DISSERTATION

ANALYSIS OF GENOME-WIDE TARGETS OF ARABIDOPSIS SIGNAL RESPONSIVE 1 (AtSR1) TRANSCRIPTION FACTOR AND ITS TRANSCRIPT STABILITY IN RESPONSE TO STRESS

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ABSTRACT

ANALYSIS OF GENOME-WIDE TARGETS OF ARABIDOPSIS SIGNAL RESPONSIVE 1 (AtSR1) TRANSCRIPTION FACTOR AND ITS TRANSCRIPT STABILITY IN RESPONSE TO STRESS

Abiotic and biotic stresses cause significant yield losses in all crops. Acquisition of stress tolerance in plants requires rapid reprogramming of gene expression. SR1/CAMTA3, a member of signal responsive transcription factors (TFs), functions both as a positive and a negative regulator of biotic stress responses and as a positive regulator of cold stress-induced gene expression. Using high throughput RNA-seq, we identified ~3000 SR1-regulated genes. Promoters of about 60% of the differentially expressed genes have a known DNA binding site for SR1, suggesting that they are likely direct targets. Gene ontology analysis of SR1-regulated genes confirmed previously known functions of SR1 and uncovered a potential role for this TF in salt stress. Our results showed that *SR1* mutant is more tolerant to salt stress than the wild type and complemented line. Improved tolerance of *sr1* seedlings to salt is accompanied with the induction of salt-responsive genes. Furthermore, ChIP-PCR results showed that SR1 binds to promoters of several salt-responsive genes. These results suggest that SR1 acts as a negative regulator of salt tolerance by directly repressing the expression of salt-responsive genes. Overall, this study identified SR1-regulated genes globally and uncovered a previously uncharacterized role for SR1 in salt stress response.

Soil salinity, one of the most prevalent environmental stresses, causes enormous losses in global crop yields every year. Therefore, it is imperative to generate salt tolerant cultivars. To achieve this goal, it is essential to understand the mechanisms by which plants respond to and cope

with salt stress. Stress-induced reprogramming of gene expression at multiple levels contributes to the survival of plants under adverse environmental conditions. The control of mRNA stability is one of the post-transcriptional mechanisms that is highly regulated under stress conditions leading to changes in expression pattern of many genes. In this study, we show that salt stress increases the level of *SR1* mRNA, by enhancing its stability. Multiple lines of evidence indicate that ROS generated by NADPH oxidase activity mediate salt-induced *SR1* transcript stability.

Furthermore, cycloheximide (CHX), a protein synthesis inhibitor, also increased *SR1* mRNA stability, albeit to a higher level than in the presence of salt, suggesting a role for one or more labile proteins in *SR1* mRNA turnover. Similar to salt, ROS generated by NADPH oxidase is also involved in CHX-induced *SR1* mRNA accumulation. To gain further insights into mechanisms involved in salt- and CHX-induced *SR1* stability, the roles of different mRNA degradation pathways were examined in mutants that are impaired in either nonsense-mediated decay (NMD) or mRNA decapping pathways. These studies have revealed that neither the NMD pathway nor the decapping of *SR1* mRNA is required for its decay. However, decapping activity is required for salt- and CHX- accumulation of *SR1* mRNA. To identify any specific regions within the open reading frame of the *SR1* transcript (~3 kb) that are responsible for the salt-induced accumulation of *SR1* mRNA, we generated transgenic lines expressing several truncated versions of the *SR1* coding region in the *sr1* mutant background. Then, we analyzed accumulation of each version in response to salt stress and CHX. Interestingly, we identified a 500 nts region in the 3' end of the *SR1* coding sequence to be required for both salt- and CHX-induced stability of *SR1* mRNA. Potential mechanisms by which this region confers *SR1* transcript stability in response to salt and CHX are discussed.

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Amira Abdel-Hameed, PhD

DEDICATION

To Soul of My Father, My Mother, and Brothers

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CHAPTER 1

INTRODUCTION

The Impact of Enviromental Stresses on Crop Productivity and Food Security

Plants sustain almost all forms of heterotrophs by being the primary source of food and feed as well as being a major source for medicines, chemicals, renewable materials and biofuels. However, plants are continuously subjected to environmental stresses including abiotic stresses such as drought, cold, heat and salinity, as well as biotic stresses caused by pathogenic bacteria, viruses, fungi and insects, which limit plant productivity and crop yields[1-4]. In addition to these natural calamities, the emission of green gases including CO_2 and CH_4 by a range of human-made activities has significantly increased global temperatures[5,6]. These higher temperatures can negatively affect crop yield directly by affecting the physiology and biochemistry of plants as well as indirectly by enhancing weed, pathogen and pest proliferation. The yield of our most important food, feed, and fiber crops significantly decreases at temperatures higher than 30°C[7]. Additionally, global warming will alter precipitation patterns, change soil moisture profile and increase the incidence of drought[5,6,8,9]. Moreover, glaciers are expected to melt and rise sea levels leading to more flooding and salinity intrusion into coastal croplands[5]. Over the last decade, it has been largely documented that crop production everywhere runs some risk of being negatively affected by these climatic changes[9-12].

Another challenge in agriculture is the shrinking of water resources in many parts of the world. During the past few decades, the available fresh water per person has significantly decreased by about four folds[13], while about 70% of the available water is already used for agriculture[14]. Many rivers no longer flow all the way to the sea and around 50% of the wetlands

around the world have disappeared[15]. A further complication is that while sustaining the already stressed environment, the global agricultural system is under the pressure to accelerate crop yield about 70% to feed the rapidly increasing world-population that is estimated to be 9 billion by 2050[16,17]. At the same time, the amount of arable land is limited and is continuously subjected to decline due to urbanization, salinization, desertification, and environmental degradation[18-21]. Thus, it is not possible to simply convert more land for cultivation to meet production needs. Moreover, most of the crop losses due to environmental stresses occur after the plants are fully grown and the land as well as the water required to grow the crop has been already invested[22]. Reducing crop losses due to these adverse conditions is equivalent to creating more land and more water. So, it is timely and imperative to generate adaptation strategies to improve crop-health and productivity under these adverse conditions.

Climate impacts and adaptation strategies are increasingly becoming major areas of scientific research[23-30]. In this regard, plant science has an important role to play to meet the global food demands over the next several decades. Research in plant genetics, biotechnology, physiology, breeding and agronomy should be able to develop a new generation of agricultural crops tolerant to diverse stresses and new crop management practices to enhance crop yield under adverse conditions[31]. Agronomists are working with farmers around the world to develop new crop production practices that will enhance the sustainability of our farms in the predicted future environment[31,32]. At the same time, plant breeders are using genetic and biotechnological approaches to adapt the existing food crops to increasing temperatures, water shortage, flooding, pathogen and insect threats as well as rising salinity[33-37]. The modified crop varieties can enhance plant resistance to extreme climate and they can also facilitate cultivation of non-arable land such as degraded soils, which will consequently increase the crop yield and increase food availability

in the future[38]. Advances in plant genetics have provided new knowledge and technologies required to address these challenges. However, there is still much to be learned about the biology of plant-environment interactions.

As plants in their environments are continuously subjected to biotic and abiotic stresses, they have evolved various physiological and biochemical mechanisms which rely mainly on changes in gene expression for stress adaptation[1-4,39]. Sensing of biotic and abiotic stresses by plants leads to activation of complex signaling cascades that differ from one stress to another[40]. Upon perception of the stress, ion channels as well as kinase cascades are activated. Also, reactive oxygen species (ROS), and phytohormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) are accumulated[41,42]. These signals activate downstream signaling pathways that cause reprogramming of the genetic machinery by altering the expression of specific genes, which contribute to enhanced stress tolerance[39]. Understanding how plants sense and respond to environmental stresses is a prerequisite for biotechnology approaches and plant breeding, as this will help to determine which physiological trait(s) and gene(s) of the plant should be targeted to improve plant adaptability[43-46].

Calcium Signaling Pathways

In plants, calcium (Ca^{2+}) ion plays a key role in almost all aspects of development and regulatory processes. It is an essential ion required for plasma membrane function and structure as well as maintaining the structural rigidity of the cell walls[47]. Also, Ca^{2+} is involved in regulating the dynamics of microtubules, which is essential for the movement of chromosomes during anaphase[48,49]. Therefore, it has a key role in growth and development of the actively dividing meristematic tissues such as root and shoot tips as well as pollen tube growth and elongation. Ca^{2+} is also used as a second messenger to elicit plant responses to diverse stimuli, including many

biotic and abiotic signals[47,50-53]. Indeed, Ca^{2+} is the most prominent messenger in plants. Almost every signal including developmental, hormonal, and stress signals causes changes in plant cellular Ca^{2+} [54].

