional isoform, which greatly contributes to the ABA hypersensitive phenotype in *roa1* mutants (Zhan et al., 2015). In all, we

demonstrated that alternative splicing is crucial for plant ABA responses, and ROA1 is required during this process.

One of the research questions followed up is that other than ROA1's function in ABA signaling, is ROA1 required in other biological processes? Our recent work suggests that ROA1 is also involved in plant cold stress responses. The expression of *ROA1* is responsive to cold treatments. Cold phenotypic studies showed that the *roa1* mutants are hypersensitive to chilling temperature and slightly more sensitive to freezing temperatures than the wild type. Decades of studies mainly focused on plant freezing stress tolerance. For instance, the CBF-mediated signaling pathway is one of the well-studied mechanisms in plant freezing tolerance. CBFs are the key transcription factors that are positively or negatively regulated by upstream regulators, including ICE1, MYB15, etc. We demonstrated in this study that ROA1 is critical for alternative splicing under cold stress and is required for chilling stress responses in plants.

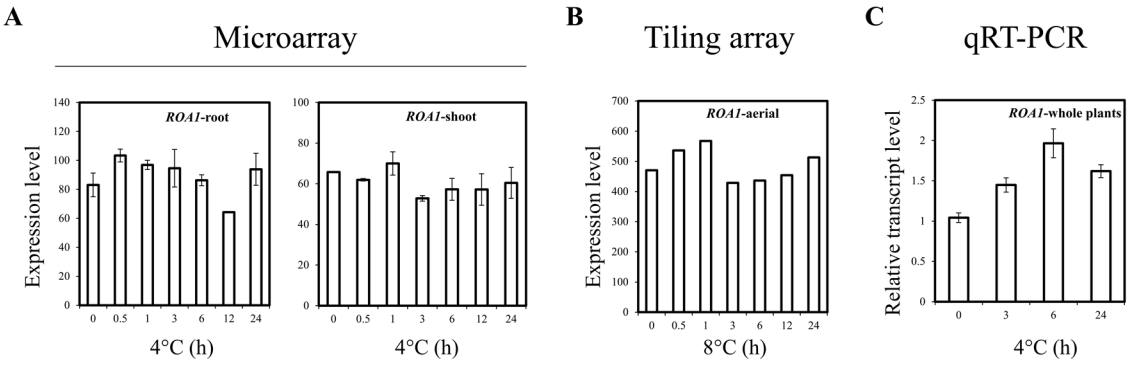


Figure 1. 1 *ROA1* is responsive to cold stress.

Data in (A) and (B) are gained from public databases. **(A)** Microarray data from eFP-browser (Schmid et al., 2005) **(B)** Tilling array data (Zeller et al., 2009) **(C)** *ROA1* expression is detected at 4°C for 0 h, 3 h, 6 h, and 24 h treatments. Values are means ± SD (n = 2 in A, 4 in C).

Results

In our previous study, *roa1-1* (*SALK_064472*) and *roa1-2* are functionally characterized in ABA response in *Arabidopsis*. *ROA1* encodes a splicing factor, which is the ortholog of a splicing factor called HsRBM25 in humans (Zhan et al., 2015). Both *roa1-1* and *roa1-2* mutations are recessive, and these two alleles were appeared to be null alleles of *ROA1* (Zhan et al., 2015).

In my dissertation project, in addition to *roa1-1* and *roa1-2*, we ordered an additional T-DNA insertion line (*SAIL_1292_E05*) in *ROA1* from ABRC and named it *roa1-3* (Figure 1. 2A). *roa1-3* is a knockout mutant (Appendix A. 2). Together, three *roa1* mutants are tested in this study (Figure 1. 2A).

Loss of *ROA1* expression leads to chilling hypersensitivity

Microarray data in the public database (Schmid et al., 2005; Zeller et al., 2009) and qRT-PCR results indicated that *ROA1* is responsive to cold stress (Figure 1. 1). Based on this knowledge, we hypothesized that ROA1 is involved in plant cold stress responses. Cold stress includes responses to chilling stress and freezing stress. We

then conducted chilling germination and post-germination assay at 4°C, by testing the growth (measurements of fresh weight, chlorophyll content, etc.) of young seedlings in response to low-temperature stress. And a freezing assay called electrolyte leakage was conducted to study the freezing phenotype in *roa1* mutants. The higher value of electrolyte leakage indicates the detections of leaked electrolyte caused by freezing-induced cell damage. And it suggested that the plants are more sensitive to freezing stress. In the cold stress phenotypic studies, we found that the *roa1* mutants are hypersensitive to chilling stress. Under chilling stress, *roa1* mutants showed reduced chlorophyll content and reduced fresh weight under light conditions (Figure 1. 2B, C, and D). *roa1* mutants also showed a slightly increased sensitivity to freezing stress compared with the wild type (Figure 1. 2E). At the same time, *roa1* complementation lines are not sensitive to cold stress conditions (data not shown here), demonstrating that the increased cold sensitivity observed in the *roa1* mutants is due to the defects in the *ROA1* gene.

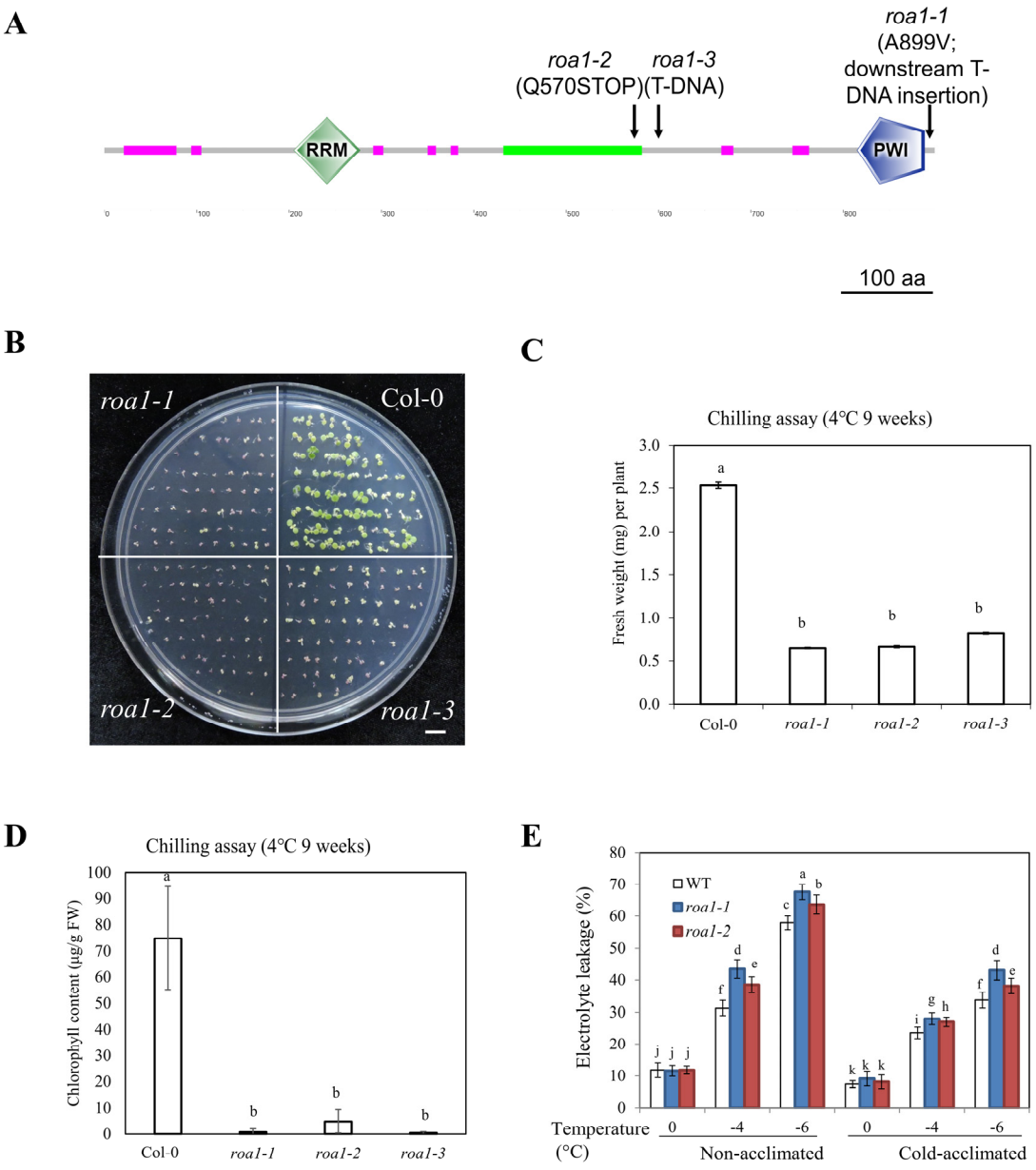


Figure 1. 2 Sensitivity of the *roa1* mutants to chilling and freezing stresses.

(A) ROA1 structure with annotated motifs. Motifs are predicted by SMART database (http://smart.embl-heidelberg.de/). Pink bar: low complexity region; Green bar: Coiled coil region. RRM: RNA recognition motif. PWI: PWI, domain in splicing factors.

(B) Chilling phenotype of the wild type and *roa1* mutants. Seeds in MS medium plates with 0.6% agar were placed horizontally under light in the growth chamber set at 4°C immediately after sowing and allowed to germinate and develop for the indicated time points. **(C)** Fresh weight of individual plant on the average. **(D)** Chlorophyll content. In (C) & (D), one-way ANOVA (Tukey-Kramer test) was applied ($p < 0.001$). Values are means ± SD ($n = 4$ [number of MS media plates; there are 60-80 seedlings for each genotype in each plate]). (D) Freezing tolerance assay of soil-grown *roa1* mutants. Values are means ± SD ($n = 12$). One-way ANOVA (Tukey-Kramer test) was performed, and statistically significant differences are indicated by different lowercase letters ($p < 0.001$).

ABA signaling pathway cross-talks with the plant cold stress signaling pathways (Shi et al., 2014). In our previous study, HAB1 was characterized as the key splicing target of ROA1 in ABA signaling (Zhan et al., 2015). Therefore, whether the function of ROA1 in cold stress response is related to its regulatory role in ABA signaling or not is a question that needs to be addressed. To this end, we studied the chilling stress responses of the *hab1-1* plant. Under normal conditions (21°C), no significant differences between the wild type (Col-0) and *hab1-1* are detected in several physiological parameters including chlorophyll content, and anthocyanin content, etc. (Appendix A. 3). No altered chilling response was detected in *hab1-1* compared with the wild type under chilling conditions (Appendix A. 3). These results suggested that altered chilling stress responses in *roa1* mutants are not due to the defects in ABA

signaling, specifically are not due to the lack of functional *HAB1* expression in *roa1* mutants.

Overexpression of *ROA1* leads to enhanced chilling tolerance.

To further study the function of ROA1 under cold stress, we generate the construct of *35S*:*HA-ROA1* in pEarleyGate 201 and transformed it into wild-type plants. The relative expression level of *ROA1* in the overexpression lines was confirmed by qRT-PCR (Figure 1. 3A). The chilling phenotype at early developmental stages, determined by the rate of cotyledons greening, showed that the overproduction of *ROA1* could enhance the chilling tolerance in *Arabidopsis* (Figure 1. 3B and C).

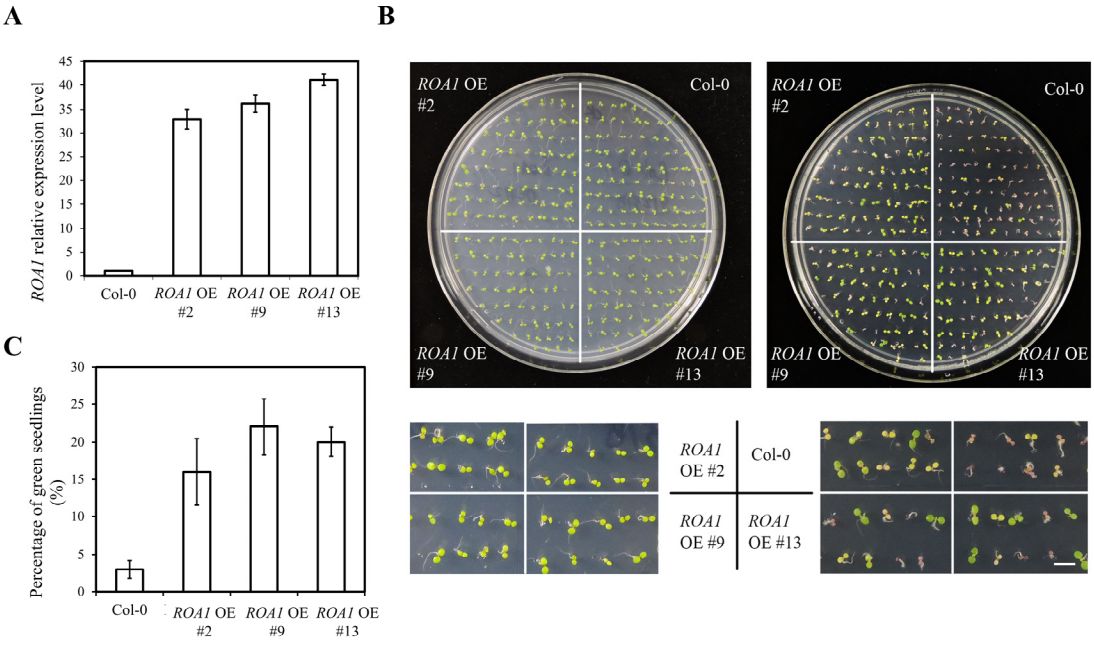


Figure 1. 3 Overexpression of ROA1 confers chilling tolerance.

(A) Relative *ROA1* expression levels in plants of the wild type and *ROA1* OE lines. **(B)** Chilling stress assay of the *ROA1* overexpression lines. Controls (left): 5-day-old seedlings grown at 20°C. Chilling stress tolerance assay (right): seeds were directly germinated in MS media plates at 4°C and allowed to grow for a total of 42 days. Bottom panels: enlarged images corresponding to the original plates in the top panels. Bar = 5 mm. **(C)** Quantification of seedlings with green cotyledons under cold stress as shown in (B). Values are means ± SD (n = 4 in A and 5 in C [number of MS medium plates; there are 70-80 seedlings per genotype for each plate]).

Taken together, *roa1* mutants are hypersensitive to chilling stress, and the overexpression of *ROA1* leads to chilling tolerance in *Arabidopsis*, suggesting that ROA1 can positively regulate plant chilling stress tolerance.

ROA1 regulates gene expression and alternative splicing under cold stress

ROA1 controls the expression of many genes under cold stress

Based on the knowledge that ROA1 is a splicing factor in *Arabidopsis* (Zhan et al., 2015), we performed RNA-seq experiments to analyze the transcription profiles in *roa1-1* under cold stress at the whole genome scale (Figure 1. 4). Based on the RNA-seq data, we found that the expression levels of many genes were affected by the *roa1-1* mutation under cold stress (Figure 1. 4B, C). Compared with the wild type, there are 290 up-regulated genes and 298 down-regulated genes in the *roa1-1* under 4°C treatment (Figure 1. 4B, C). Analysis of the *cis*-elements in those genes showed

common patterns in the promoter region in most of the up-regulated or down-regulated genes (Figure 1. 4D). This result indicates that differentially expressed genes, either up-regulated or down-regulated in *roa1-1*, may be controlled by two types of transcription factors that can bind to these *cis*-elements. GO term annotation indicated that 30% and 19.5% of the up-regulated and down-regulated genes, respectively, are responsive to stress (Figure 1. 4E).

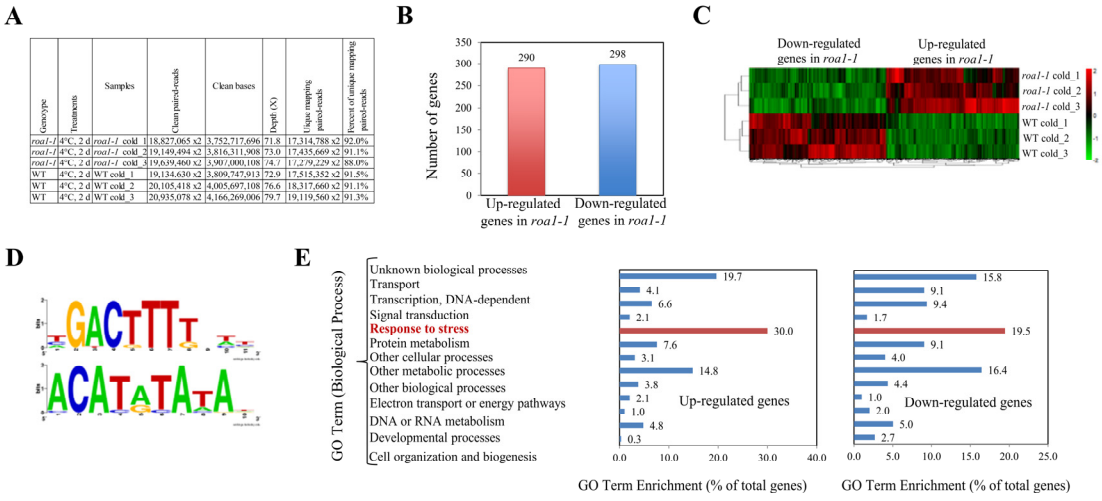


Figure 1. 4 Differential gene expression (DEG) in *roa1-1*.

(A) Summary of RNA-seq experiments. The wild type and *roa1-1* mutant were exposed at 4°C for 2 days. **(B)** Number of genes that are up-regulated or down-regulated in *roa1-1* compared with the wild type. **(C)** Differential gene expression profile shown as heatmap. **(D)** Common *cis* promoter elements present in 200 out of 290 total up-regulated genes in *roa1-1* (upper) and in 235 out of 298 down-regulated genes in *roa1-1* (lower) **(E)** Gene ontology (GO) analysis of DEGs in *roa1-1*.

ROA1 regulates alternative splicing under cold stress

Based on the results that the *roa1* mutants are hypersensitive to cold stress, and the fact that ROA1 is a splicing factor, we are interested to determine the effects of the *roa1* mutation on alternative splicing under cold stress. RNA-seq data showed that 755 genes are differentially alternatively spliced in *roa1-1* plants under cold stress at 4°C for two days compared with the wild type (Figure 1. 5A). Intron retention is the major event that occurred among the differentially alternatively spliced genes in *roa1* mutants, followed by cassette exon/exon skipping (Figure 1. 5B). Based on the gene ontology (GO) analysis, nearly 20% of these genes are responsive to stress (Figure 1. 5C). We conducted RT-PCR experiments to validate the RNA-seq results (Figure 1. 5D, E). It appeared that some of the mis-splicing events in *roa1* mutants can only be detected under cold stress (Figure 1. 5E), suggesting that alternative splicing is required for cold stress responses. Furthermore, according to the microarray data from public databases, a large portion of the genes with splicing defects in the *roa1-1* mutant are cold-responsive in the wild type (Appendix A. 4). This suggested that ROA1 splicing targets are involved in plant cold stress responses.

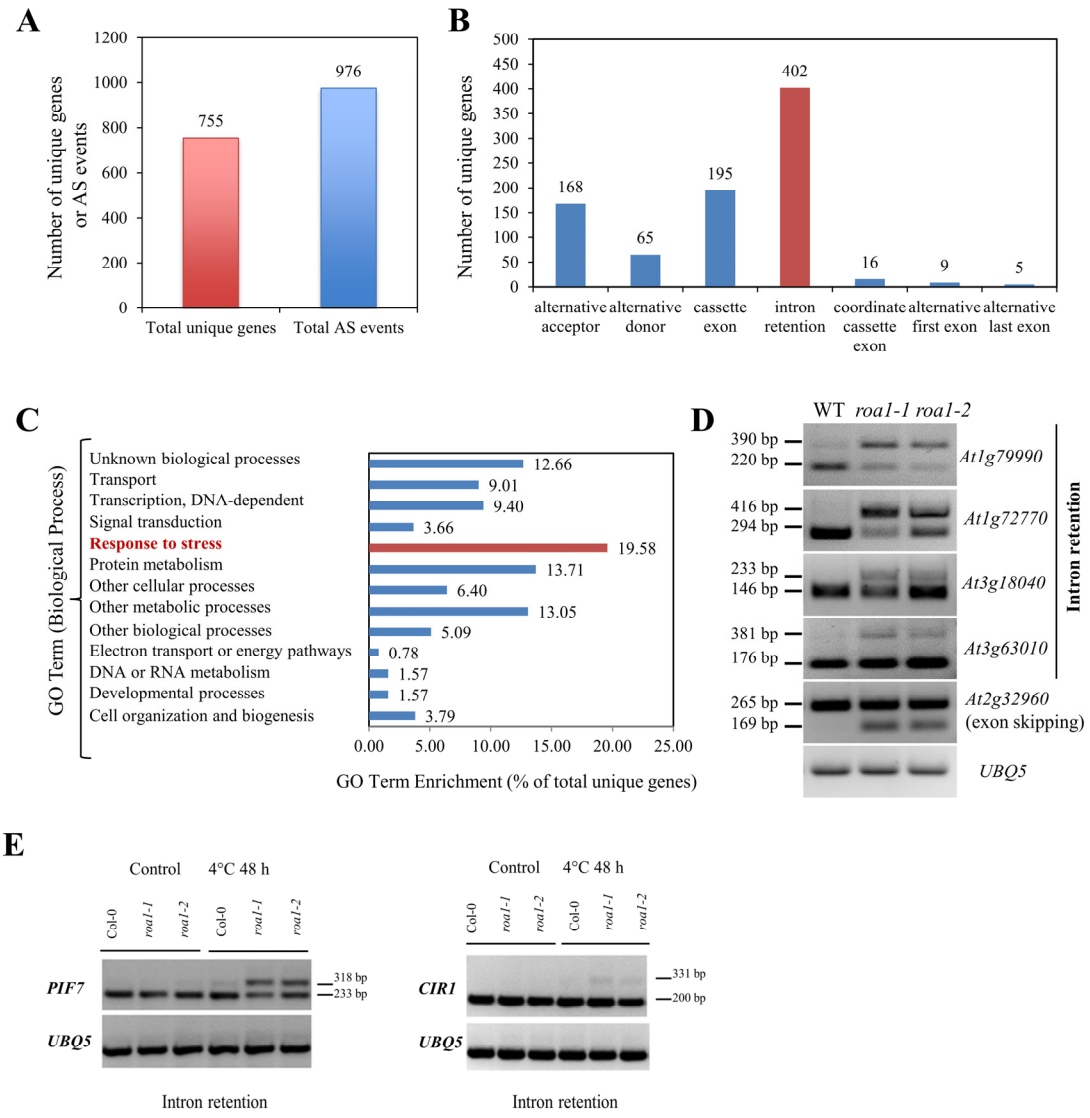


Figure 1. 5 The *roa1-1* mutation causes defective alternative splicing of pre-mRNAs.

(A) Summary of genes whose transcripts with abnormally spliced patterns in *roa1-1* as determined by the RNA-seq experiments. **(B)** Genes with defects in different types of alternative splicing patterns in *roa1-1* as determined by the RNA-seq experiments. **(C)** GO Term enrichments of differentially spliced genes in *roa1-1* under cold treatment as determined by the RNA-seq experiments. **(D)** Validation of intron retention and exon

skipping events in WT, *roa1-1*, and *roa1-2* plants under cold stress (4°C for 48 h) as determined by RT-PCR analysis. The DNA bands were shown in inverted color. **(E)** AS events in *roa1* mutants under normal and cold conditions. RT-PCR validation of *PIF7*, and *CIR1* transcripts in wild-type, *roa1-1*, and *roa1-2* under normal and cold conditions. Two-week-old plants were grown on MS medium in light, and then grown for additional 48 h at 21°C (control) or 4°C in cold treatment groups. Four biological replicates for each genotype and RT-PCR was conducted at least three times. The DNA bands were shown in inverted color.

ROA1 targets are required for plant cold responses

We selected candidate genes based on the significance of alternative splicing defects under chilling stress in *roa1* mutant and their annotated functions in plants, following three criteria: 1) their transcripts have severe defects in alternative splicing in *roa1* mutants; 2) they are cold-responsive genes; 3) ideally, they have established function in cold stress responses. In addition, we choose additional candidate genes referred to a high throughput phenotype screening (Song et al., 2013) on the SALK lines with altered responses in abiotic stress characterized by Song et al. (2013) (with very limited information on phenotypes).

Based on the classification of gene functions, we selected four groups of top ROA1 splicing target genes. Group 1: transcription factors including PRR7, PIF7, CIR1, etc. Group 2: splicing factors (RNA-binding protein). Group 3: genes related to photosynthesis, chloroplast function, and chloroplast development. Other genes with

poorly studied functions in plant cold response are classified as Group 4 (Appendix A. 5).

We ordered T-DNA insertion lines of loss-of-function lines of ROA1 splicing targets from ABRC and studied chilling stress-related phenotypes (Appendix A. 5). Among four groups of ROA1 splicing targets, we found that T-DNA insertion lines in *CIR1* (*Circadian1*), and *SNRF1* (*At2g14285*, which encodes a small nuclear ribonucleoprotein family protein) showed chilling sensitive phenotypes in the hypocotyl elongation assay (Figure 1. 6A) while *traf1b* (*TRAF-like superfamily protein 1b*), *wd40rl* (*transducin/WD40 repeat-like superfamily protein*), *atosa1* (*A. thaliana oxidative stress-related ABC1-like protein 1*), *upi* (*unusual serine protease inhibitor*), and *dsp4* (*plant and fungi atypical dual-specificity phosphatase 4*) mutant plants showed chilling sensitive phenotypes in the chilling germination and post-germination assays (Figure 1.6C). In addition, the *atdi19-4* (*drought-responsive family protein19-4*) showed increased chilling sensitivity in both hypocotyl assays and chilling germination and post-germination assays (Figure 1. 6B, C). In contrast, *pif7-1* (*phytochrome-interacting factor7*), *prr7* (*pseudo-response regulator 7*), and *lhcb7* (*light-harvesting complex b7*) showed chilling-tolerant phenotype in germination and post-germination assays (Figure 1. 6D). We found that *pif7-1* single mutant and *pif4-2* single mutant was able to confer the chilling tolerant phenotype. This result indicates that in contrast to the redundancy of PIF7 and PIF4 in the CBF-mediated cold acclimation pathway (Lee and Thomashow, 2012), PIF7 and PIF4 may not necessarily to function redundantly in chilling stress responses.

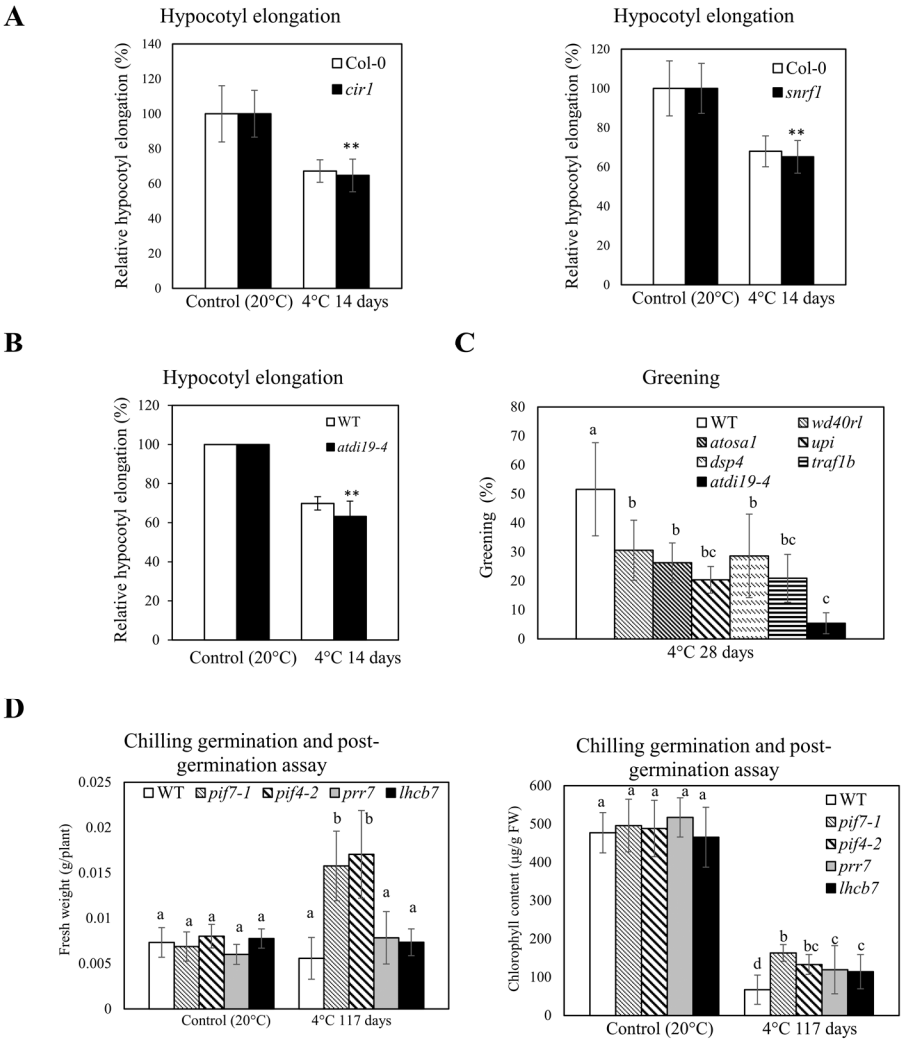


Figure 1. 6 Chilling phenotypes of ROA1 splicing targets loss-of-function lines.

(A) Percentage of relative hypocotyl elongation in loss-of-function mutant of *cir1*, and *snrf1* (*At2g14285*). The hypocotyl length of wild-type seedlings and the mutant are set as 100% respectively in control condition. Values are means ± SE (n = 3 for control group, n = 6 or 8 for chilling treatment [there are 6-8 biological replicates and each replicate contains 20-30 seedlings per genotype in each experiment]). (**, $p < 0.01$ in a Student's t-test) **(B)**

Percentage of relative hypocotyl elongation in *atdi19-4*. The hypocotyl length of wild-type seedlings and *atdi19-4* seedlings are set as 100% respectively in control condition. Values are means ± SD (n = 3 for control group, n = 6 for chilling treatment [there are 20-30 seedlings per genotype in each experiment]). (**, $p < 0.003$ in a Student's t-test) **(C)** Chilling germination and post-germination assay. Percentage of green seedlings from the total was calculated. Values are means ± SD (n = 7 [number of petri dishes and there are 30-40 seedlings per genotype in each petri dish]). Experiments were repeated at least three times. One-way ANOVA (Tukey-Kramer test), $p < 0.01$. **(D)** Chilling germination and post-germination assays. Fresh weight and chlorophyll content were measured in the wild type, *pif7-1*, *pif4-2*, *prr7*, and *lhcb7*. Values are means ± SD (n = 3 for control group, n = 10 for chilling treatment [number of petri dishes and there are 30-40 seedlings per genotype in each petri dish]). One-way ANOVA (Tukey-Kramer test), $p < 0.002$ (Fresh weight); $p < 0.036$ (Chlorophyll content). All chilling assays were repeated at least three times.

Ectopic expression of *AtDi19-4* and *SNRF1* (*At2g14285*) in *roa1* mutants can restore their chilling sensitive phenotype

In order to learn which ROA1 targets play the key role under cold stress, we generated transgenic lines overexpressing the coding region of ROA1 splicing targets of interest in *roa1-1* and *roa1-3*, respectively. With hypocotyl elongation assay, the ectopic expression of *At2g14285* (*SNRF1*) in *roa1-1* could partially restore the *roa1-1* mutant phenotype (Figure 1. 7A). The transgenic lines overexpressing *AtDi19-4* in *roa1-3* could partially restore the *roa1-3* mutant phenotype (Figure 1. 7B). These

results suggested that splicing defects in *SNRF1* and *AtDi19-4* contribute to the overall increased chilling sensitivity showed in *roa1* mutants.

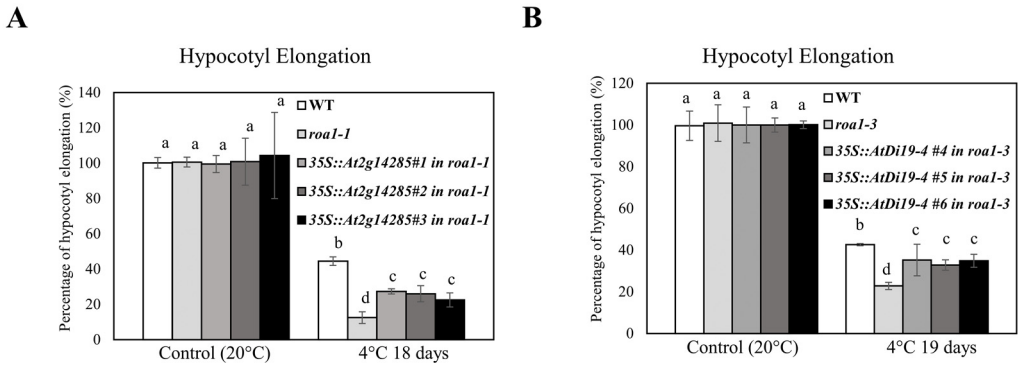


Figure 1. 7 Effects of ectopic expression of ROA1 splicing target genes in *roa1* mutants in response to cold treatment.

Percentage of hypocotyl elongation in the wild type and the transgenic lines overexpressing ROA1 splicing target genes. Transgenic lines and the wild type were planted on MS medium with 1.2% agar. Plants were grown under control (20°C) for 15 days and chilling (4°C) conditions for 18 or 19 days. Hypocotyl elongation of chilling treatment group over control condition. Data are means ± SD (n = 3 or 5, each line has 20-30 seedlings on each medium). **(A)** *35S:At2g14285* (*SNRF1*) in *roa1-1*. One-way ANOVA (Tukey-Kramer test, $p < 0.001$). **(B)** *35S:AtDi19-4* in *roa1-1* and *roa1-3*. One-way ANOVA (Tukey-Kramer test, $p < 0.02$).

ROA1 physically interacts with two U1 snRNPs, PRP40A and U1-70K, and other splicing factors

In yeast (*Saccharomyces cerevisiae*), Snu71/RBM25, PRP40, Luc7, PRP39, etc. are the associated proteins found in U1 snRNPs revealed by a structural biology study via Cryo-electron microscopy (cryo-EM) (Li et al., 2017).

We hypothesized that ROA1/RBM25, functioning as a U1 snRNP, can interact with other splicing factors, including other U1 components, in *Arabidopsis thaliana*. To learn the interaction partners of ROA1, we conducted bimolecular fluorescence complementation (BiFC) assay. BiFC results showed that ROA1 can interact with U1-70K, PRP40A, PRP39A, and SR45 *in vivo* (Figure 1. 8). In addition, ROA1 can interact with one of the ROA1 splicing targets, SNRF1 (encoded by *At2g14285*) (Figure 1. 9). SNRF1 is a positive regulator of plant cold stress response based on our loss-of-function study (Figure 1. 6A). Furthermore, the interactions between ROA1 and U1-70K, ROA1 and PRP40A *in vivo* have been validated in our IP-Mass (Immunoprecipitation-Mass Spectrometry) analysis conducted by our collaborators (data not shown here). These results suggested that ROA1 functions together with other splicing factors and other U1 snRNPs (U1-70K, PRP40A, etc.) under cold stress.