Calcium has been evolved as a second messenger because of its unique chemical and physical properties[54]. Compared to any other divalent ion, Ca^{2+} is a faster binding agent and can easily interact with proteins and organic acids[55]. Because of its ability to form different coordination bonds (from six to nine), Ca^{2+} has a high-affinity for carboxylate oxygen, rapid binding kinetics, and complex geometries[56,57]. Because of this, high concentrations of Ca^{2+} are toxic to the cell as it will cause precipitation of phosphates and trigger aggregation of proteins and nucleic acids as well as affecting the integrity of plasma membranes[57]. As a result, plants have evolved multiple mechanisms to keep the cytosolic free calcium $[Ca^{2+}]_{cyt}$ at a very low level (submicromolar). In a typical plant cell, free Ca^{2+} concentration in the cytoplasm is 100–200 nM compared to 10 mM in the apoplasm, 0.2–10 mM in the vacuole, 1 mM in the endoplasmic reticulum, and 2–6 μM in chloroplast stroma[56,58]. The requirement of this tight control of cellular Ca^{2+} concentration has paved the way for the emergence of Ca^{2+} signaling.

The extremely low $[Ca^{2+}]_{cyt}$ concentration provides a unique cellular environment in which Ca^{2+} concentration can be rapidly and highly elevated upon sensing stress. By using large electrochemical potentials either at the plasma or organelle membranes the $[Ca^{2+}]_{cyt}$ concentration can be raised up to 10 or 20 fold within few seconds[59]. The function of Ca^{2+} as a second messenger in plants has been first documented in the green algae Chara[60] followed by many reports demonstrating transient elevation in plant $[Ca^{2+}]_{cyt}$ in response to almost every known biotic and abiotic stress including osmotic, salinity, drought, anoxia, soil acidity, ozone, oxidative, heat and cold stresses as well as gaseous pollutants, mechanical cues, light, plant hormones, bacterial and

fungal pathogens[51,61-72]. However, the changes in Ca^{2+} levels elicited by each environmental stress and developmental cue is unique, which elicits an appropriate physiological response to each stimulus. This specificity is determined by the magnitude and duration of Ca^{2+} elevation as well as its subcellular location (e.g., cytosol, nucleus, organelles), and whether a single Ca^{2+} transient or multiple repetitive spikes occur. The magnitude of $[Ca^{2+}]_{cyt}$ elevations is highly specific and dependent on the external stimulus as was demonstrated for salinity[73], ozone[74], hypo-osmotic shock[75], H_2O_2[76], and high temperatures[77,78].

Originally, the cytosol was thought to be the only site involved in Ca^{2+}-dependent processes and other organelles were considered to be storage compartments out of which Ca^{2+} is released. However, it is now clear that Ca^{2+}-regulated processes also occur in different cellular organelles[59,79]. Calcium signals can also be generated from the organelles surrounded by a double membrane including mitochondria, chloroplasts and nuclei[79]. Additionally, changes in Ca^{2+} levels in specific subcellular organelles/compartments (e.g. the vacuole, nucleus, mitochondria, chloroplasts, surrounding nuclear envelope or combinations of the aforementioned stores) in response to stimuli has been documented[59]. In addition to the magnitude and subcellular location of Ca^{2+} elevation, the number of spikes as well as its duration and the lag time between the spikes could encode specific stimuli information and vary depending on the type of stress experienced and the severity[51,73,80-83]. The specific pattern of cellular Ca^{2+} change that is characteristic for a particular stimulus is termed Ca^{2+} signature[84].

The specific "Ca^{2+} signatures" are formed by the activities of the Ca^{2+} channels, pumps, and exchangers present at the membranes. The activities of these channels are tightly controlled to maintain Ca^{2+} homeostasis and to bring rapid signal-specific changes in cellular Ca^{2+} in response to different stimuli[85-87]. Internal and/or external Ca^{2+} stores can be involved in $[Ca^{2+}]_{cyt}$ elevation

depending on the type of stimuli or the type of cell[86,87]. A Ca^{2+} permeable ion channel mediates Ca^{2+} influx to the cytosol either from the apoplast, across the plasma membrane, or from the intracellular organelles causing increase in $[Ca^{2+}]_{cyt}$ concentration. After the signaling event, the restoration of $[Ca^{2+}]_{cyt}$ to normal level requires active efflux to export Ca^{2+} from the cytosol against its electrochemical gradient to either the apoplast or the intracellular organelles. This is achieved by two Ca^{2+} efflux mechanisms, P-type Ca^{2+}-ATPases and the Ca^{2+}/proton antiporter systems, which occur at the plasma membrane and endomembranes. While Ca^{2+}-ATPases are high-affinity (Km = 0.1-2 μM) but low-capacity transporters, the antiporters are low-affinity (Km = 10-15 μM) but high-capacity transporters. This suggests that the antiporters are involved in the removal of Ca^{2+} after signal mediated influx, while Ca^{2+}-ATPases are involved in the maintenance of the low resting concentration of Ca^{2+} [88,89].

Decoding of Ca^{2+} Signature

The magnitude, kinetics and spatio-temporal distribution of Ca^{2+} elevations are of critical importance to determine specificity and for stimulus response coupling[82]. In the Ca^{2+} signaling pathway, the specificity is likely achieved by Ca^{2+} binding proteins that function as signal sensors[90]. The Ca^{2+} sensor proteins elicit the appropriate physiological responses to a given signal by decoding the information represented in the specific Ca^{2+} signatures and translating it into specific protein-protein interactions, phosphorylation cascades, or transcriptional responses[91-93]. Therefore, the specificity of Ca^{2+} signaling is achieved by the dynamic interplay between Ca^{2+} signatures and Ca^{2+} sensing proteins.

Plants have about 300 hundred Ca^{2+} binding proteins that can sense changes in cellular Ca^{2+} and regulate downstream targets eliciting a stress-specific physiological response[52,94,95]. Many of these Ca^{2+} sensors are coded by multiple genes and their expression is induced by

stresses[52]. Further, the majority of these Ca^{2+} sensor proteins possesses classical helix-loop-helix EF hand motif that binds Ca^{2+} ion[94,96]. The number of EF-hands ranges from one to six in different Ca^{2+} sensors. Several one, two, and three EF-hand–containing proteins show Ca^{2+} binding at physiological concentrations[97]. Binding of Ca^{2+} leads to conformational changes in the sensor proteins that promote either their own catalytic activity or their interaction with target proteins modulating their functions which in turn regulate a plethora of cellular processes, including ion transport, metabolism, post-translational protein modifications and gene expression.

Ca^{2+} sensors are divided into three families: i) calmodulin (CaM) and calmodulin-like proteins (CMLs), ii) Ca^{2+}-dependent protein kinases (CDPKs) and iii) calcineurin B-like proteins (CBLs)[52]. While CaM is highly conserved in all eukaryotes, CML, CDPK and CBL proteins are specific to plants and some bikont protists[94,98,99]. Plant Ca^{2+} sensor proteins have been classified into two groups, sensor relays and sensor responders based on how they function[87,92]. The sensor relays do not have any known enzymatic or other functional domains. However, upon binding to Ca^{2+}, they undergo conformational changes, which trigger their interaction with other proteins and regulate their activities. This group include CaMs (with one exception CaM7), CaM-like proteins (CMLs), and calcineurin B-like proteins (CBLs)[52,91,97,100,101]. CaMs and CMLs interact with diverse proteins, whereas CBLs interact with a specific family of protein kinases called CBL-interacting protein kinases (CIPKs)[102-105]. On the other hand, sensor responders contain a catalytic domain in addition to the EF-hands. Binding of Ca^{2+} to the EF-hand motif regulates the activity of the catalytic domain. Ca^{2+}-dependent protein kinases fall into this group[94,99]. CDPKs are sensor responders because they combine a Ca^{2+} sensing domain (EF hand motifs) and a catalytic domain (protein kinase activity) within a single protein. These proteins sense Ca^{2+} signatures and translate it into phosphorylation events of specific target proteins. In contrast to that, CaMs, CMLs, CBL

proteins belong to sensor relay proteins due to the absence of any catalytic activity. However, as CBLs interact specifically with a family of protein kinases called CBL-interacting protein kinases (CIPKs), CBL-CIPK complexes could be considered as bimolecular sensor responders[106].

In addition to EF-hand–containing Ca^{2+} binding proteins, plants also possess Ca^{2+}-binding proteins that do not have this EF-hand motif. Examples of these proteins include calnexin, calreticulin, annexins, and C2 domain-containing proteins[97,107-109]. Annexins are sensor responders as they function as enzymes and contain other functional domains. Annexins have been documented to be key regulators of several plant stress responses[108]. Under salt stress, these proteins can undergo Ca^{2+}-mediated relocation from the cytosol to membranes[110], where they exert their enzymatic functions (e.g., peroxidase activity) or create Ca^{2+}-permeable transport pathways[111,112]. On the other hand, calreticulins are involved in brassinosteroid signaling, whereas calnexins are involved in drought stress responses[113,114]. This extended set of Ca^{2+} sensor proteins with diverse Ca^{2+} affinities, subcellular localizations and downstream target specificities likely increases the complexity of Ca^{2+} signaling in plants, and thereby allows plants to elicit appropriate response to changes in the environment.

Translating Ca^{2+} Signatures into Phosphorylation of Signaling Proteins

Calcium and phosphate ions represent the two major currencies of signal transduction in the cell. Ca^{2+} binding to sensor proteins changes their charges and confirmations. Similarly, phosphorylation of proteins imparts a negative charge, changing protein confirmations and interactions[115-117]. Ca^{2+}-dependent protein kinases and protein kinases regulated by interaction with Ca^{2+} binding proteins combine these two cellular currencies of signal transduction and translate Ca^{2+} signatures directly into phosphorylation events which in turn regulate downstream signaling responses[118,119]. Plants possess three families of Ca^{2+}-regulated protein kinases,

Ca^{2+}/CaM-dependent protein kinases (CCaMKs), CDPKs, and CBL-interacting protein kinases (CIPKs)[92,119,120]. CDPKs and CCaMKs (the later appear not to exist in the Arabidopsis genome) are sensor responders, whereas the CIPKs are targets of CBLs, which are sensor relay proteins[98,104].

The CDPK Signaling System

Calcium-dependent protein kinases (CDPKs), also designated as CPKs, are unique Ca^{2+}-regulated kinases present in plants and apicomplexa[121]. The Arabidopsis genome encodes 34 CDPKs beside eight CDPK-related kinases[122]. In addition to the kinase domain, CDPKs have CaM-like domain, which has four EF hand motifs for Ca^{2+} binding[123,124]. In all CDPKs the kinase and CaM-like domain are separated by a junction domain (autoinhibitory domain). At basal levels of Ca^{2+}, the autoinhibitory domain physically interacts with the kinase domain blocking its active site. Upon elevation of cellular Ca^{2+} concentration, Ca^{2+} binds to the CaM-like domain and triggers conformational changes leading to the displacement of the autoinhibitory domain and activation of the kinase[125]. The displacement of the autoinhibitory domain is usually followed by autophosphorylation for full activation of the CDPKs[125,126]. Additionally, the several CDPKs have been reported to be modified by two lipid modifications, myristoylation and/or S-acylation, at their N-termini. These two modifications have a role in membrane attachment and localization of these proteins[127,128]. Furthermore, CPK4 from Arabidopsis has been detected in various locations inside the cell including the cytoplasm, nucleoplasm, the cytoskeleton, the endoplasmatic reticulum and peroxisomes[129-131]. Thus, CDPKs can regulate diverse targets in various cellular contexts.

The CDPKs family is involved in the regulation of a wide range of physiological and developmental processes[127,132-135]. It has been documented to have a role in regulating ion transport processes involving the regulation of Ca^{2+} and potassium homeostasis[136-138]. Also, it has a function in regulating carbon and nitrogen metabolism[139-141] as well as salt and drought stress responses[136].

Functions of CDPKs were also established in plant responses to pathogens, while CDPK2 and 3 in tobacco are activated in response to pathogens like *Cladosporium fulvum*[142,143] and are involved in the adaptive regulation of the transcriptome[144]. Additionally, CDPKs can modulate MAP kinase pathway, which has a key role in pathogen response[145]. It also activates NADPH oxidases by phosphorylation, which results in production of reactive oxygen species that function in plant defense[146-148]. Moreover, CPK1 from Arabidopsis induces the production of SA for resistance[149].

CDPKs are also involved in additional mechanisms including signaling and mediating plant responses to hormones. For example, CPK4, CPK11 and CPK32 phosphorylate ABA-responsive bZIP transcription factors like ABF1 and ABF4[150,151], which mediate ABA signaling during drought and salt stress[152]. Additionally, in response to gibberellic acid, CDPK1 in Tobacco phosphorylates the transcriptional activator Repression of Shoot Growth (RSG) at Ser-114, which enables RSG binding of 14-3-3 proteins. Binding of 14-3-3 proteins to RSG triggers its translocation from the nucleus into the cytosol thereby repressing its function[153]. Interestingly, these Ca^{2+} dependent kinases can also counteract the function of other Ca^{2+} sensor proteins. For example, the CPK1 can phosphorylate the Ca^{2+} ATPase ACA2 within the N-terminal regulatory domain and inhibit its activity[154]. Whereas, the activation of the ACA2 depends on binding of Ca^{2+} activated CaM[155]. In this way, different Ca^{2+} sensors fine-tune the speed of Ca^{2+} extrusion out of the cell, thereby contributing to the generation of a specific Ca^{2+} signature.

The CBL/CIPK Signaling System

Calcineurin B-like proteins (CBLs) are small Ca^{2+}-binding proteins that interact specifically with the serine-threonine kinases known as CBL-interacting protein kinases (CIPKs)[156,157]. The Arabidopsis genome encodes 10 CBL proteins and 26 CIPK proteins[104]. CBL proteins and their interacting protein kinases (CIPKs) were first identified in Arabidopsis[156,157].

Subsequently, 10 CBLs and 30 CIPKs were identified in the genome of rice[104,158]. The multiplicity of CBL and CIPK proteins helps to generate specificity in the Ca^{2+} signaling pathway. Preferential interaction of distinct CBLs with specific CIPKs governs the regulation of specific physiological targets[119,158,159]. In contrast to the CDPK sensor responders, the CBL-CIPK signaling network consists of two modules in which the CBL proteins act as sensor relay while the CIPKs provide the response activity[105,160]

All CBL proteins harbor four EF hand Ca^{2+} binding sites which are arranged in completely invariant spacing within the protein[158]. On the other hand, plant CIPKs consist of kinase domain at their N-terminal part and a junction domain (asparagine–alanine–phenylalanine domain, also referred to as NAF domain) as well as additional functional domains at the C-terminal part[157,159]. The NAF-domain is evolutionary conserved and functions as an auto-inhibitory domain which block the active site of the kinase domain[98,159]. Binding of CBL proteins to the NAF domain displaces it and releases active kinase domain[161,162]. The activation process of CIPKs is further enhanced by autophosphorylation as well as trans-phosphorylation by an unidentified kinase[161,162]. Recently, phosphoproteomic screens have identified two MAP kinases as potential kinases of CIPK[163]. At the same time, CIPKs phosphorylate their interacting CBLs, which enhances the CBL-CIPK interaction[164,165].

Forward and reverse genetic screens indicated that CBLs and CIPKs contribute to plant responses to different stresses including salt, cold and drought stresses[166-171]. Analysis of *cbl1* loss-of-function mutants revealed that CBL1 has a key role in the regulation of plant responses to abiotic stresses like drought, cold, and salt[169,172]. Additionally, forward genetic screens aiming to identify critical components of plant salt tolerance have identified a key role of the CBL Ca^{2+} sensor SOS3 (CBL4) and the CIPK-type kinase SOS2 (CIPK24) in salt stress adaptation by

regulating the Na^+/H^+ antiporter SOS1[166-168]. CBL4-CIPK24 (SOS3-SOS2) complexes at the plasma membrane to activate the H^+/Na^+ antiporter SOS1 and thereby contributes to the regulation of Na^+ extrusion[166-168,173,174]. Also, CBL10 mutant shows enhanced sensitivity of plants to salt stress[171,175]. Lee *et al.*, (2009) have indicated a critical role for another CIPK (CIPK15) in O_2 deficiency tolerance in rice under flooded conditions[176].