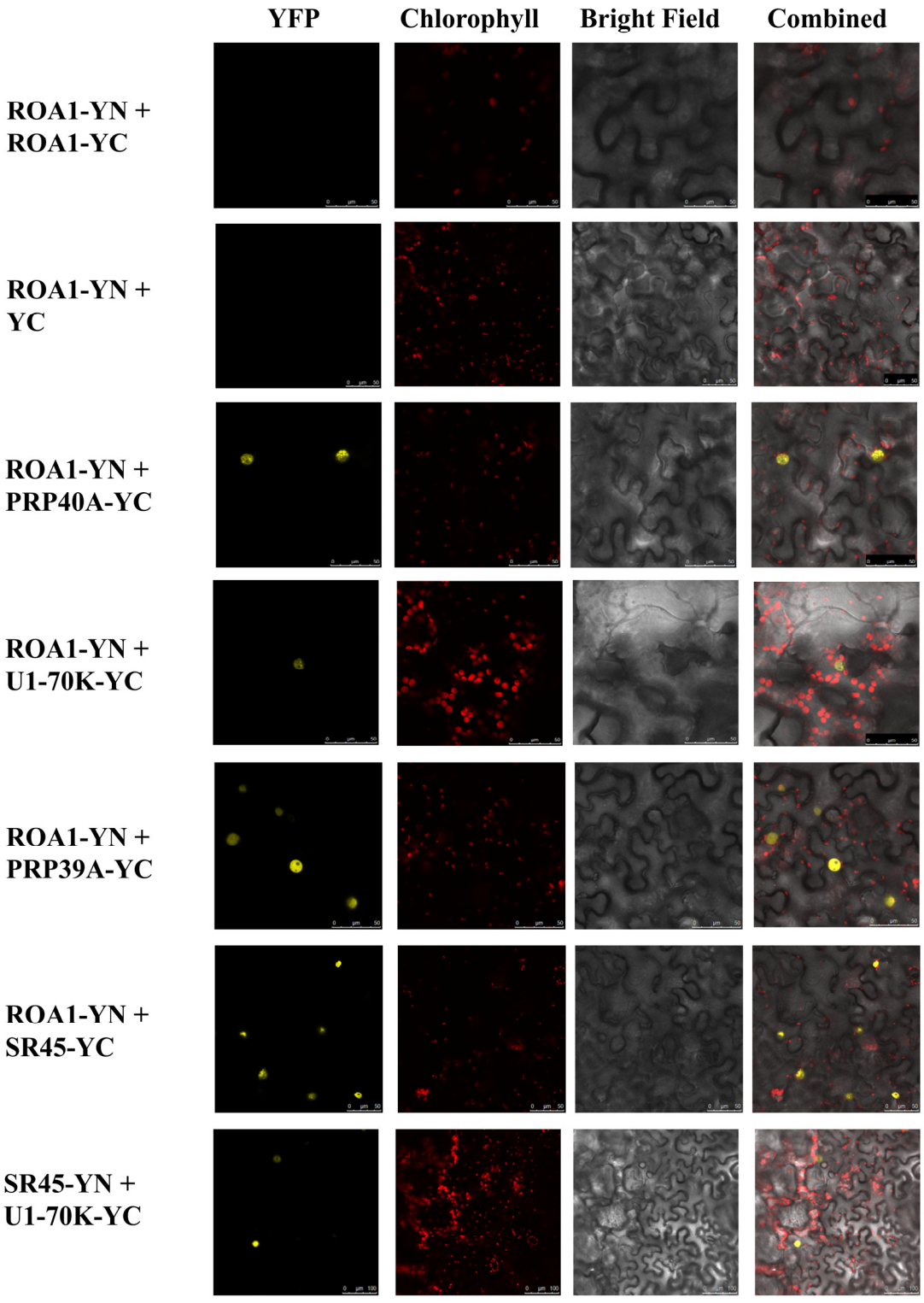


Figure 1. 8 Determination of ROA1 interaction partners by BiFC assays.

BiFC assays to show interactions between ROA1 and other U1 snRNP components. C-YFP is an empty vector. The coding sequence from genes of interest was cloned into the entry vector and recombined into the destination vector pEarleyGate202 YFP-C-terminal (YC) and/or pEarleyGate201 YFP-N-terminal (YN). Negative control: ROA1-YN + ROA1-YC (Bar = 50 μm). Positive control: U1-70K-YC + SR45-YN (Bar = 100 μm). Control group with empty vector: ROA1-YN + YC (Bar = 50 μm). The different combinations of plasmids were transformed into three-week-old tobacco leaves and the YFP signals were detected with a Leica SP5X confocal microscope (63×/1.2 NA water/0.22 mm WD). All experiments were repeated at least three times.

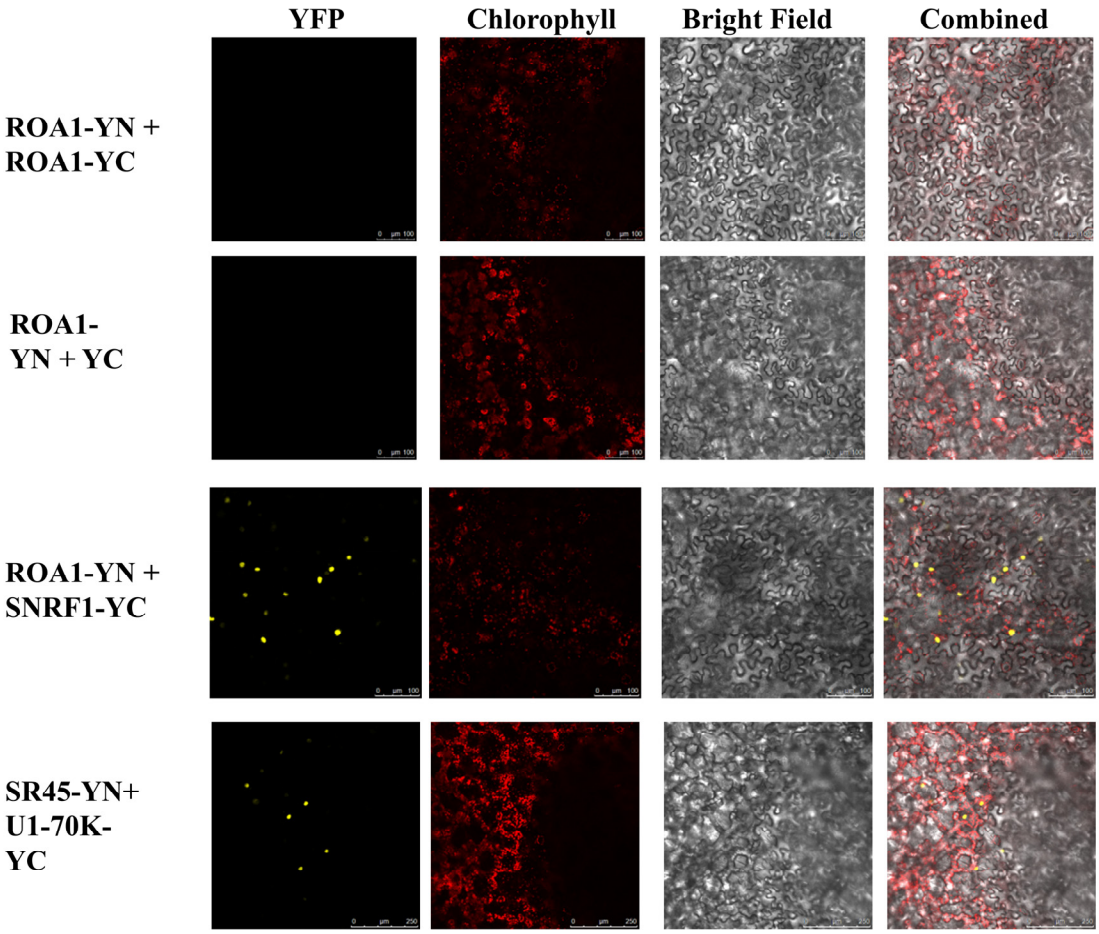


Figure 1. 9 Interaction between ROA1 and SNRF1 determined by BiFC assays.

Interaction between ROA1 and SNRF1 (encoded by *At2g14285*). The different combinations of plasmids were transformed into three-week-old tobacco leaves and the YFP signals were detected with a Leica SP5X confocal microscope (20×/0.8 NA multi-imm/0.26 mm WD). Bar = 100 μm or 250 μm.

Discussion

Extensive studies have been conducted focusing on the mechanisms of plant freezing stress tolerance. Remarkably, CBF-dependent and CBF-independent pathways are well studied, and they are the key regulatory pathways in plant cold stress responses. However, how plants respond to chilling stress and the underlying regulatory mechanisms remain unclear. In this study, we characterized the function of ROA1/RBM25, one of the U1 snRNPs in *Arabidopsis thaliana*. *roa1* mutants are hypersensitive to chilling stress and slightly more sensitive to freezing stress (Figure 1. 2). ROA1 overexpression lines showed improved chilling tolerance compared with the wild type (Figure 1. 3), indicating that ROA1 is required for chilling tolerance in *Arabidopsis*.

Considering the function of ROA1 as a U1 snRNP, we conducted RNA-seq experiments. RNA-seq analysis indicated that ROA1 regulates alternative splicing of many genes in response to chilling stress (Figure 1. 5). In *roa1-1*, over 700 genes showed differential alternative splicing events compared to the wild type (Figure 1. 5A), and many ROA1 target genes are responsive to cold stress (Appendix A. 4). Although the *roa1-1* mutation leads to differential gene expressions (DGE), we mainly focused on ROA1's function in RNA splicing since DGE may be an indirect effect of the mutation. When analyzing different AS events, we noticed that intron retention and cassette exon are two major types of AS events found in the *roa1-1* under cold stress. In addition, some ROA1 splicing targets showed AS event

specifically under cold stress (e.g. *PIF7* and *CIR1*) (Figure 1. 5E), This suggested that ROA1 has some specificity in controlling mRNA splicing under cold stress in plants.

Phenotypic study on loss-of-function lines of ROA1 splicing target genes showed chilling sensitive or tolerant phenotypes (Figure 1. 6) demonstrating that ROA1 target genes are important for chilling stress responses. These genes encode proteins with diverse functions. Some of the ROA1splicing target genes (*SNRF1* and *AtDi19-4*) are critical for ROA1 function under chilling stress because ectopic expression of *SNRF1* and *AtDi19-4* in *roa1* mutants can partially restore their chilling sensitive phenotype (Figure 1. 7).

We also conducted protein-protein interaction assays to study ROA1 interaction partners in *Arabidopsis thaliana*. Consistent with other species, such as humans and yeast, *Arabidopsis* ROA1/RBM25 is highly conserved among different species as one of the U1 snRNPs. These results suggest that ROA1 may function together with other U1 snRNP components to regulate alternative splicing under cold stress. ROA1 is specifically required in alternative splicing under cold stress because other U1 snRNP components such as U1A are not involved in such a process (Gu et al., 2018).

However, how ROA1 function precisely under cold remains unknown. My hypothesis is that cold stress triggers signal transduction and protein kinases or phosphatases may control the activity of ROA1 or its interacting partners to regulate alternative splicing. For instance, serine/arginine-rich (SR) proteins may bind to the exon-specific site called exonic splicing regulators and interact with U1 snRNPs to choose the alternative splicing sites (Reddy and Ali, 2011).

Taken together, we proposed a working model of ROA1 in chilling stress responses (Figure 1. 10). ROA1, functioning as a U1 splicing factor, positively regulates plant chilling stress tolerance. It can interact with U1 snRNPs complex components, such as PRP40A, and U1-70K. It can interact with and regulate SNRF1, a splicing factor that is required in plant cold stress response. And ROA1 regulates transcription factors by alternative splicing. In addition, ROA1 control the alternative splicing of other components that are required in plant cold stress responses.

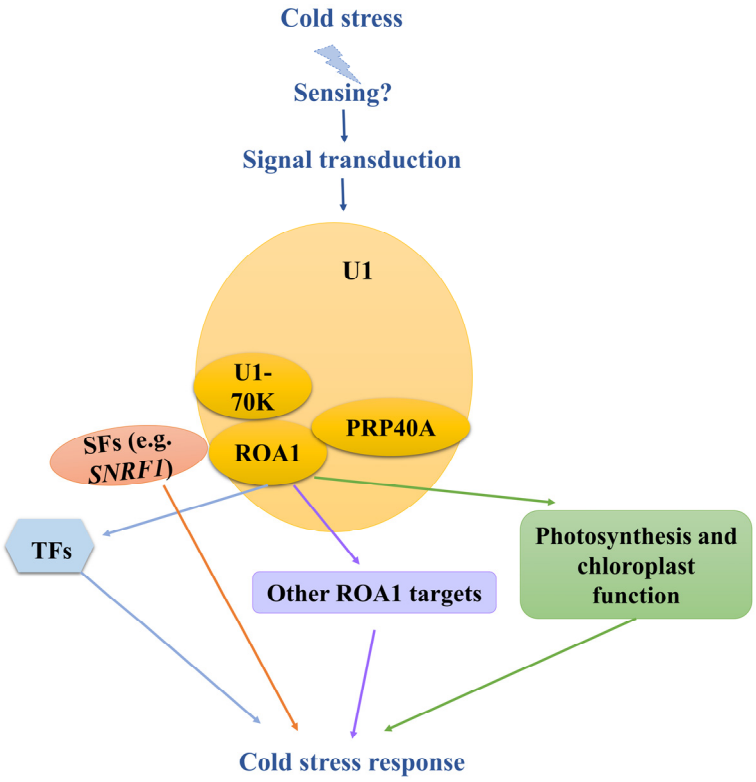


Figure 1. 10 A working model of ROA1 function in plant chilling stress responses. ROA1 is one of the U1 snRNPs in *Arabidopsis*. It can interact with other U1 snRNPs (e.g. U1-70K, PRP40A). Under chilling stress, ROA1 regulates gene expressions via alternative splicing and positively contributes to chilling tolerance. ROA1 target genes include

transcription factors (TFs, e.g. PIF7, CIR1, etc.), genes that related to photosynthesis and chloroplast function, splicing factors (SFs, e.g. SNRF1 encoded by *At2g14285*), etc.

Further studies could focus on the function of other U1 snRNPs in plant cold responses and other abiotic stress responses. For instance, the function of PRP40A and U1-70K in chilling stress responses. And looking for splicing regulators that interact with ROA1 and other U1 snRNPs to learn the selection of splicing sites more in-depth. In addition, more ROA1 targets can be characterized in detail to understand their functions in cold stress responses. We will be able to understand the mechanisms of alternative splicing by comparing the splicing profiles among these splicing factors, including exon recognition patterns and profiles. Future studies can also focus on how cold signal is perceived by plants and the function of the spliceosome in response to cold stress. Since U1 snRNPs are highly conserved among species, I speculate that manipulation of these genes in crops may improve chilling tolerance.

Methods

Plant materials and growth conditions

T-DNA insertion lines were obtained from Arabidopsis Biological Resource Center (ABRC; Columbus, OH): *roa1-1* (*SALK_064472*) and *roa1-2*, *roa1-3* (*SAIL_1292_E05*); *SALK_051843C* (*cir1*), *SALK_074896C* (*cir1*), *SALK_087851C*,

pif7-1, *SALK_052537C* (*snrf1*), *SALK_076524C* (*At2g14285*), *SALK_005077C* (*At4g10110*), *lhcB7* (*SALK_018360C*), *rve1* (*SALK_025754C*), *atosa1* (*SALK_045739C*), *SALK_096470C*, *SALK_009988C*, *SALK_072556C*, *SALK_138056C* (*AtDi19-4*), *SALK_129269C*, *SALK_080753C*, *SALK_057639C*, *CS2103560* (*prp40a prp40b*: *GK-156D11-013211*; *GK-791F02-024937*), *hab1-1* (*SALK_002104*). Seedlings were grown on Murashige & Skoog (MS) Medium (*Phyto*Technology Laboratories) under cool, white light (~100-120 μmol m^{-2} S^{-1}) with a 16-h-light/8-h-dark photoperiod at 21 ± 1°C. Soil-grown plants were grown in LC1 potting mix (Sun Gro Horticulture, Bellevue, WA) under cool, white light (~100-120 μmol m^{-2} S^{-1}) with a 16-h-light/8-h-dark photoperiod at 21 ± 1°C.

Plant chilling stress response assays

Plant chilling stress response assays include hypocotyl elongation and germination and post-germination assay in *Arabidopsis thaliana*. For hypocotyl elongation assay under cold stress, seeds from the wild type and mutants of interest were sawn in square Petri dishes (10 cm x 10 cm) containing MS medium (1 × Murashige & Skoog Basal Medium with Vitamins from *Phyto*Technology Laboratories, 1.2% agar, 2% sucrose, pH 5.7). The Petri dishes were kept in darkness either at 21 ± 1°C for one week or under chilling condition (4°C) for 20 to 40 days. Photos of seedlings grown under different conditions were taken by Nikon COOLPIX P600 with a 60× zoom lens. Hypocotyl elongation was measured by ImageJ 1.52a (Schneider et al., 2012). For germination and post-germination assay, seeds from the wild type and mutants of

interest were sawn in round Petri dishes (diameter 15 cm, height 1.5 cm) containing MS medium (Murashige & Skoog Basal Medium with Vitamins, from *Phyto*Technology Laboratories, 0.6% agar, 2% sucrose, pH 5.7). The Petri dishes were placed under cool, white light (~100-120 μmol m^{-2} S^{-1}) with a16-h-light/8-h-dark photoperiod either at normal growth temperature (21 ± 1°C) for one week or under chilling condition (4°C) for 30 to 60 days. Fresh weight and chlorophyll content were measured. The greening rate (%) was calculated as a percentage of (number of green seedlings)/total (number of seedlings).

Detection of chlorophyll content and anthocyanin content

Chlorophyll content was measured as described (Lichtenthaler and Wellburn, 1983) with some modifications. Whole plants were collected and incubated with 80% acetone overnight in darkness at 4°C. Chlorophyll content was calculated as [$7.49A_{665}$ + $20.3A_{648}$]/[(fresh weight) × (volume of 80% acetone)] (μg/g FW). Anthocyanin was extracted by incubating ground seedlings in 1% HCl/ methanol overnight in darkness at 4°C with continuous shaking. Anthocyanin content was calculated as A_{530}-$0.33A_{657}$ (Mancinelli and Walsh, 1979).

RNA-seq analysis

Two-week-old wild-type and *roa1-1* seedlings grown on MS medium (1 × MS salt, 0.6% agar, 2% sucrose, pH 5.7) were treated at 4°C for two days. Plants grown at normal conditions and plants after cold treatment were collected and extracted for total RNA as described (Zhan et al., 2015). There are three biological replicates per

genotype. Illumina sequencing was performed in the Shanghai Center for Plant Stress Biology with an Illumina HiSeq 2500 System. RNA-seq data analyses were performed as described (Zhan et al., 2015).

qRT-PCR and RT-PCR analyses

For qRT-PCR analysis, 5 μg of total RNA was used for the first-strand cDNA synthesis (ThermoFisher). Three to four biological replicates and two technical replicates were used in each experiment with a Bio-Rad CFX96 platform. The comparative quantification method (fold change = $2^{-\Delta\Delta Ct}$) was applied to calculate relative gene expression levels, and *UBQ5* was used as a reference gene.

For RT-PCR analysis, specific pairs of primers were designed based on RNA-seq data to detect alternative splicing events. The wild-type, *roa1-1*, and *roa1-2* were grown at normal growth conditions (at 21°C) for 2 weeks and treated at 21°C or 4°C for an additional 48 h. Five μg of total RNA was used for the first-strand cDNA synthesis (ThermoFisher), and *UBQ5* was used as a reference gene.

Construction of the *35S:AtDi19-4*, *35S:CIR1* and *35S:At2g14285* plasmids.

cDNA clones of *AtDi19-4* (*U21513*), *CIR1* (*S63636*) were ordered from ABRC. The coding region of *At2g14285* (*SNRF1*) was cloned from the wild-type plants by reverse transcription (ThermoFisher, RevertAid First Strand cDNA Synthesis Kit). Gateway system (Invitrogen) was applied and entry clones were generated with pDONR/Zeo (Invitrogen). The *35S:AtDi19-4*, *35S:CIR1*, and *35S:At2g14285* plasmids were constructed by LR reactions with destination vector pEarleyGate201.

The relevant plasmids were transformed into *roa1-1* or *roa1-3* plants by floral dip method with *Agrobacterium tumefaciens* strain GV3101.

Bimolecular fluorescence complementation (BiFC) assay

Gateway cloning was performed to generate the BiFC constructs. The coding sequence (CDS) from these genes was cloned into the entry vector pDONR/Zeo (Invitrogen). The entry vectors were recombined into the destination vector pEarleyGate202 containing the YFP-C-terminal (YC) and/or pEarleyGate201 containing the YFP-N-terminal (YN). The resulting plasmids are ROA1-YN, ROA1-YC, SNRF1 (AT2G14285)-YC, PRP40A-YC, U2A'-YC, U1-70K-YC, FIB2-YC, SR45-YC, PRP39A-YC, U170K-YC, and SR45-YN. Constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. Transformed *Agrobacteria* cultures were grown 24 h at 28°C in LB medium. The cultures were collected with short centrifugation, and resuspended in freshly made infiltration medium (10 mM $MgCl_2$, 10 mM MES, 150 μM acetosyringone, pH 5.6) to a final concentration of OD_{600} = 0.5 and incubated for 2 h. Fully expanded leaves from three-week-old tobacco (*Nicotiana benthamiana*) were chosen for infiltration. Equal volumes of cell suspension were carefully infiltrated into the back of tobacco leaves in several places. The plants were covered with plastic covers to keep humidity immediately after the infiltration. After 48 h of growth, the leaves were examined under a Leica SP5X confocal microscope.

Chapter 2: RSA4, a cellulose synthase, is required for plant salt stress tolerance in *Arabidopsis*

Abstract

Salt stress is one of the abiotic stresses that severely affect agricultural production. Our lab had previously identified a salt-sensitive mutant and named it as *rsa4* (*short root in salt medium4*). We subsequently found that *rsa4* mutants are sensitive to drought stress. Positional cloning revealed that *RSA4* is *CESA6* (*AT5G64740*), which encodes cellulose synthase 6 (CESA6) functioning in primary cell wall synthesis. Overexpression of *RSA4* increased salt tolerance. ROS overaccumulation was found in the mutant under NaCl treatment, indicating ROS detoxification capacity was affected in *rsa4* and it may lead to the salt stress hypersensitivity. Furthermore, the previous study done by our lab revealed that the stomata density is not significantly different between the wild type and *rsa4* mutant. We conducted the Toluidine Blue staining assay to examine cuticle integrity and the results suggested that *rsa4* mutation affects cuticle deposition. By qRT-PCR analysis, we found that the expression of *KCS1* and *TWS1*, two cuticle-related genes, was reduced in *rsa4-1* under drought stress. Overall, our results suggest that RSA4 is a positive regulator of salt and drought stress responses in plants through maintaining redox status and cuticle integrity under stressful conditions.

Introduction

Drought stress and salt stress responses in plants

Water stress, caused by drought stress or salt stress, severely affects plant growth and development. Responses to drought and salt stress share overlapping responses in plants (Zhu, 2002).

Salt stress includes primary stress such as osmotic stress and ionic stress. Hence, the mechanisms of salt stress responses in plants include detoxification of excessive ions, repair of cellular damage caused by salt stress, and regulation on plant growth in response to salt stress, etc. (Zhu, 2002). SOS (Salt Overly Sensitive) pathway plays a central role in Na^+ detoxification and ion homeostasis, and it extrudes excessive Na^+ ions from the cell.

Reactive oxygen species (ROS) are a group of molecules that contain oxygen in an unstable state, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), superoxide ($O_2^{\bullet-}$), etc. Abiotic stresses can dramatically induce ROS production and an excessive level of ROS will damage the cell even to death. On the other hand, ROS accumulation functions in signal transduction in response to abiotic stresses.

Drought stress is one of the abiotic stresses that adversely affect plant growth and development. Water deficiency could cause wilting and even death of the plant. Plant stress hormone abscisic acid (ABA) is induced under drought stress, and it regulates stomata closure to control stomatal transpiration. Drought stress signal transduction can be grouped as ABA-dependent and ABA-independent pathways, and the central

transcription factors of these signal transduction pathways are ABA-responsive element (ABRE) binding factors and dehydration-responsive element binding factor 2 (DREB2), respectively (Shinozaki and Yamaguchi-Shinozaki, 2007).

Plant morphology change is also important in drought stress responses. The plant cuticle is the hydrophobic layer of the shoot in plants. Under drought stress, cuticular wax production is induced to avoid water loss by transpiration (Kosma et al., 2009). In addition, root growth is also important in response to drought stress.

Cell wall and cellulose synthase

The cell wall is a barrier which not only provides support to the cell but also functions as a barrier in response to different environmental stresses. Tenhaken et al. (2015) found that the primary cell wall can be remodeled in response to different abiotic stresses (Tenhaken, 2015). The plant cell wall consists of pectin, hemicellulose, and cellulose. Cellulose, a major component of cell wall polysaccharides, is synthesized by cellulose synthase complexes (CSCs). CSCs, a rosette-like structure, located at the plasma membrane. Genes encode cellulose synthases (CESA) are called *CESA*. Three CESAs (CESA1, CESA3, and CESA6) synthesize the primary cell wall, and a different group of CESAs (CESA4, CESA7, and CESA8) synthesize the secondary cell wall (Hill et al., 2014). Chen et al. (2005) found that CESA8 is involved in plant drought stress and osmotic stress responses in *Arabidopsis*. *lew2-1* and *lew2-2*, two mutant alleles of *CESA8*, showed tolerant phenotypes under those stress conditions. *lew2* accumulated a constantly higher level of ABA and osmolytes under different

water conditions including normal condition and drought stress, etc. *lew2* endured the stress condition when such condition is normal to the wild type. Taken together, their results suggested that cellulose synthesis is important for plants to cope with different stress conditions such as drought stress and osmotic stress (Chen et al., 2005).

We identified the RSA4/CESA6 through a forward genetic screen for salt-sensitive mutants. We showed evidence that RSA4 is required for salt and drought stress responses in plants through maintaining redox status and cuticle integrity.

Results

rsa4-1 mutant is hypersensitive to salt, drought, and osmotic stress

EMS mutant screen identified *rsa* mutants that showed reduced root growth on salt medium in a modified root bending assay (Guan et al., 2013a). The mutants were first grown on MS medium vertically and then transferred to MS medium supplied with 100 mM NaCl. After the inversion of the vertical medium plate, the short-root mutants were easily selected from the population. And we named the mutants as *rsa* (*short root in salt medium*) (Wu et al., 1996; Guan et al., 2013a). Among the mutants, we found that *rsa4-1* is hypersensitive to different stresses, including salt stress, drought stress, and osmotic stress.

rsa4-1 is hypersensitive to salt stress

The growth of *rsa4-1* on NaCl containing MS medium showed its hypersensitivity to salt stress, including yellow leaves, significant loss of fresh weight, and reduced root

and shoot growth compared with the wild type (Figure 2. 1A-D). Furthermore, seed germination assay and the soil-grown plants under salt stress indicated that the *rsa4-1* mutant was also hypersensitive to salt stress at seed germination and late developmental stages (Figure 2. 1E, F, G).

The mechanisms of salt stress tolerance in plants include different aspects, such as the response to ionic stress and osmotic stress (Zhu, 2016). Therefore, we subsequently examined the effect of the *rsa4-1* mutation on these stresses.

To learn whether the hypersensitivity in *rsa4-1* is specific to Na^+, we tested the responses of *rsa4-1* plants to several additional salts including KCl, LiCl, and CsCl. The results showed that *rsa4-1* was hypersensitive to KCl and LiCl, but not to CsCl (Figure 2. 2). To determine whether increased salt sensitivity in *rsa4-1* is caused by imbalanced Na^+ homeostasis, we measured the Na^+ content in wild-type and *rsa4-1* mutant under normal conditions and salt stress conditions. We found that there is no significant difference in the Na^+ content between wild-type and *rsa4-1* plants with or without NaCl treatment (Figure 2. 1H, I), indicating that the *rsa4-1* mutation did not affect Na^+ homeostasis and RSA4 may function in the salt stress pathways independent of the SOS pathway.

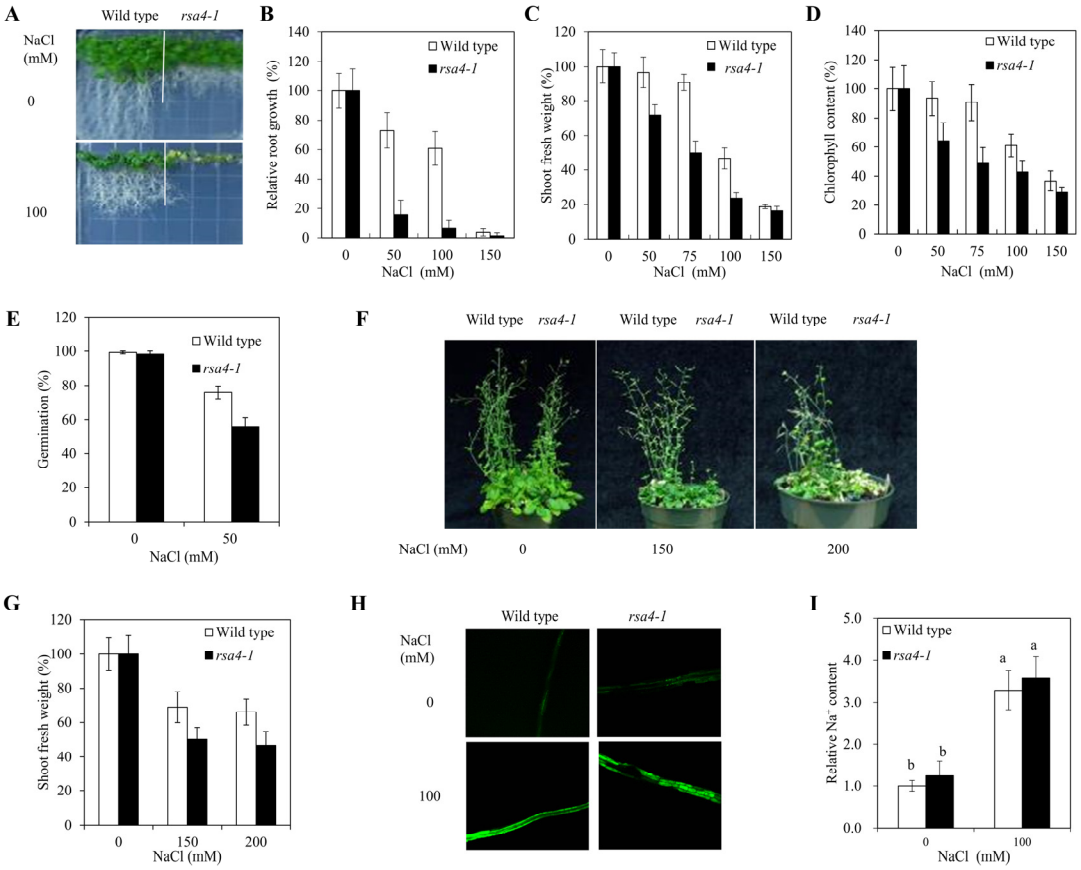


Figure 2. 1 *rsa4-1* plants are hypersensitive to NaCl at different developmental stages.

(A) Sensitivity of the *rsa4-1* mutant to salt stress. The wild-type (left) and *rsa4-1* (right) seedlings were grown on MS medium with 0 mM or 100 mM NaCl for 22 days. **(B)** Quantification of root growth of seedlings under normal and salt stress conditions. Root growth under normal growth conditions in wild-type and *rsa4-1* seedlings was considered as 100%. **(C)** Quantification of shoot fresh weight of seedlings under normal conditions and salt stress conditions. Shoot growth under normal conditions in wild-type and *rsa4-1* was set as 100%. **(D)** Quantification of chlorophyll content of seedlings under normal condition and salt stress conditions. Chlorophyll content of seedlings grown under normal condition in wild-type and *rsa4-1* seedlings was considered as 100%. **(E)** The seed germination rate of the wild

type and *rsa4-1* under normal condition and 50 mM NaCl treatment. Each biological replicate has 80-150 seeds per genotype. Seeds with emerged radical through the seed coat were considered as germinated seeds. **(F)** Soil-grown plants under normal conditions and salt stress conditions. **(G)** Quantification of shoot fresh weight of seedlings shown in (F). **(H)** Na^+ contents in wild-type and *rsa4-1* seedlings were treated with 100 mM NaCl for 3 days. Fluorescence indicates the presence of Na^+ contents. **(I)** Quantification of Na^+ contents in wild-type and *rsa4-1* seedlings shown in (H) with an image analyzer. One-way ANOVA (using Duncan's multiple range test) was performed and statistically significant differences are indicated by different lowercase letters ($p < 0.05$). Values are means ± SD ($n = 30$ in [B], 24-48 in [C] and [D], 30 in [E], 48 in [G], 24 in [I]).

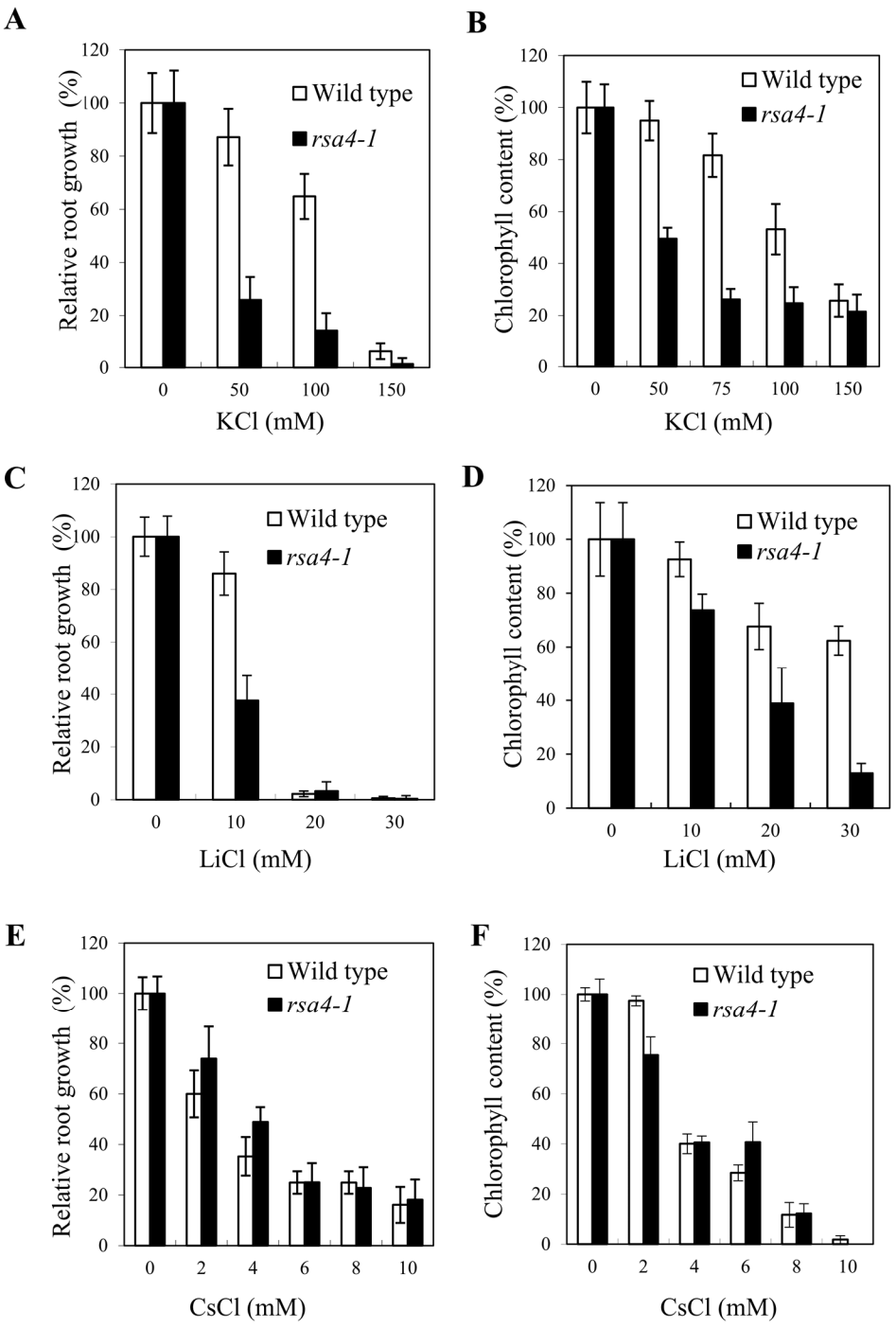


Figure 2. 2 *rsa4-1* is hypersensitive to KCl, LiCl, but not to CsCl.