Furthermore, Xu *et al*, (2006) established a role of the CBL/CIPK system in regulating K^+ homeostasis by phosphorylating ion channels[177]. Consistently, the CBL3/CIPK9 complex has been reported to function in regulating K^+ homeostasis under low-K^+ stress[178]. Moreover, the kinase CIPK23 can be targeted to the plasma membrane by CBL1 and CBL9[177,179], where it can regulate the activity of the Shaker-like potassium channel AKT1[177,180,181]. Furthermore, CIPK23 can also regulate the activity of the plasma membrane nitrate transporter CHL1 (also named NRT1.1)[182]. The CBL/CIPK system also contributes to plant responses to the phytohormone ABA[179,183-185]. CBL9/CIPK3 complex was found to be involved in modulating ABA responses[184]. In concert with that, a loss of function mutant of *CBL9* renders plants hypersensitive to ABA[185]. Additionally, CBL-CIPK participates in the regulation of polar growth processes including pollen germination and tube elongation[186]. CBL1 and CBL9 were found to be essential for proper pollen germination and pollen tube growth.

Converting Ca^{2+} Signals into Transcriptional Responses

Precise regulation of gene expression to express the right genes in the right cells at the right time is critical for growth and development as well as environmental responses. Almost all stresses are coupled with reprogramming of gene expression in plants to enhance stress signaling and adaptation[187-192]. In this process, transcription factors (TFs) represent the master-switches that target stress-responsive genes and regulate their expression[193,194]. The fact that the signal-specific

changes in cellular Ca^{2+} always followed by signal-induced changes in gene expression proposed that Ca^{2+} is likely to mediate these changes in gene expression[51,195-197]. Additionally, the localization of several Ca^{2+} sensors (e.g. CaMs, CDPK3, and CDPK4) to the nucleus as well as the translocation of others to the nucleus in response to stresses (e.g., At-CDPK2 in response to osmotic stress and Mc-CDPK1 in response to salt stress) suggest a role for these proteins in regulating gene expression[131,198-202]. In this regard, the elevated levels of cytosolic and nuclear Ca^{2+} in response to stress have been demonstrated to modulate gene expression[187,196,203,204]. Moreover, artificial Ca^{2+} manipulations have been found to change the expression of numerous stress responsive genes[196]. However, the mechanisms by which Ca^{2+} mediates regulation of gene expression in response to stresses are not well understood[205].

The conversion of Ca^{2+} signatures into transcriptional regulation may be achieved by several pathways. Calcium can bind directly to some TFs, such as the downstream regulatory element antagonist modulator (DREAM) proteins, and modulate their activities which in turn regulate gene expression[206]. Furthermore, activated Ca^{2+} sensors can bind to *cis*-elements in the promoters of specific genes and regulate their expression directly[207]. Also, activated Ca^{2+} sensors can bind to or phosphorylate DNA binding TFs and activate or inactivate them which in turn will activate or suppress downstream genes[208].

Ca^{2+}-Binding Transcription Factors

Calcium can regulate gene expression by binding directly to some TFs. *Arabidopsis thaliana* NaCl-inducible gene 1 (*AtNIG1*) was the first identified Ca^{2+}-binding TF[209]. AtNIG1 harbors an EF-hand motif at its N-terminal region and a bHLH domain at its C-terminal region. AtNIG1 has a key positive role in regulating salt stress response[209]. It binds to the canonical E-box element (CANNTG) in the promoter region of several salt stress-responsive genes and regulates

their expression[210,211]. Additionally, one of the Arabidopsis CaM isoforms, CAM7 (also named ZBF3) was identified as Ca^{2+}-binding TF[212-214]. CAM7 was found to interact directly with promoters of genes involved in seedling development[212]. CAM7 participates in the Ca^{2+}-mediated light-signaling pathway by regulating the expression of light-responsive genes and photomorphogenesis. It binds directly to Z-/G-box elements (ATACGTGT/CACGTG) located in the promoter of light-responsive genes, including *CAB1* and *RBCS1A*, and triggers their expression[212]. The binding of Ca^{2+} to the EF-hand motif of CAM7 leads to a conformational change of this region, which, in turn, enables the DNA-binding activity of CAM7[213,214].

Ca^{2+}-Mediated Transcriptional Regulation via Phosphorylation/Dephosphorylation

The regulation of TFs by reversible phosphorylation is a key post-translational modification that can alter their stability[215], translocation[216], oligomerization, DNA-binding affinity[217] and interaction with other proteins[150]. Protein phosphorylation has been demonstrated to be a key mechanism involved in the regulation of ABA and stress-responsive pathways in plants[218-222]. In response to ABA and stress-induced signals, several bZIP factors are phosphorylated, mostly by CDPKs, which in turn induces expression of ABA-responsive genes[152,220,223]. The basic leucine-zipper (group A bZIP) TFs (ABFs) are the key factors mediating ABA-regulated gene expression by binding to the ABA-response element (ABRE) located in the promoters of many ABA-responsive genes[152,224]. The activation of CDPKs by Ca^{2+} elevation in response to many biotic and abiotic stresses[123,225,226] as well as the phosphorylation of bZIP factors by CDPKs suggest that CDPKs might decode Ca^{2+} signals and enhance plant adaptation to stresses, as well as plant growth and development through the ABA-signaling pathway[135,150,151,227,228]. Growing evidence indicates that phosphorylation of Ser residues at the N-terminus of bZIP TFs is essential for ABA-induced activation of gene expression. In this regard, AtCPK32 was found to

interact and phosphorylate a highly conserved serine (Ser^{110}) of the ABA-induced TF ABF4 *in vitro*[150]. Choi *et al*., (2005) established that AtCPK32 is a positive regulator of ABF4 function and the phosphorylation of ABF4 by AtCPK32 is essential for ABF4 activity and ABA-dependent transcriptional regulation[150]. Moreover, the phosphorylation of ABF1 by AtCPK4 and AtCPK11 positively regulates ABA signaling[151,221]. Additionally, AtCPK4 and AtCKP11 may regulate stomatal aperture by phosphorylating ABF1 and ABF4[151].

Ca^{2+}-dependent phosphorylation is responsible for modulation of the DNA-binding activity of GT-1, a *trans*-acting factor[229]. GT-1 is a light-modulated DNA-binding protein interacting with BoxII in *rbcS-3A* promoter. Analysis of recombinant GT-1 mutants demonstrated that phosphorylation of Thr-133 is accountable for a 10–20-fold stimulation of DNA-binding activity. Treatment with calf intestine alkaline phosphatase of extracts prepared from light-grown plants reduced the GT-1 DNA-binding activity, suggesting that GT-1 may act as a molecular switch modulated by Ca^{2+}-dependent phosphorylation in response to light signals[229].

Ca^{2+}/CaM-Mediated Transcriptional Regulation

CaM is a small (17 kDa), acidic, highly conserved Ca^{2+} sensor in eukaryotes[230-233]. Multiple forms of CaM are reported in plants[100,230,232,234]. One mechanism by which plant cells may transduce Ca^{2+} signals to elicit specific physiological responses involves the differential expression of these CaM isoforms. In Arabidopsis, there are seven genes encoding four CaM isoforms (CaM1/4; CaM2/3/5; CaM6; CaM7) that differ only in one to five amino acid residues, which likely determine target specificity[230,232,235-238]. In addition to CaM, plants also contain several CaM-like (CML) proteins that show 16 to 75% amino acid identity with CaM but exhibit some structural divergence and harbor different numbers of EF hands ranging from 1 (CML1) to 6 (CML12)[100]. In Arabidopsis genome, 50 isoforms of CML have been identified[100]. CaM is

localized mainly in the cytosol but also can be found in in peroxisomes, plastids, mitochondria, the extracellular matrix, and nuclei[187,198,200,239-247], indicating potentially diverse roles for it.

Calmodulin consists of two globular domains connected by a flexible helical region. Each of the globular domains has a pair of EF-hands. Binding of Ca^{2+} to the EF-hands triggers conformational changes, which result in the exposure of hydrophobic surfaces that form high-affinity binding sites for downstream target proteins[100,231,248,249]. In the target proteins, CaM binds to a small region composed of a stretch of 16-35 basic and hydrophobic amino acids that form a basic amphiphilic-helix[249]. The CaM-binding domain (CaMBD) that is often encountered binds to CaM in the presence of Ca^{2+}. However, another type of CaMBD named the IQ motif, which binds CaM in the absence of Ca^{2+} and dissociates in its presence was also identified[249-251].