The sensitivity of the *rsa4-1* mutant to different salt. The wild type and *rsa4-1* were grown on MS medium with a gradient of KCl, CiCl, or CsCl for 22 days. **(A, C, E)** Quantification of relative root growth (%) of seedlings under normal conditions and salt stress conditions. **(B, D, F)** Quantification of relative chlorophyll content (%) of seedlings under normal conditions and salt stress conditions. Values are means ± SD (n = 30).

RSA4 **encodes a cellulose synthase, which is required for primary cell wall synthesis**

Positional cloning revealed that *RSA4* is *CESA6* (*CELLULOSE SYNTHASE 6*, *At5g64740*) (Figure 2. 3A). CESA6 is one of the cellulose synthases responsible for primary cell wall synthesis. We ordered two additional alleles of *CESA6* (*rsa4-2* and *prc1-1*) from ABRC. *prc1-1* is a point mutation with a premature stop codon ahead of six transmembrane domains at C-terminus (Fagard et al., 2000).

Salt stress assay revealed that additional *rsa4* mutants (*rsa4-2* and *prc1-1*) are hypersensitive to 100 mM NaCl treatment, and the complementation lines of *rsa4-1* restored the wild type phenotype (Figure 2. 3B-D). These results indicate that we cloned the correct *RSA4* gene.

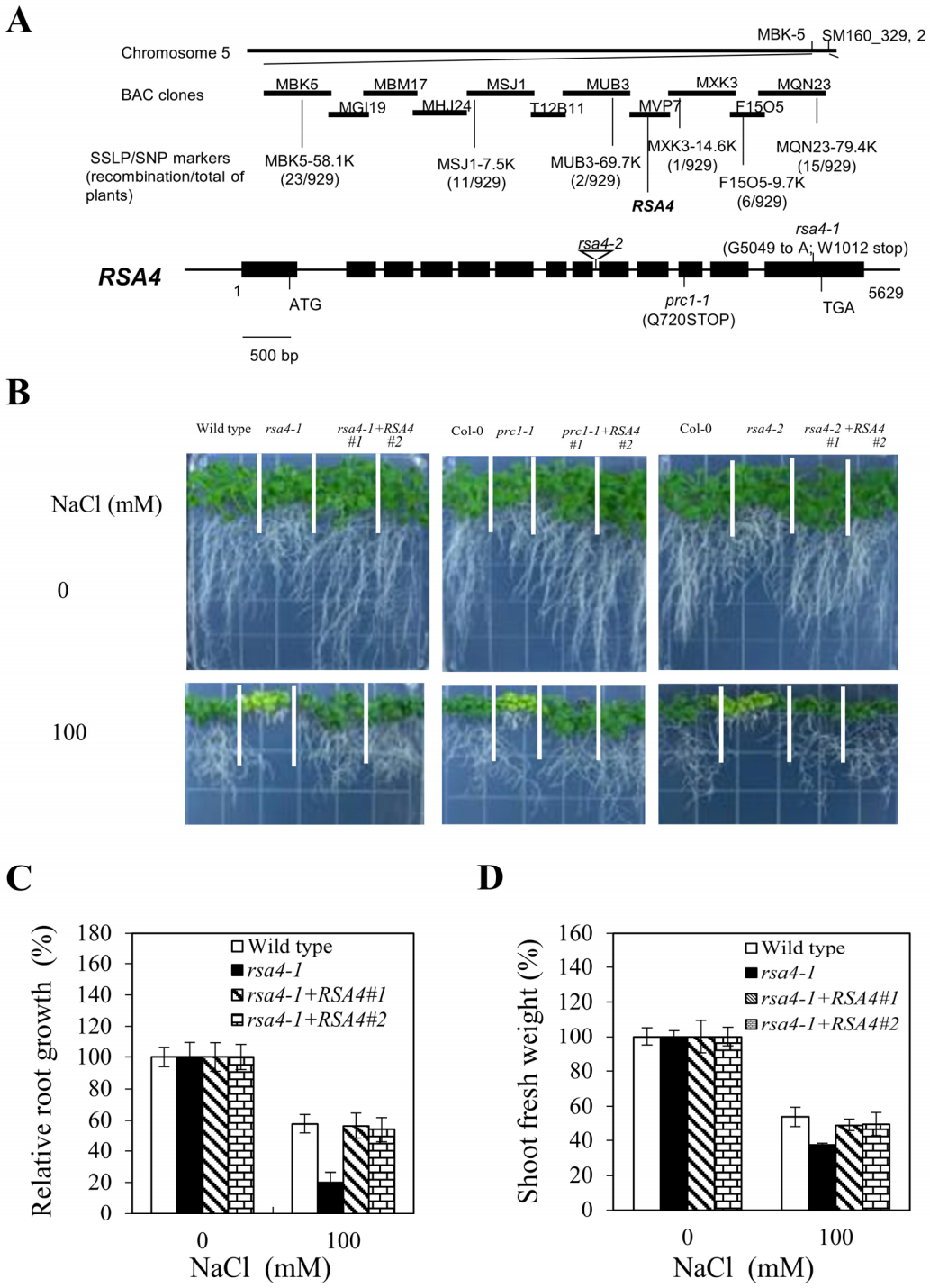


Figure 2. 3 Molecular cloning of *RSA4*.

(A) Positional cloning identified *RSA4* as the gene encodes for CELLULOSE SYNTHASE 6. **(B)** Complementation analysis of *rsa4-1*. Responses of the *rsa4* mutants and *rsa4-1* complementation lines to salt stress. **(C)** Quantification of root growth of wild-type, *rsa4-1*, and *rsa4-1* complementation lines. **(D)** Quantification of shoot fresh weight of wild-type, *rsa4-1*, and *rsa4-1* complementation lines. Values are means ± SD (n = 30).

Soil-grown *rsa4* mutant plants are hypersensitive to drought stress

Drought stress is the other water stress which severely affects plant growth. To test whether *rsa4* mutants are sensitive to drought stress, we applied dehydration treatments on soil-grown plants. We tested the survival rate in two types of drought treatments: one is three weeks of drought treatment, and the other is a 2-week water resumption after the 3-week drought treatment (resume water after plant leaves begin to wilt). The survival rate of *rsa4-1* was significantly reduced under three weeks (18 days) of drought treatment. In addition, after water resumption, most of the wild type plants survived while *rsa4-1* mutants were not able to recover from the drought treatment (Figure 2. 4A). Similar results were seen in *prc1-1*, another allele of the *RSA4* gene, in the water-resumption assay (Figure 2. 4B, C). These results showed that RSA4 is required for drought stress tolerance.

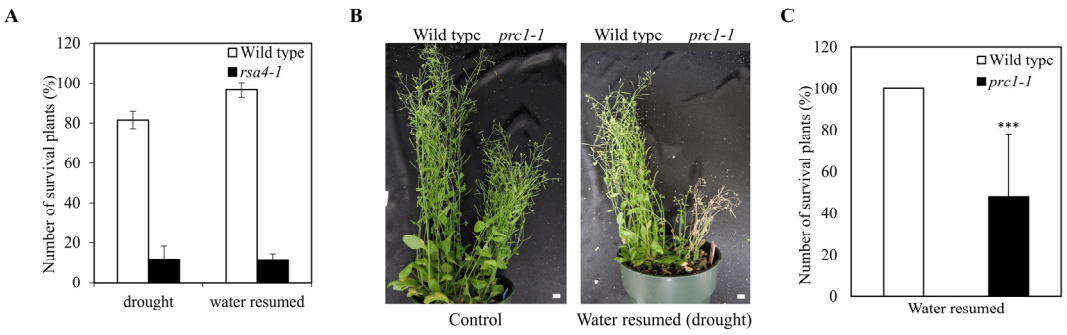


Figure 2. 4 Soli-grown *rsa4* plants are hypersensitive to dehydration conditions.

(A) Two-week-old wild-type and *rsa4-1* plants grown in soil in the same pots were either well-watered or deprived of water for an additional 18 days. Quantification of survival rates of plants when water was withdrawn (drought) for 18 days or when water was resumed. **(B)** Two-week-old wild-type and *prc1-1* were transferred into soil side-by-side. Pictures of the control group (well-watered) were taken after an additional five weeks. The water-resumed group was first deprived of water for additional three weeks, and then water was resumed for two weeks. **(C)** Quantification of survival rates of plants shown in (B). This experiment was repeated at least three times. Error bars represent the standard deviation (n = 80 in A, 24 in C [number of pots], 4-6 plants per genotype per pot). ***, $p < 0.001$ in a Student's t-test.

rsa4-1 is hypersensitive to osmotic stress

Because *rsa4-1* mutant plants are sensitive to multiple salts (Figure 2. 2), we suspect that *rsa4-1* may be sensitive to general osmotic stress. Indeed, the *rsa4-1* plants are sensitive to the general osmotic stress conferred by 150 mM mannitol (Figure 2. 5).

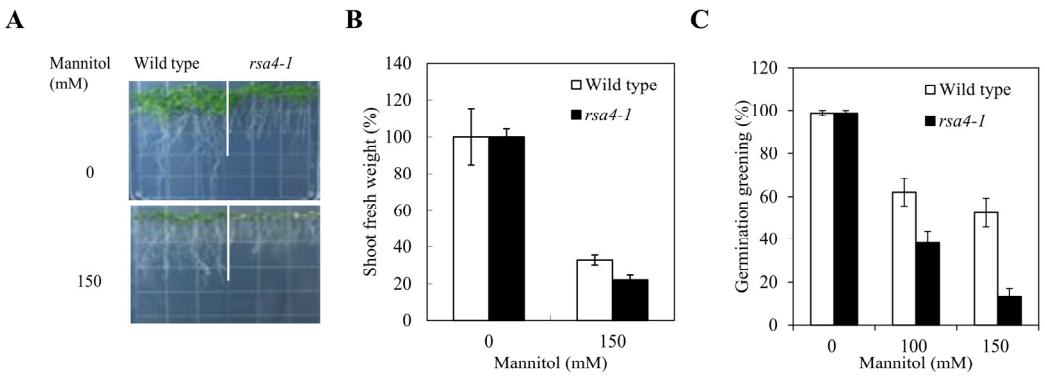


Figure 2. 5 *rsa4-1* plants are sensitive to general osmotic stress.

(A) The wild type and *rsa4-1* seedlings were grown on MS medium with 0 mM or 150 mM mannitol for 12 days. **(B)** Quantification of shoot fresh weight of the wild type and *rsa4-1* shown in A. Shoot fresh weight of the plants under normal condition was set as 100%. **(C)** Quantification of green seedlings post-seed germination shown in A. Values are means ± SD (n = 30).

Osmotic stress induces the ABA accumulation in plants (Zhu, 2002). ABA is one of the plant hormones required for plant abiotic stress responses. ABA-induced stress response includes stomata closure, stress-sensitive gene expression, etc.

rsa4 mutants were hypersensitive to exogenous ABA compared to the wild types in a wide range of exogenous ABA concentrations (Figure 2. 6A), with fresh weight loss and less chlorophyll accumulation (Figure 2. 6B, C). These results suggest that ABA signaling may be involved in *rsa4-1* responses to osmotic stress.

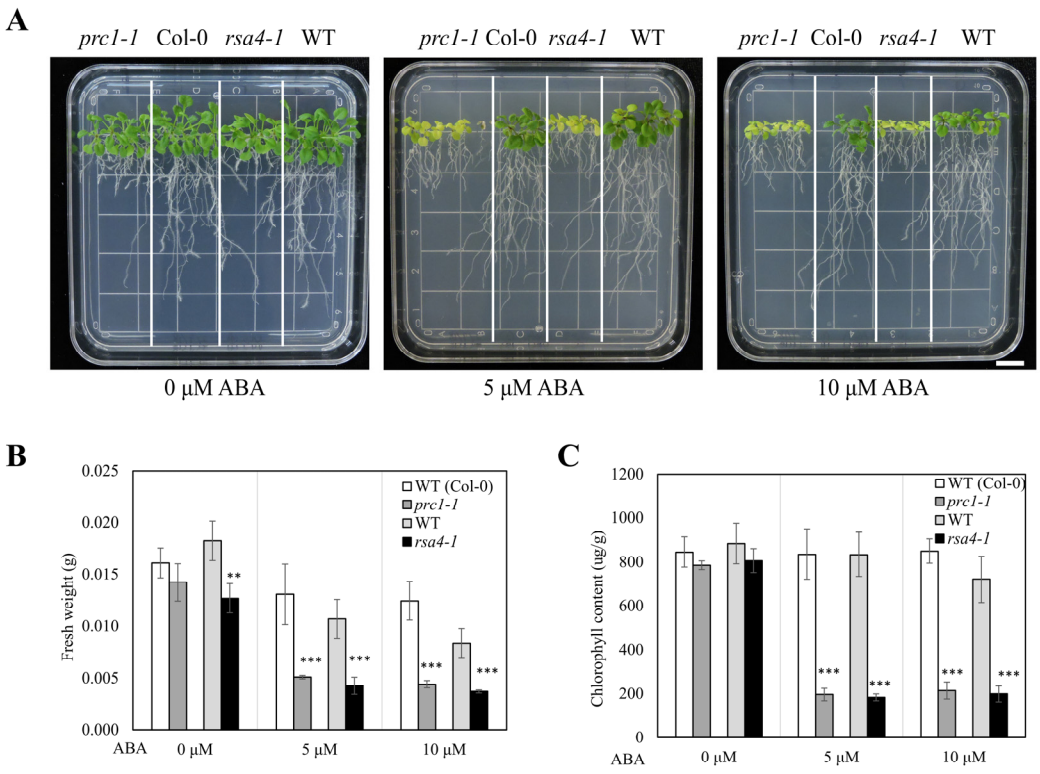


Figure 2. 6 Responses of the wild type and *rsa4* mutant plants to exogenous ABA.

(A) Four-day-old plants were transferred to MS medium containing various levels of ABA (0 µM, 5 µM, and 10 µM ABA) and allowed to grow for an additional 17 days. Bar = 1 cm. (B) Shoot fresh weight of different plants shown in (A). (C) Chlorophyll content of different plants shown in (A). Values are means ± SD (n = 3; there are 25-35 seedlings per genotype). (** $p < 0.0022$, *** $p < 0.0001$ in Student's t-tests).

Overexpression of *RSA4* leads to enhanced salt stress tolerance

Previous results showed that RSA4 is a positive regulator for salt stress. We overexpressed *RSA4* in the wild type (Col-0). In this study, the germination rate is

defined by the emergence of cotyledons under normal conditions. The wild type and *RSA4* overexpression lines showed no significant difference in germination rates under normal conditions (Figure 2. 7A, B). *RSA4* overexpression lines showed a salt-tolerant phenotype compared with the wild type with increased rates of fully established seedlings under salt stress conditions (Figure 2. 7C).

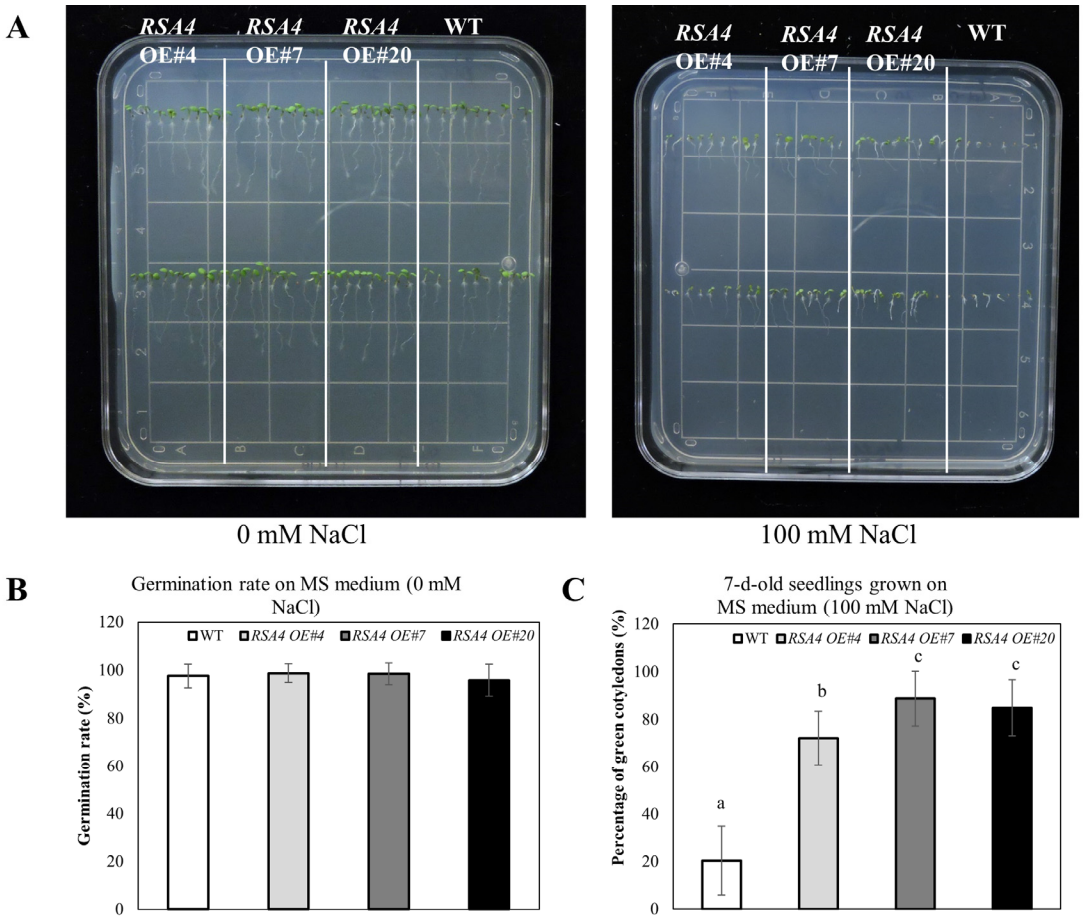


Figure 2. 7 *RSA4* overexpression lines are salt-tolerant during early seedling development.

(A) Seeds of the wild type and *RSA4* overexpression lines were sawn on MS medium containing 0 or 100 mM NaCl and incubated in a growth chamber for an additional seven

days. **(B)** The germination rate of the wild type and *RSA4* OE lines on MS medium (without NaCl). **(C)** Percentage of seedlings with green cotyledons in the wild type and *RSA4* OE lines on MS medium containing 100 mM NaCl. Values are means ± SD (n = 5 in control condition, n = 16 in NaCl treatment [number of MS medium plates; there are 16-20 seedlings per genotype per MS medium plate]). One-way ANOVA (Tukey-Kramer test) was performed ($p < 0.0004$).

Reactive oxygen species (ROS) are over accumulated in *rsa4-1* under salt stress

Since the *rsa4-1* mutation does not alter Na^+ homeostasis, RSA4 is not likely to be involved in the SOS pathway. To answer the research question of how RSA4 mediates salt stress, we hypothesized that ROS detoxification may be affected in the *rsa4-1* mutant.

ROS is rapidly induced by abiotic stresses. An excessive level of ROS will damage the cells. ROS detoxification is a way to keep it at a moderate level. Meanwhile, the accumulation of ROS can become the cellular signaling molecule to regulate downstream stress responses. We measured ROS levels in the wild type and *rsa4-1* and found that *rsa4-1* accumulated more total ROS than the wild type under both normal and salt stress conditions (Figure 2. 8A). H_2O_2 level was also increased in the *rsa4-1* under salt stress (Figure 2. 8B).

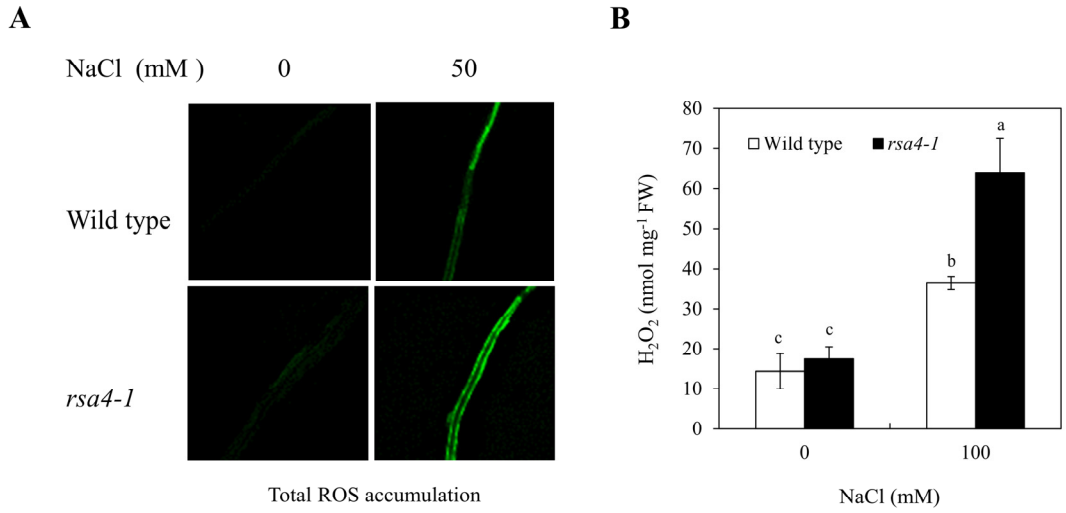


Figure 2. 8 ROS are over accumulated in *rsa4*-1 plants.

(A) Total ROS accumulation in roots of 5-day-old wild-type and *rsa4-1* seedlings subjected to 0 or 50 mM NaCl for 12 h. Bars = 200 mm. Seedlings were incubated with 5-(and 6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) for 30 min and washed with distilled H_2O to remove excess CM-H_2DCFDA. Fluorescence images were obtained with a Leica SPX5 confocal microscope (Leica Microsystems) (B) H_2O_2 content of wild-type and *rsa4-1* plants subjected to 0 or 50 mM NaCl for 12 h. Values are mean ± SD (n = 30).

Drought stress response and cuticle deposition in *rsa4* mutants

In plants, controlling water loss is crucial for plants. Two major ways of transpiration are stomata transpiration and cuticular transpiration. By the precise regulation on stomata closure, plants are able to exchange gases essential for photosynthesis and meanwhile avoid excessive water loss. Cuticle is the epidermal structure on plant

leaves and stems to keep plants from water loss. It is made of waxes and lipids, functioning as a barrier in response to abiotic and biotic stresses. Preliminary study done by our lab showed that stomata density and size were not significantly different between wild-type and *rsa4* mutants (data not shown here). Hence, we hypothesized that defects of cuticle deposition in *rsa4* mutants may be one of the causes of the drought sensitivity shown in *rsa4* mutants.

We used plant cuticle staining with a hydrophilic dye, Toluidine Blue O (TB), to determine cuticle deposition in *rsa4* mutants (Tanaka et al., 2004). Since plant cuticle is composed of hydrophobic molecules, the complete cuticle layer will not be stained by TB dye. Therefore, the percentage of TB staining can be used as an indicator of the completeness of cuticle layer.

We then collected the third- and forth-true leaves from wild-type and *rsa4* mutants (*rsa4-1*, and *prc1-1*) to compare the differences between the wild type and *rsa4* mutants. We chose these leaves according to the preliminary test that we found these leaves were more sensitive to TB treatment (Figure 2. 9A, B). *rsa4-1* showed stronger cuticle incompleteness under both control and drought conditions (Figure 2. 9C), indicating loss-of-function in *RSA4* gene affects cuticle deposition in *Arabidopsis*.

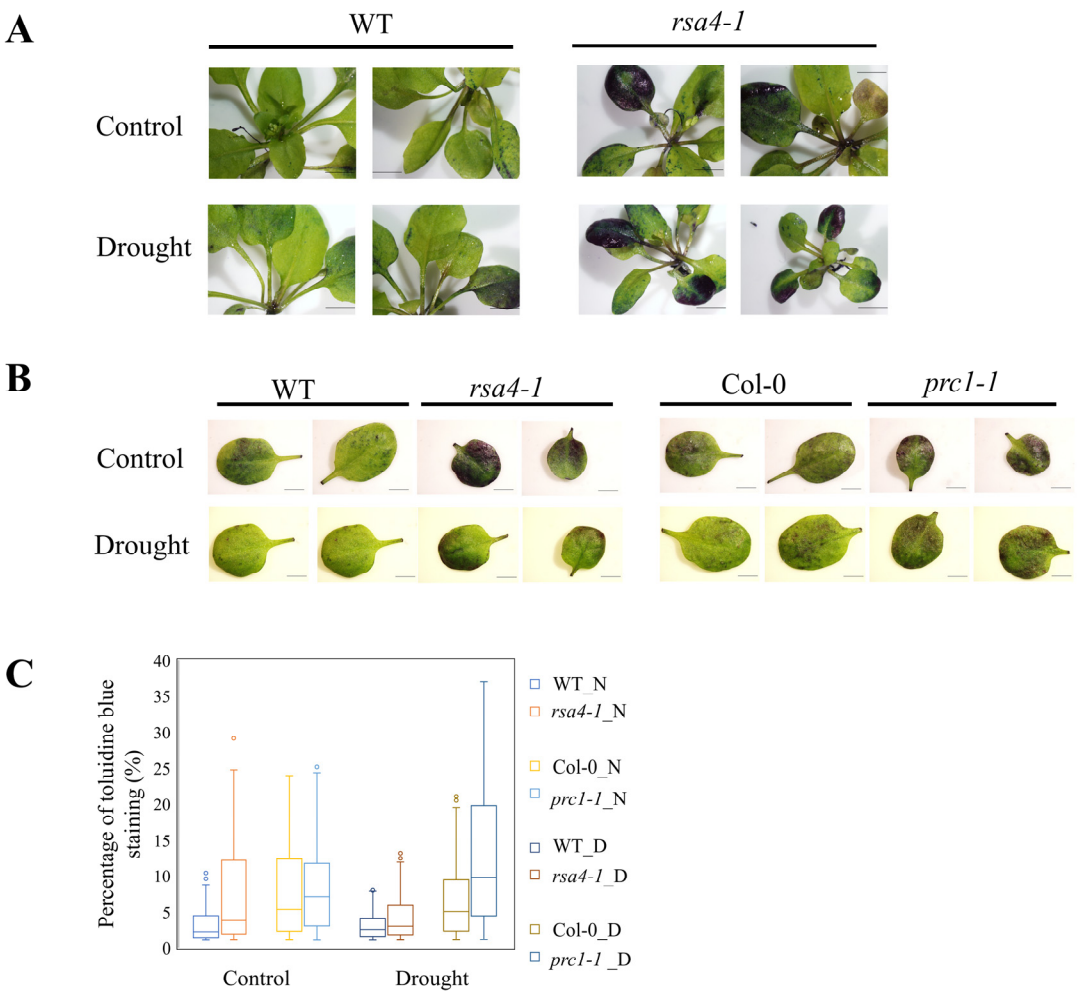


Figure 2. 9 The *rsa4* mutations affect cuticle deposition in *Arabidopsis*.

Toluidine Blue (TB) staining of *rsa4* mutants and wild-type plants. **(A)** Whole shoot TB staining. The shoots were detached from the plants at rosette stage for TB staining. **(B)** TB staining of the excised 3rd and 4th true leaves from 4-week-old soil-grown plants (Control-welled watered; Drought-watered withheld for one-week). **(C)** Quantification of the TB staining of leaves shown in (B). The experiment was repeated at least three times (there are 100-300 leaves per genotype in each experiment). Percentage of Toluidine Blue (TB) staining was measured as (TB-stained pixels collected with Adobe Photoshop)/(Pixels of total leave

area collected with Adobe Photoshop) × 100. WT_N, wild type under control condition, WT_D, wild type under drought stress condition. Student's t-tests were performed to compare the mean values of the wild type and *rsa4* mutants under different conditions. p values are 0.00327 (WT_N vs. *rsa4-1*_N), ns (Col-0_N vs. *prc1-1*_N), 0.00000853 (WT_D vs. *rsa4-1*_D), 0.00000561 (Col-0_D vs. *prc1-1*_D).

To understand how the *rsa4-1* mutation affects cuticle deposition, we examined the expression of several wax-synthetic or cuticle-related genes in *rsa4-1* plants subjected to drought stress. We found that the expression of *KCS1* and *TWS1* was reduced in *rsa4-1* under drought treatment (Figure 2. 10A, B). KCS1, also named 3-KETOACYL-COA SYNTHASE 1, is a membrane-localized enzyme for the synthesis of very long-chain fatty acids in wax synthesis in *Arabidopsis* (Todd et al., 1999). *TWS1* is highly expressed in seeds and expressed during later plant growth phases. This single copy gene encodes a small protein (81 amino acids) which is required in cuticle deposition (Fiume et al., 2016). These results indicate that the reduction of cuticle-related gene expression may lead to cuticle incompleteness in the *rsa4-1* plants. In addition, we tested the expression of a drought-inducible gene *RD29A*. We found that under drought stress conditions, *rsa4-1* showed less induction of *RD29A* transcripts compared with the wild type (Figure 2. 10C). The detection of drought induced *RD29A* transcripts confirmed the effectiveness of the drought treatment in our experiments.

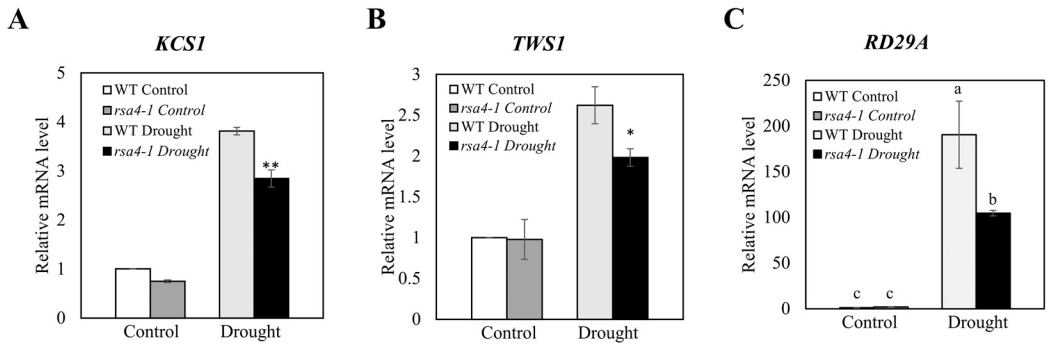


Figure 2. 10 qRT-PCR analysis of cuticle-related gene expression in soil-grown *rsa4-1* plants.

The third and fourth true leaves from 3-week-old soil-grown wild-type and *rsa4-1* plants were collected for total RNA extraction. Expression of the cuticle- or wax-related genes (*KCS1*, *TWS1*) and *RD29A* in soil-grown wild-type and *rsa4-1* plants (Control-well watered; Drought-water withheld for 4 days). **(A)** Expression of *KCS1*. **(B)** Expression of *TWS1*. The student's t-test was applied in (A) and (B). *, $p < 0.05$; **, $p < 0.01$. **(C)** Expression of *RD29A*. One-way ANOVA (Tukey-Kramer test) was applied ($p < 0.001$). Data were shown as mean ± SE (n = 4 [number of experiments; there are 4 biological replicates in each experiment]).

Discussion

RSA4 encodes the cellulose synthase 6 that is responsible for the synthesis of the primary cell wall. Loss of *RSA4* function affects the plants to cope with salt stress and drought stress.

The *rsa4-1* mutant plants are hypersensitive to NaCl, however, the Na^+ contents in the wild type and the mutant are not different both under normal growth conditions

and salt treatment. This result suggested that RSA4 functions in pathways under salt stress independent of the SOS pathway (Figure 2. 1H, I). *RSA4* overexpression lines increased salt stress tolerance in the early seedling developmental stage (Figure 2. 7), indicating that RSA4 positively regulates salt stress tolerance. ROS scavenging is one means that plants detoxify excessive ROS and plants can utilize moderate ROS in downstream signal transduction. We found that *rsa4-1* over accumulated ROS under salt stress conditions (Figure 2. 8). The results suggest that ROS detoxification is affected in *rsa4-1* and it may be the cause of salt hypersensitivity in *rsa4-1*.

The mechanism of salt stress includes two primary aspects: one is ionic stress, and the other is osmotic stress (Zhu, 2002). We applied mannitol to determine the osmotic stress responses in *rsa4-1* and found that *rsa4-1* is hypersensitive to osmotic stress conferred by mannitol (Figure 2. 5). The *rsa4* mutants also showed hypersensitivity to exogenous ABA treatment (Figure 2. 6). Osmotic stress induces ABA accumulation in plants. These results suggest that osmotic stress responses in *rsa4* mutants may be dependent on ABA signaling. We did not examine the expression levels of ABA biosynthetic genes and other regulatory genes in ABA signaling in the *rsa4* mutants. If possible, the results will be able to help us understand the relationship between osmotic stress response to ABA signaling in *rsa4* mutants.

The *rsa4* mutants are sensitive to drought stress. Water loss in plants is regulated by different compartments, including stomata and cuticle. These two structures are both very important for the regulation of water transpiration in plants. A preliminary study done by our lab showed that there is no significant difference in stomata of *rsa4*

mutants compared with the wild type. We then applied Toluidine Blue staining to test on the cuticle layer of *rsa4* mutants and found the mutants showed severe cuticle incompleteness compared with the wild type, both under normal and drought stress conditions (Figure 2. 9). We also found that under drought stress, the wild type plants appeared to show less TB staining compared with normal growth condition, which may be explained by the fact that drought stress could promote cuticle production to reduce water loss caused by transpiration.

To study the mechanism of how *rsa4* mutation affects cuticle deposition, we tested several cuticle-related genes expression under both normal and drought stress conditions. Among the cuticle synthetic genes and regulators in cuticle deposition, we found two genes, *KCS1* and *TWS1* showed reduced expression level (Figure 2. 10A, B). KCS1 is an enzyme involved in very long-chain fatty acids synthesis and TWS1 is a small protein that regulates cuticle deposition. These results suggested that the incompleteness of cuticle deposition may be caused by a reduced expression level of cuticle related genes under drought stress. Drought stress responses in *rsa4* mutants may be affected by the incomplete cuticle deposition. Because cuticle (cutin and wax) biosynthesis and deposition are very complex (Yeats and Rose, 2013), additional evidence is required to have a better understanding of how RSA4 affects cuticle deposition in plants.

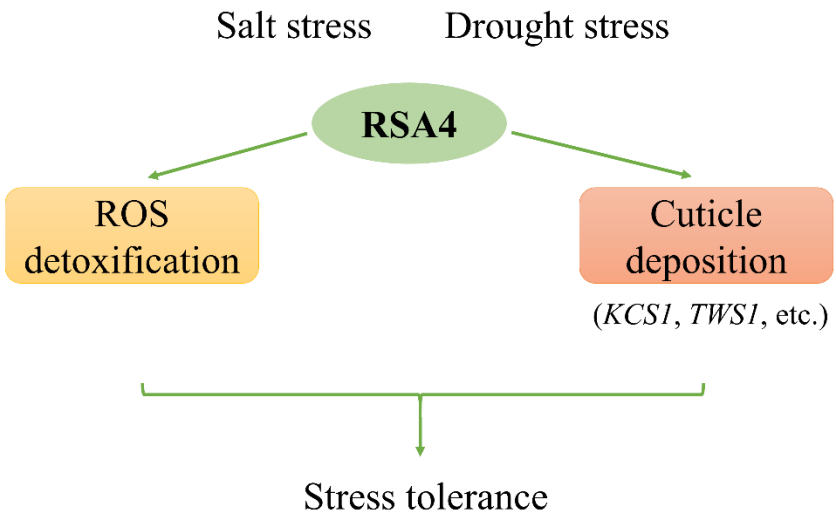


Figure 2. 11 A working model of RSA4 function in respond to salt stress and drought stress.