A large number of CaM-binding proteins with diverse cellular functions have been identified by screening cDNA expression libraries with labelled CaM[244,252-254]. CaM binding proteins were also identified by probing protein chips containing proteomes representing mostly TFs and signaling proteins with CaMs/CMLs[255]. The CaM-binding proteins include several kinases, phosphatases, ion transporters, cytoskeleton-associated proteins, metabolic enzymes and TFs[252,254,256] indicating that CaM regulates a wide variety of cellular events. Accordingly, molecular and genetic studies have demonstrated specific roles of CaM/CMLs in diverse aspects of plant development and stress responses by converting Ca^{2+} signals into transcriptional responses, protein phosphorylation or metabolic changes[235]. Consistent with this hypothesis, it has been shown that environmental challenges rapidly up-regulate the expression of different CaM isoforms in various plants. For example, the expression of CaM genes is differentially up-regulated by touch[257-259], cold shock, wind[187], wounding, pathogens[260,261], auxin and salinity[262]. Additionally, various studies have revealed involvement of CaM in regulation of metal ions uptake[263,264],

generation of reactive oxygen species[247,265] and modulation of several kinases/ phosphatases[120,266,267] as well as TFs[268-271]. Also, CaM has been implicated in Ca^{2+}-dependent responses to light, gravity, mechanical stress, phytohormones, pathogens, osmotic stress, salinity, heavy metals, xenobiotics, anoxia, oxidative stress, heat shock and chilling[50,231,259,272,273].

CaMs/CMLs play important roles in gene regulation. Ca^{2+}/CaM can interact with target proteins in the cytosol and induce their transduction to the nucleus, which subsequently transduces a signal to the nucleus. In addition, Ca^{2+}/CaM can interact with target proteins in the nucleus itself[231]. Environmental signals have been shown to alter the distribution of petunia CaM53[274] and rice OsCaM61[243] between the plasma membrane and the nucleus by influencing their prenylation. Transcription factors are among the target proteins for Ca^{2+}/CaM in plant cell nuclei[244,268-271]. Several TFs such as calmodulin binding transcription activators (CAMTAs; also referred to as signal-responsive proteins, SRs), WRKYs and MYBs are regulated by direct interaction with CaMs[275-277]. Also, CaMs can regulate TFs indirectly. For example, an Arabidopsis CaM-dependent kinase (AtCBK3) and a CaM-dependent phosphatase (AtPP7), which are activated by CaM binding, can subsequently regulate heat shock TFs positively by phosphorylation or negatively by dephosphorylation[266,278]. Moreover, the TGA3 TF, a member of the basic leucine zipper TF family, was first identified by its physical interaction with Ca^{2+}-loaded CaM[270,279]. The binding of the Ca^{2+}/CaM complex to TGA3 enhances its DNA-binding activity. On the other hand, the TGA3 protein binds to the C/G-box sequence elements found in the promoter of the Arabidopsis CaM isoform CaM3. In addition to TGA3, another 17 bZIP family members were identified as CaM binding proteins[279].

Additionally, Ca^{2+}/CaM also regulates MYB TFs, which are known to be involved in the regulation of several aspects of plant growth and development[280]. Several members of the MYB

class of TFs were found to bind Ca^{2+}/CaM[279]. Moreover, The DNA-binding and transcriptional activities of the R2R3- MYB2, an upstream regulator of salt stress and dehydration responsive genes, was found to be enhanced by interaction with a salt stress induced isoform of soybean (*Glycine max*) CaM (GmCaM4) in a Ca^{2+}-dependent manner[281-284]. The accumulation of GmCaM4 protein in response to salt stress enhances the expression of MYB2 target genes that encode protective proteins such as *PYRROLINE-5-CARBOXYLATE SYNTHASE 1* (P5CS1), *ALCOHOL DEHYDROGENASE 1* (ADH1), and *RD22*, which in turn confers salt tolerance[281]. On the other hand, another CaM isoform, GmCAM1 was found to inhibit the DNA-binding activity of R2R3-MYB2 indicating differential and specific roles of CaM isoforms. Overexpression of *GmCaM4* in Arabidopsis resulted in a concomitant increase in salt stress tolerance, whereas overexpression of *GmCAM1* had no effect[281]. The simultaneous positive and negative regulation of biological responses by Ca^{2+}/CaM/CMLs suggests that these proteins are important for dynamically fine-tuning different responses. Interestingly, the expression of *GmCaM4* is also induced by pathogens. Constitutive expression of *GmCaM4* in tobacco plant enhances plant resistance to bacterial, fungal and viral pathogens by inducing the expression of wide range of defense-related genes[260]. Moreover, other MYBs including MYB62 and MYB78 also bind to GmCaM1 and GmCam4[281].

Ca^{2+}/CaM pathway is also involved in the regulation of the WRKY family of TFs. The WRKYs mainly regulate immune responses[285], abiotic stress adaptation as well as growth and development of plants[286,287]. One of the Arabidopsis WRKY family members, AtWRKY7, was recently reported to interact with CaM in a Ca^{2+}-dependent manner via a conserved region called the C-region in the N-terminal part of the protein[288]. WRKY7 is induced in response to pathogens and SA and negatively regulates plant defense responses[288]. Interestingly, The CaMBD present in the AtWRKY7 is distinct from the classical CaMBDs described until now and is conserved in

several WRKY proteins including WRKY11, 15, 17, 21, 39, and 74 which were also found to interact with Ca^{2+}/CaM[288,289]. Additionally, a global analysis of Ca^{2+}/CaM binding proteins in Arabidopsis using protein microarrays has identified several additional WRKYs (WRKY43, 45, 50, and 53) that interact with different isoforms of CaM in a Ca^{2+}-dependent manner[279].

Moreover, a plant-specific family of CaM binding proteins called CaM Binding Protein 60 (CBP60) was first isolated from maize[290] and then from tobacco[291], Arabidopsis[244], and bean[292]. CBF60 proteins are differentially expressed in response to biotic stresses and elicitors of plant defense[292,293]. One of the members of this family, CBP60g, interacts with Ca^{2+}/CaM and induces the expression of Iso-Chorismate Synthase 1(ICS1), an enzyme involved in SA synthesis. Thereby, Ca^{2+}/CaM activated CBP60g enhances plant defense response by triggering SA biosynthesis[293,294]. Also, CBP60g-overexpressing Arabidopsis plants show increased tolerance to drought stress[294]. Another class of TFs with Ca^{2+}/CaM-regulated members is the trihelix GT element binding proteins (GTLs)[295,296]. One GTL family member, GTL1 (GT-2 LIKE 1), a negative regulator of water use efficiency and drought stress, was found to interact with Ca^{2+} activated CaM[297,298]. Another example of transcriptional regulation by CaM/CML proteins is provided by the Ca^{2+}CaM/CML binding to the nuclear protein IQD1 (IQ-domain 1) that regulates the expression of glucosinolate genes, which are involved in plant defense against pathogen or herbivory attack[299].

The Ca^{2+}/CaM complex also has a role in transcriptional regulation during plant development by regulating NAC domain [a region conserved in NAM (No Apical Meristem), ATAF (Arabidopsis Trancription Activation Factor, and CUC (Cup-Shaped Cotyledon) proteins] -containing TFs. NAC proteins have been reported to play a key role in the development of embryos, shoot apical meristems, and floral organs as well as in auxin-mediated lateral root formation. They also have a crucial role in plant adaptation to biotic and abiotic stresses[300-302]. A

NAC domain-containing TF, termed CBNAC (CaM-Binding NAC Protein) was identified recently as a CaM-binding protein *via* the screening of an Arabidopsis cDNA expression library. CBNAC acts as a transcriptional repressor of target gene expression and has a CaM-binding domain at its C-terminus, which binds to CaM in Ca^{2+}-dependent manner. Binding of CaM enhances the transcription repressor activity of CBNAC[303]. Together these findings indicate a crucial role for the Ca^{2+}/CaM regulated pathways in the regulation of gene expression in response to developmental as well as biotic and abiotic stresses signals in plants.

Calmodulin-Binding Transcription Activators (CAMTAs)

Calmodulin-Binding Transcription Activators (CAMTAs; also referred to as Signal-Responsive Proteins, SRs) are a small family of highly conserved TFs. This family of TFs was first identified in plants in a screen for CaM binding proteins and was named CAMTAs (also called EICBPs or SRs) to emphasize their CaM-binding property[247,268,269,271]. After first being identified in Arabidopsis and tobacco[247,269,271] the CAMTA family has been identified in various plant species including[268], rice (*Oryza sativa* L.)[304], sorghum[305], rapeseed[306], tomato (*Solanum lycopersicum*)[307], grapevine (*Vitis vinifera*)[308], soybean (*Glycine max*)[309], *Zea mays*[310], and Medicago (*Medicago sativa*)[311]. Additionally, CAMTAs have been also identified in humans, Drosophila and worms[275,312,313].This family of TFs is evolutionarily conserved from plants to humans at the sequence level, which suggest their essential role in the cell[247,268,275,304]. CAMTAs may act as transcription activators, as demonstrated by yeast one-hybrid analysis[268], and by *trans*-activation in plant protoplasts[304] and cell cultures[314], as well as stress-induced expression and activation *in planta*[310,315]. However, CAMTAs may also act as transcription repressors *in planta*[316].

All CAMTAs have nuclear localization signals (NLSs - bipartite signal) and are localized to nucleus. In Arabidopsis, CAMTAs have only one NLS at the N-terminus[247]. However, in

OsCBT (CAMTA ortholog in rice) there are two NLSs, one at the N-terminus and one at the C-terminus[304]. Additionally, members of SR/CAMTA family possess at their N-terminal a sequence-specific DNA-binding domain called CG-1, which binds to CGCG or CGTG core motifs. The core CGCG sequence was first identified as a binding site for a TF isolated from a parsley (*Petroselinum crispum*) cDNA library, giving the name CG-1 to the DNA binding domain of protein interacting with this motif[317]. Further analysis of the *cis*-element for this family of TFs in different species identified two basic core CAMTA binding motifs, CGCG and CGTG[247,304]. The CGCG-core consensus motif is (A/C)CGCG(C/G/T), whereas the CGTG-core consensus motif is (A/C)CGTGT[247,315,316,318,319]. Interestingly, the core DNA binding motif CGCG is part of a rapid stress response element (RSRE - *VCGCGB*), which was previously established as a functional general stress response (GSR) motif[320,321]. The RSRE is a *cis*-element enriched in the promoters of genes that are rapidly induced in response to diverse stresses, including biotic (herbivory by *Pieris rapae*, and infection by *Botrytis cinerea*) and abiotic (mechanical wounding and cold) stresses[320]. Together, these data suggest a role of CAMTAs in biotic and abiotic stress responses *via* binding to RSRE element and regulating downstream genes expression. Recent genetic screens confirmed that CAMTA3 is an important component in RSRE-driven gene expression[322,323]. Also, CGCG *cis*-element was identified in the promoters of a large number of rapidly (≤5 min) stress-induced genes, suggesting CAMTA proteins as early stress sensors[320].

Additionally, the consensus motif (A/C)CGTGT encompasses in its sequence a classical ABA-responsive element (ABRE motif, ACGTGT). The ABRE motif is found in the promoters of genes involved in ABA signaling and is recognized by bZIP proteins[324]. ABA is mainly involved in regulating plant tolerance to drought stress by regulating stomatal aperture and root growth as well as by activating Ca^{2+} signaling under drought stress[190]. Collectively, these data

suggest a role for CAMTAs in the regulation of drought stress response by modulating the expression of ABA responsive genes *via* binding to CGTG boxes in their promoter regions. A microarray analysis of drought-treated *camta1* mutant showed that CAMTA1 positively regulates the expression of many ABA-related genes[325].

In addition to the CG-1 domain, CAMTAs contain a transcription factor immunoglobulin (TIG) domain involved in non-specific DNA binding and several ankyrin repeats that confer the ability to interact with other proteins. Also, CAMTAs possess two different types of CaM-binding domains; a Ca^{2+}-dependent CaM-binding domain (CaMBD) and a Ca^{2+}-independent CaM-binding domain, named the IQ motif[93,247,268,316]. The role of CaMBD in regulating the activities of CAMTAs has been characterized in various CAMTA members[150]. For example, by co-expressing OsCBT with a CaM gene in protoplasts, Choi *et al.*, (2005) showed that Ca^{2+}/CaM binding to the CaMBD negatively regulates the transcriptional activity of OsCBT[150]. On the other hand, Ca^{2+}/CaM binding was found to be required for the transcription repressor activity of CAMTA3[315,316]. However, the role of IQ motif in the function of CAMTAs still to be elucidated. The binding of CaM to the IQ motif in Ca^{2+}-free conditions was only verified in an *in-vitro* CaM overlay assay using the rice CAMTA homolog, OsCBT[304].

In Arabidopsis, there are six CAMTAs (CAMTA1 to CAMTA6), which are differentially expressed in response to multiple stresses[247,326]. For example, *CAMTA1* and *CAMTA3-6* transcript levels are induced upon cold and heat treatment, whereas *CAMTA1-4* and *CAMTA6* are induced by salinity[247]. Also, *CAMTA* genes responds differentially to phytohormones and secondary messengers mediating plant responses to biotic and abiotic stresses, such as abscisic acid (*CAMTA2* and *CAMTA4-6*), methyl jasmonate (*CAMTA1, 3,* and *4*), ethylene (*CAMTA1, 3,* and *4*), H_2O_2 (*CAMTA2-6*), salicylic acid (*CAMTA2* and *CAMTA4-6*), and auxin (*CAMTA1*)[247,277,327].

Moreover, the expression of *SlSRs* (CAMTA/SR orthologs in tomato) was found to be developmentally regulated during fruit development and ripening[328]. Also, a large number of stress-related elements have been reported in the promoter regions of some *ZmCAMTA* genes, indicating regulated expression of those genes in response to stress. Additionally, abiotic stresses (drought, salt, and cold), stress-related hormones [abscisic acid, auxin, salicylic acid (SA), and jasmonic acid] as well as biotic stress [rice black-streaked dwarf virus (RBSDV) infection] was found to enhance the expression of *ZmCAMTA* genes[310]. Collectively, the induction of *CAMTAs* in response to these stimuli suggests their involvement in multiple signal transduction pathways and stress responses.

One of the environmental stresses that is considered to be a major cause of losses in crop yields is drought[329]. CAMTAs have been demonstrated to have a role in regulating plant response to water deficiency stress. In this regard, drought stress up-regulates one member of CAMTA family in cotton[330,331] and rice[332] roots as well as tomato[333]. Also, SISR1L, a CAMTA member from tomato was identified as a positive regulator of drought tolerance[333]. Silencing of SlSR1L affects the tolerance to drought and accelerates water loss in leaves. Additionally, in Arabidopsis, CAMTA1 has been demonstrated to be a positive regulator of drought stress response. *camta1* mutant shows higher sensitivity and reduced survivability under drought stress. Pandey *et al*., (2013) established that CAMTA1 probably regulates drought recovery by regulating root development under drought stress, as root growth in the *camta1* mutant was inhibited under stress conditions, which reduced plant survival[325]. Also, microarray analysis revealed that CAMTA1 regulates a wide variety of genes in response to drought stress[325]. Those genes are mainly related to ABA signaling pathway and involved in processes related to potassium import/homeostasis, cell expansion, root hair tip growth, lipid peroxidation, protein phosphorylation and signal transduction.

CAMTA1 acts as a positive and negative regulator of expression of specific TFs, e.g. AP2-domain TFs, ERF13, DRE/CBF2, DRM1 or WRKY33[325]. Also, Galon *et al*., (2010) have investigated the effect of salt stress on the expression pattern of *CAMTA1*. By using *CAMTA1*promoter::GUS line as a visual tool, they reported the induction of *CAMTA1* in the leaves under salt stress suggesting a role for CAMTA1 in salt stress response[319].

Also, the role of CAMTAs in biotic stress responses has been well established. In rice, OsCBT was identified as a negative regulator for plant defenses against both the bacterial pathogen *Xanthomonas oryzae pv. oryzae* and the rice blast fungus *Magnaporthe grisea*[334]. Consistently, CAMTA transcription factors in tomato, SlSR1 and SlSR3L, also negatively regulate plant resistant against bacterial and fungal pathogens[335]. In Arabidopsis, the *camta3/sr1* mutant exhibits enhanced tolerance to both fungal and bacterial pathogens indicating a negative role of CAMTA3 in plant immunity[318]. On the other hand, CAMTA3 was also reported to be a positive regulator for plant defense against insect herbivory in Arabidopsis. Additionally, CAMTA3 acts as a positive regulator of cold-stress response by enhancing the expression of *CBF* genes. CAMTA1 and CAMTA2 and CAMTA5 work in concert with CAMTA3 to regulate CBF genes expression[315]. Recently, a *CAMTA* gene (LOC_Os01g69910) was found to be associated with the phenotype of cold tolerance in rice[336]. Additionally, another member of CAMTA, CAMTA2, has a positive regulatory role in the metal toxicity adaptation processes of plants. Under aluminum (Al) toxicity stress, CAMTA2 activates the expression of aluminum-activated malate transporter 1 (AtALMT1), which encodes efflux transporter for an Al chelator, malate[337,338].