In summary, we showed evidence that RSA4 is required for salt and drought stress responses in plants through maintaining redox status and cuticle integrity (Figure 2. 11).

Methods

Plant materials and growth conditions

Arabidopsis plants with *RD29A::LUC* reporter system in Columbia *glabrous1* (*gl1*) background were selected under salt stress in an EMS mutant screen as described (Guan et al., 2013a). *rsa4-1* was selected with short root phenotype in a root-bending assay in salt medium (100 mM NaCl) as described (Guan et al., 2013a). EMS line (*prc1-1*) and T-DNA insertion line *SALK_024384C* (*rsa4-2*) were obtained from the

Arabidopsis Biological Resource Center (ABRC). Plants were grown on MS medium in a vertical orientation (1 × MS salts, 1.2% agar, 2% sucrose, pH 5.7), and soil-grown plants were grown in the LC1 potting mix (Sun Gro Horticulture, Bellevue, WA). Plants were grown under cool white light (~100-120 μmol m^{-2} S^{-1}) with a 16-h-light/8-h-dark photoperiod at 21 ± 1°C.

Overexpression of *RSA4*

The coding region of *RSA4*was cloned from the wild-type plants by reverse transcription (ThermoFisher, RevertAid First Strand cDNA Synthesis Kit). Gateway system (Invitrogen) was applied and entry clones were generated with pDONR207 (Invitrogen). The *35S:RSA4* plasmids were constructed by LR reactions with destination vector pEarleyGate203. The relevant plasmids were transformed into the wild type plants by floral dip method with *Agrobacterium tumefaciens* strain GV3101.

Growth assay under ABA treatment

Five-d-old *Arabidopsis* seedlings (*rsa4-1*, WT [gl1]; *prc1-1*, and Col-0) were transferred to a new MS medium (1 × MS salts, 1.2% agar, 2% sucrose, pH 5.7) supplemented with 0 μM, 5 μM, and 10 μM ABA and to grow vertically for an additional 15-20 days. Fresh weight of whole plant and chlorophyll content were measured to quantify the phenotype. Chlorophyll content measurements as described (Lichtenthaler and Wellburn, 1983) with some modifications. Whole plants were collected and incubated with 80% acetone overnight in darkness at 4°C. Chlorophyll

content was calculated as [7.49A_{665} + 20.3A_{648}]/[(fresh weight)*(volume of 80% acetone)] (μg/g FW).

Detection of Na^+ intensity

The Na^+ content was measured using fluorescent dye CoroNa Green as described (Park et al., 2009a). One-week-old seedlings grown on 1.2% agar MS medium were transferred to MS medium containing 0 or 100 mM NaCl and allowed to grow for an additional three days. To measure the sodium content, we incubated untreated and salt-treated seedlings with 50 μM CoroNa Green (Molecular Probe, Carlsbad, CA, USA) for 3 h at room temperature in darkness. These seedlings were then washed three times with distilled water to remove residual unbound CoroNa Green. CoroNa Green-dependent fluorescence was observed with a Leica SP5 confocal microscope (Leica, Germany). Average fluorescence intensity was quantified after subtracting the corresponding image background of each sample detected with ImageJ software (NIH).

Determination of ROS levels and H_2O_2 content in *Arabidopsis*

ROS levels were measured as described (Guan et al., 2013a). Seedlings were grown vertically on MS medium (1 × MS salts, 1.2% agar, 2% sucrose, pH 5.7). Five-d-old wild-type and *rsa4-1* seedlings were transferred to MS medium with 0 or 50 mM NaCl for 12 h. Seedlings were incubated with 5-(and 6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) for 30 min and

washed with ddH_2O to remove excess CM-H_2DCFDA. Fluorescence images of the root were obtained with a Leica SPX5 confocal microscope.

H_2O_2 content of 5-d-old wild-type and *rsa4-1* plants subjected to 0 or 50 mM NaCl growing for an additional 12 h was measured by Amplex red hydrogen/peroxidase assay kit (Invitrogen).

Plant cuticle staining by Toluidine Blue (TB) under drought stress conditions

The third and fourth true leaves of *rsa4-1*, wild-type; *prc1-1*, and Col-0 plants were used in cuticle completeness assay with TB staining. The third- and fourth- true leaves (100 to 300) from soil-grown 1-month-old plants, in control and drought treatment groups, were collected for TB staining. For drought treatment groups, irrigation was stopped in 3-week-old plants, the start date of water termination was defined as Day 0, leaves were collected at Day 4 or Day 6, depending on the rate of water loss in soil. Toluidine Blue O (Sigma-Aldrich, cat. No. 198161) solution (0.05% [w/v]), dissolved in distilled deionized water [ddH_2O)) was then filtrated by 0.2 μm nylon syringe filter. The leaves were soaked in 0.05% TB solutions for 15 minutes and then rinsed by excessive ddH_2O. Photos of all leaves were taken by Olympus DP71 (software: DP manager version 3, 1, 1, 208) with a dissecting microscope (Diagnostic Instruments). The ratio of TB staining area was quantified by Photoshop (Adobe Photoshop CC 2018) in color pixel. The total leaf area was selected by the quick selection tool, and the number of pixels was recorded as the total leaf area. After deselection, one TB stained color was then selected by the

eyedropper tool. To keep the consistency of TB assay, this selected TB stained color was applied to all measurements. TB staining area was quantified by Color Range in the Select section, also shown in pixel. TB staining ratio (%) = ([TB staining pixel]/ [total pixel]) ×100%, in which indicates TB staining status in different genotypes under control and drought conditions.

qRT-PCR analysis

Total RNA was extracted from the third and fourth true leaves of soil-grown plants under normal growth conditions and drought stress conditions (4 days of drought treatment). Five μg of total RNA was used for the first-strand cDNA synthesis (ThermoFisher). Four biological replicates and two technical replicates were used in each experiment with a Bio-Rad CFX96 platform. The comparative quantification method (fold change = $2^{-\Delta\Delta Ct}$) was applied to calculate relative gene expression levels, and *UBQ5* was used as the reference gene.

Chapter 3: Conclusions and future perspectives

This dissertation aims to elucidate the role of two molecular components ROA1 and RSA4 that are critical for plant abiotic stress tolerance.

In the first study, we characterized the function of ROA1/RBM25, a U1 snRNP, in response to cold stress in *Arabidopsis*. Under the low-temperature condition, *roa1* mutants showed chilling hypersensitivity and slight freezing sensitivity. In addition, *roa1* mutants showed defects in alternative splicing events compared with the wild type, mainly in intron retention events, followed by cassette exon (exon skipping) events. The changes in alternative splicing may lead to the altered transcriptome seen in *roa1* mutants. ROA1 splicing targets include transcription factors (CIR1, PIF7, etc.), splicing factors (SNRF1, encoded by *At2g14285*), molecular components that are required in photosynthesis (LHCB7) and chloroplast functions, and other molecular components that are responsive to stress conditions (AtDi19-4) or with poorly studied functions. Furthermore, BiFC assay and IP-MS showed that ROA1 can interact with other components in the U1 snRNPs and splicing factors. Taken together, our results revealed the critical function of a splicing factor ROA1 for alternative splicing of pre-mRNAs under cold stress and cold stress tolerance in plants.

Many research questions still need to be answered in future studies. The first question is how cold signal (or other stress signals) is perceived and regulates splicing factors in response to the stress condition? Several studies showed the crucial role of

U1 snRNP proteins in different stress responses (Zhan et al., 2015; Amorim et al., 2018; Gu et al., 2018). Is there any correlation among those splicing factors in response to stress conditions? How do they distinguish different stress signals and contribute to stress tolerance? The future perspectives may focus on identifying the role of other splicing factors and U snRNA (spliceosomal RNA) in stress tolerance and looking for the binding motifs patterns or the splice site patterns of downstream targets via bioinformatics strategies.

The second study functionally characterized a cellulose synthase RSA4 in salt and drought stress responses. *rsa4* mutants showed increased sensitivities in salt stress, general osmotic stress, exogenous ABA application, and drought stress. Over-accumulated ROS level was detected in the *rsa4* mutant under NaCl treatment. However, no significant differences were observed in stomatal density and stomatal closure under the control of ABA (data not shown here). Cuticle incompleteness was detected in *rsa4* mutants under normal and drought stress conditions. Furthermore, the expression of two cuticle biosynthesis genes in *rsa4* mutants is altered compared with the wild type under drought stress. These results suggested that plant primary cell walls may be important for osmotic and ionic stress responses, as well as ABA-dependent stress responses. It may also affect cuticle accumulation, which contributes to water stress response via cuticular transpiration.

The question is how a cellulose synthase mutant affects cuticle accumulation in plants? We hypothesis that cell wall integrity may contribute to cuticle accumulation especially under osmotic stress conditions because of the physical localization of cell

wall and cuticle. Under osmotic stress, the turgor pressure inside the cell decreases, and the cell wall remodeled (Tenhaken, 2015). However, the potential wall-associated osmosensor and cuticle biosynthetic and transportation pathways remain unclear. Future studies may focus on cuticular wax transportation and distribution. The knowledge gained from cuticle deposition in plants will help answer the question of the role of cellulose synthase and cell wall in cuticle deposition.

Appendices

Appendix A. 1 Primers used in the ROA1 study.

Name of primer	Primer Sequence (5' to 3')	Purpose
PROK2 LBa1	TTTTTCGCCCTTTGACGTTGGAG	Genotyping of SALK lines
SALK_037582 LP	AGATACCAATCCACAATTAGACCATG	Genotyping
SALK_037582 RP	ACGAAATTCGATAGGGTTGAGAGTCG	
SALK_091569 LP	AGCTGTGGATGTTGATGACAGTCC	Genotyping
SALK_091569 RP	TGAAGCGAAGTGCTTCCTAAGTTCC	
pif7-1 RP	CATCCTCTGGTTTATCCTATCACGCCG	Genotyping [referred to Leivar P, Monte E, Al-Sady B, et al. Plant Cell. 2008;20(2):337-352.]
pif7-1 LP	CCGTTCATGGTCTAGGCG	
EMO48	TGATAGTGACCTTAGGCGACTTTTGAACGC	
pif4-2 LP	AGGATCAATGAGAGAATGAAGGCTTTGC	Genotyping
pif4-2 RP	TGGCTCACCAACCTAGTGGTCC	
SALK_018360C RP	ACCTGTCGTTTCTAGCTTCCTGAGC	Genotyping
SALK_018360C LP	TGGATCTAACGATGTGGCCTGTTAC	
SALK_007551C RP	TCATCAGTATCTTCAAGGTTGTGC	Genotyping
SALK_007551C LP	ACAGTTGTAGCTGTTTCTGATGG	
SALK_030430C RP	TGAGAATTCGTCGTTCTTCAACTC	Genotyping
SALK_030430C LP	ACCAGATAAGTAAGGCATGATCACC	
SALK_052537C RP	TGTGCTATAGAGAGGCAAATCACC	Genotyping
SALK_052537C LP	TCCTAGCTACAAAAAACAAGCATGG	
SALK_005071 RP	AGATGAGTTAGCCATCATACCTCCG	Genotyping

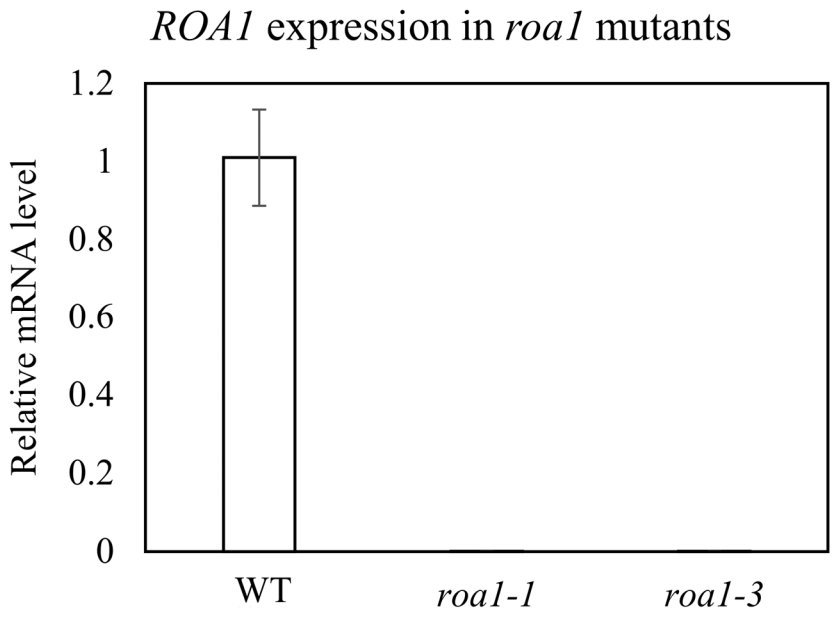
SALK_005071 LP	TGCTTTCGCTGAGTATGAGAC	
SALK_005077 RP	ACTTCCAATTGCACCGTATACATAGG	Genotyping
SALK_005077 LP	TGACAACTAGATTGAGCAGAAGGTTCTTG	
SALK_019266 RP	TGATATGGTAATCCGTATCGAATTCGG	Genotyping
SALK_019266 LP	ACCTAAAGGCTTTGCTTTCGCTGAG	
SALK_076524 RP	TGAACATAGAAGCTAAGACCATACCTG	Genotyping
SALK_076524 LP	TCGCTAATCCACGATTTCGATTCC	
SALK_072556C RP	AGATCCCTATGGATCGCTTCCACTCAAG	Genotyping
SALK_072556C LP	TGTCTGAACCGGAGATCGGATTATCG	
SALK_087851C RP	TGCTCTCTCTAGGGTAAGCGTTCGACGTC	Genotyping
SALK_087851C LP	AAGGTTCCTACTAACGTCCACAATCTG	
SALK_009988C LP	TGAGTGATTGAGACAGGTTCCTTACG	Genotyping
SALK_009988C RP	TGAACAACTACTCTCTCATGCACTGG	
SALK_025754C LP	TCCAGCAGCTCAAGAGTTCTTCTTG	Genotyping
SALK_025754C RP	TGTTCTGTTGTATTCACACAGGTTGC	
SALK_045739C LP	AGGTTGGCTGCAATCGGAGAAGAC	Genotyping
SALK_045739C RP	ACTAAACTTCCTGCAGCAACAGC	
SALK_057639C LP	ACCATGATTGATGATTCGTCAAACTCC	Genotyping
SALK_057639C RP	TGTATTGGGTTCTTCTCCACAATGAC	
SALK_080753C LP	TGCACTGGTGCAGCATCAAGAGTCC	Genotyping
SALK_080753C RP	ATGCTTGGTTGTCGGAATTCTGG	
SALK_096470C LP	AGAGGATGACAAGAAGACAACTCTC	Genotyping
SALK_096470C RP	ACCAACAAACATACACTCCTCAATCC	

SALK_129269C LP	ACTCTCTAACGTCATGATTGTATTG	Genotyping
SALK_129269C RP	AGAAAGATTTGGGTGGTTTATAAGATG	
SALK_138056C LP	TCTGCCTTCAAATCGTCTTCTCC	Genotyping
SALK_138056C RP	ACAGGCTCATATCCTAAGTATAGATAACG	
SALK_021070 LP	ACCTTCGCTCACATCCATAGCAGTCTC	Genotyping
SALK_021070 RP	TCCATGGTCTCCTCGTGGCTGTTTGAAG	
CS2103562 (GK) LP	SAME AS SALK_021070RP	
CS2103562 (GK) RP	SAME AS SALK_021070LP	
SALK_057558 LP	TCTGCCTCCTTCGTCTTTCCTCAGC	
SALK_057558 RP	TCTGGTGCAATTAGTGACACTGAGGC	
CS816258 (SAIL) LP	TCAGTGTGCTTCCTACAGAGTCC	
CS816258 (SAIL) RP	TCTGATGAAGATGTTAGTTCTGATAGGG	
SALK_032382 LP	TCTACAGACCTTTAGTCTAACGTCAGG	Genotyping
SALK_032382 RP	TGCAACAGGAGAGCTTACTGCC	
SALK_043590 LP	SAMEASSALK_151254LP	
SALK_043590 RP	SAMEASSALK_151254RP	
SALK_066044 LP	TGTCCATGTTGATTGCTTCGTAATC	
SALK_067172 LP	SAMEASSALK_066044LP	
SALK_067172 RP	SAMEASSALK_066044RP	
SALK_151254 LP	TCACAGGACCACGAGCTCAAGTC	
SALK_151254 RP	TGCTGTAACATCGACATAAGAAACCC	
M13 F Zhu lab	TGGATGTTTTCCCAGTCACGACG	DNA sequencing
M13 R Zhu lab	TGCCAGGAAACAGCTATGACCATG	