Additionally, CAMTAs may regulate abiotic and biotic stress responses by modulating hormone signaling. For example, one of the CAMTA proteins in Arabidopsis, CAMTA1, has been demonstrated to play a role in auxin signaling in growth and development[319] as well as drought

adaptation[325]. CAMTA1 repressor lines and *camta1* mutants showed enhanced responsiveness to auxin, suggesting that in wild-type plants, enhanced expression of *CAMTA1* in response to stresses suppresses the plant's responsiveness to auxin. A genetic analysis in Arabidopsis revealed that *camta1* mutant lines exhibit enhanced responsiveness to auxin compared to wild type seedlings[327]. Furthermore, transcriptome analysis of *camta1* mutant plants revealed upregulation of 17 genes, which are normally induced by auxin signaling[319]. Thus, CAMTA1 may respond to stresses by suppressing auxin response, which in turn suppress growth and development under stress conditions enabling the plant to divert resources toward stress adaptation[319,339]. Furthermore, Galon *et al*., (2010) performed microarray analysis for all *camta* mutants and showed that *CAMTA1, 2,* and *3* negatively regulate auxin signaling whereas *CAMTA4, 5,* and *6* may play a reverse role by positively regulating it[339].

Another CAMTA in Arabidopsis, CAMTA3 (SR1), participates in ethylene-induced senescence by directly regulating *non-race-specific disease resistance 1* (*NDR1*) and *ethylene insensitive3* (*EIN3*) gene expression[340]. Also, CAMTA3 suppresses SA dependent plant immunity by directly suppressing the expression of *EDS1*, appositive regulator for SA biosynthesis[316,318]. It has been shown that CAMTA1 and CAMTA2 work in concert with CAMTA3 and effectively repress the accumulation of SA by suppressing the expression of SA biosynthesis-related genes, such as *ICS1, CBP60g,* and *SARD1* at warm temperatures rather than at low temperatures[341].

Another member of CAMTAs, CAMTA5, has been reported to be involved in brassinosteroids (BRs) signaling pathway, which regulates plant growth and development[342,343]. Brassinozole resistant 1 (BZR1) and its homolog BZR2 are key transcription factors that regulate the expression of most BR-responsive genes[344,345]. Recently, CAMTA5 has been identified as one of the potential BZR1-associated proteins. Interestingly, CAMTA5 has been reported to regulate

the expression of *CBF2*, which is a BR-repressed and direct target gene of both BZR1 and BZR2 which further supports the involvement of CAMTA5 in the BR signaling pathway and the regulation of target gene expression[343]. All these findings indicate an important role of CAMTAs in regulating biotic and abiotic stress responses. therefore, CAMTA genes may be good candidates for crop breeding.

Signal Responsive 1 (SR1)

Signal Responsive 1 (SR1), also known as CAM-binding Transcriptional Activator 3 (CAMTA3) (for brevity I will use SR1 from now on) is the most studied member of the SR family. It has been reported that SR1 is involved in regulating plant immunity, insect resistance, cold/freezing tolerance and probably other functions[315,316,346]. Moreover, the core DNA binding motif of SR1 is part of a rapid stress response element (RSRE - *VCGCGB*)[320,321]. Recent genetic screens confirmed that SR1 is an important component in RSRE-driven gene expression. SR1 activates the expression of *RSRE:LUC* reporter in a Ca^{2+}-dependent manner[322]. Also, *sr1* mutant exhibited reduced RSRE:LUC activity, suggesting that SR1 is a positive regulator of early stress responses and involved in the expression of many biotic and abiotic stress responsive genes[323].

The plant innate immune system has receptors that recognize Pathogen-Associated Molecular Patterns (PAMPs) and activate the PAMP-Triggered Immunity (PTI)[347]. Successful pathogens can suppress this layer of immunity by delivering effectors into host cells, however, the host cytoplasmic Nucleotide binding Leucine-rich Repeat domain (NLR) receptors recognize these effectors and activate Effector-Triggered Immunity (ETI) leading to local host cell death known as the hypersensitive response (HR)[348,349]. There are two subfamilies of plant NLRs, based on the presence of either an N-terminal Toll/Interleukin-1 Receptor (TIR) or a coiled-coil (CC) domain[348]. Enhanced Disease Susceptebility1 (EDS1) is required for signaling by TIR-NLRs, whereas Non-

Race Specific Disease Resistance1 (NDR1) is important for CC-NLR-triggered HR[350]. Gain-of-function mutations in NLRs[351,352] or loss of genes that act as negative regulators of immunity show autoimmune phenotype including stunted growth, accumulation of ROS, and elevated defense gene expression[352-356].

SR1 negatively regulates plant resistance against a wide range of bacterial and fungal pathogens in Arabidopsis[316,318,340,357-359]. Disease resistance studies with Arabidopsis WT and loss-of-function *sr1* mutants showed that the mutants are more resistance to virulent and avirulent strains of *Pseudomonas syringae (Pst) DC3000* as well as the non-host resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*[316,318,359] Also, *sr1* showed enhanced resistance against the necrotrophic fungi *Botrytis cinereal* and *Sclerotinia sclerotiorum* and the biotrophic fungus *Golovinomyces cichoracearum*[316,357,358,360,361]. Constitutive disease resistance of fungal and bacterial pathogens in a loss-of-function mutant suggests that SR1 is a negative regulator of plant immunity. Also, transgenic plants overexpressing *SR1* exhibited enhanced susceptibility to virulent pathogens. A gain-of-function mutant of *SR1* was identified in a forward genetic screen for Systematic Acquired Resistance (SAR)-deficiency in Arabidopsis. This gain of function mutant showed compromised SAR and enhanced susceptibility to virulent pathogens. These data suggest that SR1 is a key regulator of both basal resistance and SAR[361].

The expression of defense-related genes *EDS1*, *NDR1*, *PR1*, *PR2* and *PR5*[362], was constitutively activated under low temperature in *sr1* plants[316,350,360,362]. Du *et al.*, (2009) showed that SR1 binds to the *EDS1* promoter and negatively regulates its expression[316]. Also, SR1 was found to bind to the promoter region of *NDR1* and suppresses its expression[306,350]. This indicates that the suppression of *EDS1* and *NDR1* in *sr1* plants could be the reason for the constitutive immunity. SR1 negatively regulates the accumulation of SA by repressing the expression of SA

synthesis-related genes, such as *EDS1* and *ICS1*[316,341,360], which encode positive regulators for SA biosynthesis and are critical for SA-mediated defense[363-365]. Loss of function *sr1* mutants accumulate more SA and hydrogen peroxide (H_2O_2), which cause enhanced disease resistance[316,357,360]. These data indicate that the elevated SA is a major cause of *sr1* phenotypes and disease resistance. This was also confirmed by epistasis analysis with mutants of compromised SA accumulation and disease resistance[316].

More recently, Lolle *et al*., (2017) indicated that autoimmunity in *sr1* is NLR triggered[366]. They screened for dominant-negative NLR alleles (*NLR-DN* alleles) that suppress the autoimmune phenotype of the *sr1* mutant[318] and identified *DSC1-DN* and *DSC2-DN* as a suppressor for autoimmunity in *sr1* mutant[366]. Expression of *DSC1-DN* or *DSC2-DN* in *sr1* suppressed plant resistance to *Pst* DC3000 and restored *PR1* expression almost to wild-type levels. Moreover, overexpression of *DSC1* or *DSC2* in *Nicotiana benthamiana* can trigger the HR, but co-expression of *SR1* prevented this. Thus, these two NLRs appear to be active in the absence of SR1 causing the autoimmune phenotype[366].