U1-70K attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGGAGACTCCGGCGATCC	Gateway *attB* primers of *U1-70K*
U1-70K attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC ACGAACATACTCTCGCGATTCTG	
PRP40B attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGCTAATAACCATCAATATCCTGGC	Gateway *attB* primers of *PRP40B*
PRP40B attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC ATACCGACCACATTCTCCATCTTC	
At2g41020 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGGAGAAGAGCTGCAATATCAGC	Gateway *attB* primers of *At2g41020*
At2g41020 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC ATCTGCGTCTCCAAGACCATCAC	
At2g43370 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGAGCGGCGGAGGAAACAACGTCG	Gateway *attB* primers of *At2g43370*
At2g43370 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GTCGGAAGAATGATGATCTTGACTATAGG	
PRP40A attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGCGAATAATCCTCCGCAGTC	Gateway *attB* primers of *PRP40A*
PRP40A attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTCCCCAACTTCTCCATCCTCTAGC	
PRP39A attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGGTGACAGCGAGGCCATGG	Gateway *attB* primers of *PRP39A*
PRP39A attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GTAGTACGTGTTGTAGTACGCTTGAGG	
U2A' attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGTGAAGCTCACGGCTGATTTG	Gateway *attB* primers of *U2A'*
U2A' attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTCCTCCATGGGAGCAGAGTC	
At2g14285 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGAATACAAAGGTTTTCTCGCTTCTG	Gateway *attB* primers of *At2g14285*
2g14285 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GTCTTGATCAGCATCTTCGAGTTCTTC	
At2g14285 attB1 (fc)	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCTCGCTGCAAACTCATGGCTACCATACC	Gateway *attB* primers of *At2g14285* (full length cDNA)
At2g14285 attB2 (fc)	GGGGACCACTTTGTACAAGAAAGCTGGGTC TGAAGGCCAGAACTCGAAGAAGAATATG	
SR45 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGCGAAACCAAGTCGTGGCCGTCG	Gateway *attB* primers of *SR45*
SR45 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC AGTTTTACGAGGTGGAGGTGGTGG	

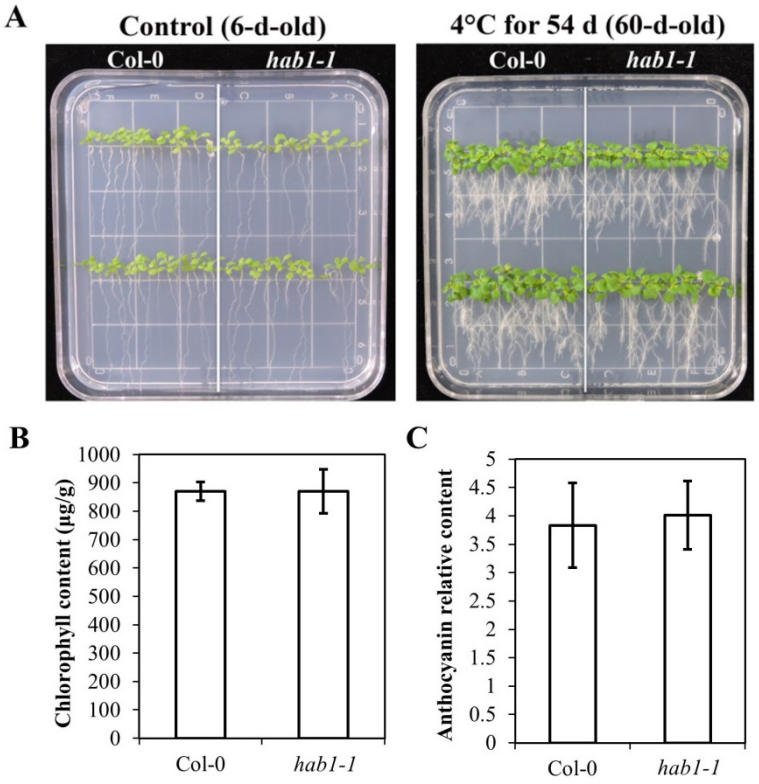
At4g22380 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGACGGGAGAGGTAGTGAACCC	Gateway *attB* primers of *At4g22380*
At4g22380 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GATCAGGAGCTTCTCAATTGCATCC	
FIB2 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGAGACCTCCTCTAACTGGAAGTGG	Gateway *attB* primers of *FIB2*
FIB2 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC AGCAGCAGTAGCAGCCTTTGGCTTC	
ALY4 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGTCTGGAGCATTGAATATGACTCTTG	Gateway *attB* primers of *ALY4*
ALY4 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC AGAGGTGTTCATGGCATCAGCGTG	
ATHSP90-1 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGCTGATGCAGAGACTTTTGC	Gateway *attB* primers of *ATHSP90-1*
ATHSP90-1 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GTCGACTTCCTCCATCTTGCTCTC	
SAIL_1292 _E05 LP	TCATGAGAACGTCTGCTCCTTTG	Genotyping
SAIL_1292 _E05 RP	AGAGAAAGAGAAAAGGAGAAGGAACG	
SALK_069 703 LP	TGGGATACTAAGCAACAAAGGAAGATAAA G	Genotyping
SALK_069 703 RP	TCAGGGAATACACCACCTCATTTGGC	
AtDi19-4 CDS attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGATTCCAATTGGATCAATTGTC	Construction of plasmids: pEarleyGate201-*AtDi19-4* in *roa1* mutants
AtDi19-4 CDS attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTAGAAATCGTCTTCAAGCATGGTTG	
AtDi19-4 comp attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCTCGCATACAAAAGATGAGCCGCTC	Construction of complementation line of pMDC107-*AtDi19-4* in SALK_138056C
AtDi19-4 comp attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GAAATCGTCTTCAAGCATGGTTGACAAC	
CIR1 CDS attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGCTATGCAGGAACGTTG	Construction of plasmids: pEarleyGate201-*CIR1* in *roa1* mutants
CIR1 CDS attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC TCACCACAAAGGATATGATAATTTTACA	
CIR1 comp attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCTGGCTGGGTACACAATTTTTTTCAC	Construction of complementation line of pMDC107-*CIR1*
CIR1 comp attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC CCACAAAGGATATGATAATTTTACATTGTC	
At2g14285 CDS attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGAATACAAAGGTTTTCTCGCTTCTG	Construction of plasmids:

At2g14285 CDS attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTAGTCTTGATCAGCATCTTCGAGTTC	pEarleyGate201-*At2g14285* in *roa1* mutants
At2g14285 comp attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCACAGAAGAGACAACACGACAGATGG	Construction of complementation line of pMDC107-*At2g14285*
At2g14285 comp attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GTCTTGATCAGCATCTTCGAGTTCTTC	
ROA1 CDS attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGGCCGACGAATCTTCTTCTCCG	Construction of BiFC plasmids: pEarleyGate202(YC)-*ROA1* and pEarleyGate201 (YN)-*ROA1*
ROA1 GEN attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC GGCTTTGGATTTTACCGGGACTCC	
ROA1 GEN attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCTGCATGTGTATTGTCATGAAACCCTAG	Construction of pGWB410-*ROA1*
RD29A qRT-F	ACATCATGAGAATGGTGCGACTAAG	qRT-PCR
RD29A qPCR R	ATGAGCCGGTGCATCGTGTC	
At5g51780 asF1	GGCAAAGGAGACAAGAAATGGCTTC	RT-PCR (validation of AS event)
At5g51780 asR1	CAACCAAACACTGACGAACCACCAC	
At5g61270 asF1	TGATGGTCCCTCCTATGGGTAAC	RT-PCR (validation of AS event)
At5g61270 asR1	GATCAATAATACTAGGTCGCTAGACTA	
At5g37260 -3I-F1	CTGATGAACTTATATCTTCCTCAGATG	RT-PCR (validation of AS event)
At5g37260 -3I-R1	CTGCGCATGGCTTCGAATCTGAACTGC	



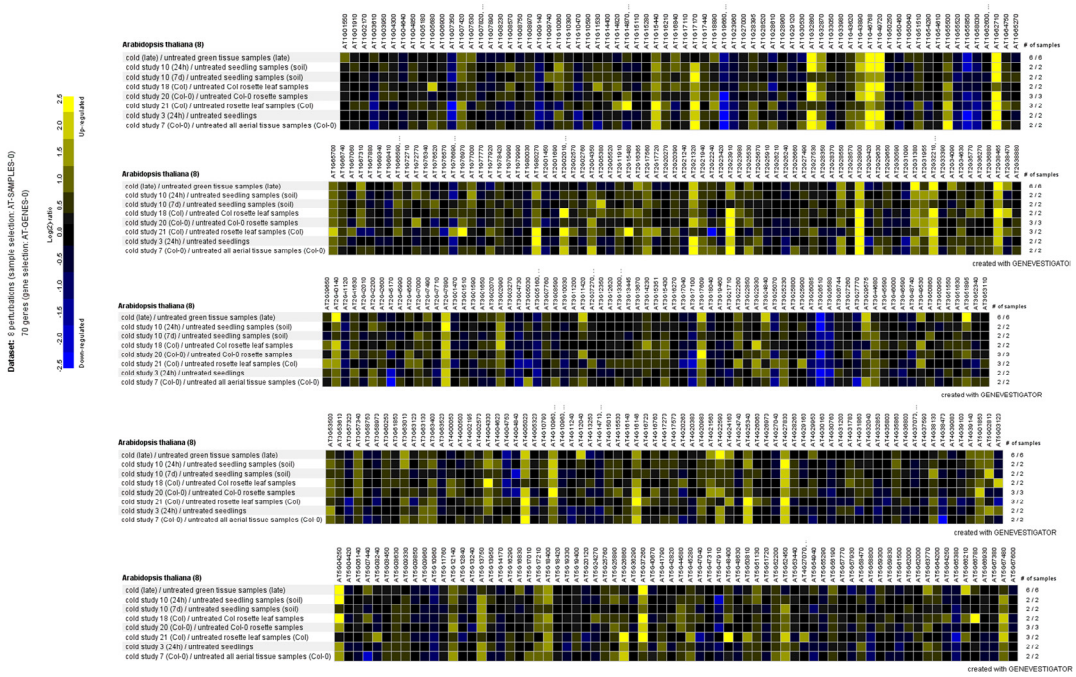
Appendix A. 2 Expression of *ROA1* in the *roa1* mutants determined by qRT-PCR analysis.

Total RNA was extracted from three-week-old wild-type and *roa1* mutants. qRT-PCR assay was repeated for at least three times. Values are means ± SD (n = 3).



Appendix A. 3 Chilling stress responses of *hab1-1*.

(A) Seeds of Col-0 and *hab1-1* were planted on MS medium with 1.2% Agar. Left panel: 6-day-old seedlings grown at 21°C, right panel: 6-day-old seedlings grown at 21°C were transferred to a growth chamber at 4°C and allowed to grow for an additional 54 days. **(B)** Chlorophyll content of cold-treated (4°C for 54 days) plants. **(C)** Anthocyanin content of cold-treated (4°C for 54 days) plants. Values are means ± SD [n = 10 in (B) and (C); there are 6-8 seedlings for each genotype grown side-by side on each MS plate].



Appendix A. 4 Most of the genes displaying splicing defects in *roa1-1* mutant are cold-responsive in WT (created with GENEVESTIGATOR).

Appendix A. 5 Categories of selected ROA1 splicing target genes.

AGI ID	Description	T-DNA lines	Alternative splicing type
Group 1. Transcription factors			
At5g02810	pseudo-response regulator 7	*SALK_091569C* (*prr7*)	intron retention
At5g17300	*RVE1*, MYB family transcription factor	*SALK_025754C*	cassette exon

At5g37260	MYB family transcription factor. *CIRCADIAN1* (*CIR1*)/*REVEILLE 2* (*RVE2*), a positive regulator of cold stress.	*SALK_051843C* (*cir1*), *SALK_074896C*	intron retention
At5g51780	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	*SALK_087851C*	alternative donor; intron retention
At5g61270	*PIF7*, phytochrome-interacting factor7	*pif7-1*	intron retention
Group 2. Splicing factors			
At2g14285	Small nuclear ribonucleoprotein family protein	*SALK_052537C* (*snrf1*), *SALK_076524C*	alternative acceptor
At4g10110	*RBM7*, RNA-binding (RRM/RBD/RNP motifs) family protein	*SALK_005077C* (*rbm7*)	intron retention
Group 3. Photosynthesis, Chloroplast function and development			
At1g76570	*LIGHT-HARVESTING COMPLEX B7*, Chlorophyll A-B binding family protein	*SALK_018360C* (*lhcb7*)	intron retention
At5g17300	*RVE1*, MYB family transcription factor	*SALK_025754C*	cassette exon
At5g64940	*AtOSA1*, ABC2 homolog 13	*SALK_045739C* (*atosa1*)	alternative acceptor

Group 4. Other genes with poorly characterized functions in plant cold stress responses			
At1g04300	*TRAF1B*, TRAF-like superfamily protein	*SALK_096470C* (*traf1b*)	intron retention
At1g78070	Transducin/WD40 repeat-like superfamily protein	*SALK_009988C* (*wd40rl*)	alternative acceptor; alternative donor
At1g79990	WD40-containing protein	*SALK_072556C*	intron retention
At3g06760	*AtDi19-4*. Drought-responsive family protein	*SALK_138056C* (*atdi19-4*)	cassette exon
At4g03960	*ATPFA-DSP4*, Phosphotyrosine protein phosphatases superfamily protein	*SALK_129269C* (*dsp4*)	cassette exon
At4g19040	ENHANCED DISEASE RESISTANCE 2	*SALK_080753C*	cassette exon
At5g43580	*UPI*, serine protease inhibitor, potato inhibitor I-type family protein	*SALK_057639C* (*upi*)	alternative acceptor

Appendix B. 1 Primers used in the RSA4 study.

Name of primer	Primer Sequence (5' to 3')	Purpose
RSA4 OE F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACACCGGTGGTCGGTTAATCG	Overexpression of RSA4 in wild type plant and *rsa4-1* mutant
RSA4 OE R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACAAGCAGTCTAAACCACAGATCTCGAG	
RSA4 clone R1	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGATCCTTTGAAAGCCGCTAAC	gene clone for 5' fragment-2 (2437)
RSA4 clone R2	GGGGACCACTTTGTACAAGAAAGCTGGGTCCAAGTACGTTTCTCGCTCGATAG	gene clone for 5' fragment-1 (1009)
RSA4 cDNA F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGCAAATGAAGTTGGAGAAG	Cloning primer for cDNA
RSA4 SDM-F	GTGCTACGTCTTGTGATTCTTGGTCTCTTCTTTCACTACCGTATTC	sense and antisense primers from 838-883 in *RSA4* full length cDNA to correct 853A to G point mutation detected in pDNOR207-*RSA4* plasmid through site-directed mutagenesis
RSA4 SDM-R	GAATACGGTAGTGAAAGAAGAGACCAAGAATCACAAGACGTAGCAC	
SRS3 comp F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCGGACTATATCTATCAACAAGTCAAAAG	complementation of *rsa4-1* mutant with genomic fragment
SRS3 comp R	GGGGACCACTTTGTACAAGAAAGCTGGGTACGTCTCCAAGCATGCGGATTGAAAATC	
CER10 F	TGGAGCTTTGGTGCTTACATTGC	qRT-PCR (cuticle-related gene expression)
CER10 R	AGCGATGTTGAATCCTAGCCATTGG	
GPAT8 F	TCGTTTGCCTGAAGAAATGACTG	qRT-PCR (cuticle-related gene expression)
GPAT8 R	AGTAAGTTCGGTGCATTCGAAG	
KCS1 F	TGATCTCAAAGATTGGCACATGG	qRT-PCR (cuticle-related gene expression)
KCS1 R	ATCCAAACGCAATCTGCCAAAG	
CER2 F	TGAACTTGAGATCAACGGAGGG	qRT-PCR (cuticle-related gene expression)
CER2 R	TGCAAGGTCTTCTTCAGGCATC	
CED1 F	TGCAAGGGACGCAATATGTGATG	qRT-PCR (cuticle-related gene expression)
CED1 R	TCTGGTGAGTAGCCTGGTGAG	
DCF F	TCTCACTCGGTGGGTTTGGTC	qRT-PCR (cuticle-related gene expression)
DCF R	ACAAGATCACTTCCCTCCCTGG	
CER3 F	TGTGTTCTTAGCTCACGGAGTTG	

CER3 R	AGATTGTTCCTGAGGGTATAGAAGC	qRT-PCR (cuticle-related gene expression)
LTPG1 F	AGCTTCTAGGGATTTCACCTAGC	qRT-PCR (cuticle-related gene expression)
LTPG1 R	TGATCCTCCCTTATCCGTGG	
TWS1 F	TCCAGTGGATCCAACTCCGACGAC	qRT-PCR (cuticle-related gene expression)
TWS1 R	TCTTGGGATTGCTGTGGAGGAGG	
HDG1 F	TGTTGATATCCCAGCAATGCAAGC	qRT-PCR (cuticle-related gene expression)
HDG1 R	AGTAACGACCCTCCTTCCTCC	
PEL3 F	AGTTCGGAGCTTCGGTGATCC	qRT-PCR (cuticle-related gene expression)
PEL3 R	ACCTCGGTGAGCTTCCCACC	
LTL1 F	TGTGACATCGAAGGTGGCATG	qRT-PCR (cuticle-related gene expression)
LTL1 R	TCCTATTGGCTTTCTCTGTCGG	
MYB16 F	TGTCGGATCATCAACTGGTGTG	qRT-PCR (cuticle-related gene expression)
MYB16 R	TCGATTGAATCACCACCGAGC	
ABCG11 F	TCCTTGGGTCAGAGGTTACATAGC	qRT-PCR (cuticle-related gene expression)
ABCG11 R	TCCATCTGTTCGTGTAGCAATG	
KCS6 F	TCATCGTTATGGTACGAGCTTAGC	qRT-PCR (cuticle-related gene expression)
KCS6 R	ACAGGGTAACGGTCGATACAATCGG	
CER8 F	ACAAGAACCCTGAACTCACTGAAG	qRT-PCR (cuticle-related gene expression)
CER8 R	TCCAAATGCTCGAGAGCAAC	
CUS1 F	TGTACGATTTGGGTGCTCGACG	qRT-PCR (cuticle-related gene expression)
CUS1 R	TCGTTGGAGTTCGGTAGCACAC	
CUS2 F	TCGTCAACAACTACTACTTGGTCCC	qRT-PCR (cuticle-related gene expression)
CUS2 R	TCGTTGGAGTTCGGTAGCACAC	
MYB96 F	TCATGAAAGACACACCCAAGGC	qRT-PCR (cuticle-related gene expression)
MYB96 R	AGAGACCCTTGTGATATTAGACCATG	

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