Expression of *PDF1.2*, and *VSP1*, marker genes of ethylene and JA defense signaling pathways, was also highly enhanced in *sr1* mutants, suggesting that SR1 negatively regulates plant defense probably by also modulating ethylene and JA defense signaling pathways[360]. In support of this, Rahman *et al*., (2016) established that SR1 negatively regulates chitin-triggered immunity to the necrotrophic pathogen *Sclerotinia sclerotiorum* by targeting *Jasmonate Insensitive 1* (*JIN1*) and *BRI1-Associated Kinase 1 (BAK1)*[367]. The *JIN1* is an important component in JA signaling pathway, which is one of the most important plant defense pathways and is essential for the resistance to *S. sclerotiorum*[368,369]. Expression of *AtJIN1* was found to be greatly enhanced in *sr1* mutant compared to wild-type plants[367]. The promoter area of *JIN1* gene was found to have a

CGCG *cis*-element in the region of −262 to −257 (CCGCGT), suggesting that SR1 may directly target *JIN1* and modulate JA signaling thereby regulating plant defense response[367]. In addition, Rahman *et al*., (2016) established that SR1 negatively regulates *S. sclerotiorum* resistance by suppressing the expression of *BAK1,* which is a central regulator of PTI[367,370]. The enhancement of the expression of *BAK1* in *sr1* and the presence of a CGCG *cis*-element in the region of –173 to –168 (ACGCGT) of its promoter suggests that SR1 negatively regulates the resistance to *S. sclerotiorum* by directly suppressing *BAK1*-mediated PTI[367].

Additionally, Cao *et al*., (2016) established that SR1 enhances the susceptibility of *Brassica napus* to *S. sclerotiorum* by directly targeting and suppressing the RNA silencing machinery[367]. The role of RNA silencing machinery in protecting plants from the viral infection is well-known[371-374]. Recently, the role of RNA silencing machinery in plant resistance to fungal and bacterial pathogens has been revealed. Similar to viruses, bacteria have also developed mechanisms to suppress RNA silencing to infect successfully[375-377]. Also, miRNAs are differentially expressed in response to inoculation with fungal pathogens, such as *Erysiphe graminis*[378], *Fusarium virguliforme*[379], *V. dahliae*[380], *V. longisporum*[381], *M. oryzae*[382], and *B. cinerea*[383]. Moreover, mutants of the RNA silencing machinery key components showed enhanced susceptibility to fungal pathogens[381]. Cao *et al*., (2016) have predicted that twenty-one out of 51 of *B. napus* RNA silencing machinery genes *Dicer-Like* (*DCL*)*, Argonaute* (*AGO*)*,* and *RNA-Dependent RNA Polymerase* (*RDR*)*,* contain CAMTA-binding site (CGCG box) in their promoters[367]. Also, they have reported that *S. sclerotiorum* inoculation highly induced the expression of *BnSR1* gene while significantly suppressed the expression of many CGCG-element-containing RNA silencing component genes. Their data suggest that SR1 may target the RNA silencing machinery and down-regulate it enhancing the plant susceptibility to pathogens.

A genome-wide analysis comparing WT and *sr1* mutant has identified 105 differentially expressed genes[357]. Among these differentially expressed genes, 99 genes were found to be up-regulated. Those up-regulated genes mainly include defense-related genes involved in defense against a wide range of fungi, bacteria, viruses and insects as well as genes involved in hypersensitive response, oxygen metabolism and oxidative stress response[357]. Amongst these are genes related to defense against different fungal strains (e.g. *HR4* against powdery mildew and *WRKY33* against *B. cinerea* and *Alternaria brassicicola*); bacteria (*WRKY-33* and *CRK5* against *P. syringae*, *PAD4* against virulent and avirulent strains of *P. syringae*); insect (e.g. *PAD4* against Green peach aphid) and viruses. Furthermore, seven of the up-regulated genes encode disease-resistance proteins with antimicrobial peptide activity (e.g. *At3g04210*, *At2g32680*, *At3g25010* and *At3g11010*). There are also three genes that are involved in the hypersensitive response (*NDR1*, *SYP122* and *PLP2*)[357].

The function of SR1 here is to provide an effective approach for the Ca^{2+} signal to reach a well-balanced defense against pathogens by preventing unnecessary over-activation of plant immunity that can result in retarded plant growth and even death[316,384-386]. Zhang *et al*., (2014) have reported that the negative regulation of plant immunity by SR1 is relieved at the time of pathogen infection by temporary depletion of this TF *via* the pathogen-induced SR1IP1–CUL3-mediated ubiquitin pathway to help establish an effective defense against the attacking pathogens[387]. They have shown that the AtSR1-interaction protein 1 (SR1IP1) acts as a substrate adaptor in CUL3-based E3 ubiquitin ligase to specifically recruit SR1 for ubiquitination and subsequent degradation by the 26S proteasome when the plants are attacked with *P. syringae*[387].

Interestingly, SR1, in addition to its roles described above, functions as a positive regulator of herbivory and wound-induced response[346,388]. Compared to wild type *sr1* mutants were more

susceptible to herbivore attack by *Bradysia impatiens*[346] and the generalist herbivore *Trichoplusia nian*[388]. Complementation of *sr1* with mutated *SR1* that is impaired in its ability to bind CaM did not restore plant resistance to herbivore attack, indicating that Ca^{2+}/CaM-binding domain has a key role in herbivore-induced wound response[346]. Consistent with SR1 role in herbivory, *sr1* mutants have decreased total glucosinolates compared to wild-type plants with the two key herbivory deterrents, indol-3-methyl (I3M) and 4-methylsulfinylbutyl (4MSOB), most affected.

In addition to its role in biotic stresses, Doherty *et al*., (2009) reported that SR1 functions in cold stress by regulating the expression of the *CRT/DRE Binding Factor (CBF*) genes, *CBF1* and *CBF2*, in response to cold[315]. *sr1* mutant showed about 50% reduction in transcript levels for *CBF1* and *CBF2* in plants exposed to low temperature for 2 hours[315]. Further, Kim *et al*., (2013) showed that CAMTA1 and CAMTA2 work together with SR1 to increase plant freezing tolerance by inducing the expression of *CBFs* genes as well as many other cold-induced genes that fall outside the *CBF* response pathway[341]. SR1 along with CAMTA1 and CAMTA2, have been reported to bind to a CAMTA DNA regulatory motif, vCGCGb, in the promoter regions of *CBF1* and *CBF2* and activate their expression. CBF1 and CBF2 TFs then induce the expression of ~100 other genes that activate multiple mechanisms to enhance freezing tolerance[315,389,390].

Comparison of WT plants and *camta* 1/2/3 triple mutant transcriptomes after exposure to low temperature showed that the three CAMTA proteins contribute to induction of ~15% of the genes that are cold-induced at 24 hours. The promoters of these CAMTA-induced genes were highly enriched in the vCGTGb and vCGCGb CAMTA-binding sites as well as the CBF-binding site, rCCGAC, suggesting that many of these genes are direct targets of the CAMTAs, confirming the role of CAMTA proteins in regulating the CBF pathway and enhancing freezing tolerance. However, out of the 128 early cold-induced genes that were CAMTA-regulated, only nine were

identified as members of the CBF regulon[391]. Thus, most of these genes appear to fall outside the CBF pathway.

In another study, analysis of the expression of *CBFs* in response to cold using plants with quintuple and sextuple mutants of *CAMTA* family genes showed that CAMTA3 and CAMTA5 are the main regulators of cold-inducible expression of *CBF1* and *CBF2*. Also, transactivation assays using protoplasts demonstrated that among the six CAMTAs, only CAMTA3 and CAMTA5 significantly enhanced the activities of reporter genes driven by the *CBF1* and *CBF2* promoters, but none of the CAMTA proteins activated expression of the reporter gene driven by the *CBF3* promoter[392]. Recently, using yeast- two-hybrid assay, Lee and Seo (2015) have proposed the MYB96–HHP (Hepta Helical Protein) module to explain how CAMTA3 is activated in response to cold stress[393]. They reported that cold stress induces the expression of the MYB96 transcription factor, which in turn induces the expression of *HHP* genes by binding to their promoters. Subsequently, the HHP2 protein interacts specifically with CAMTA3 and stimulates its transcriptional activity by triggering post-translational modifications. The activated CAMTA3 binds in turn to the promoter region of the *CBF* genes and induces its expression in response to cold[393].

CHAPTER 2

GLOBAL GENE EXPRESSION ANALYSIS USING RNA-SEQ UNCOVERED A NEW ROLE FOR SR1/CAMTA3 TRANSCRIPTION FACTOR IN SALT STRESS

Summary

Abiotic and biotic stresses cause significant yield losses in all crops. Acquisition of stress tolerance in plants requires rapid reprogramming of gene expression. SR1/CAMTA3, a member of signal responsive transcription factors (TFs), functions both as a positive and a negative regulator of biotic stress responses and as a positive regulator of cold stress-induced gene expression. Using high throughput RNA-seq, we identified ~3000 SR1-regulated genes. Promoters of about 60% of the differentially expressed genes have a known DNA binding site for SR1, suggesting that they are likely direct targets. Gene ontology analysis of SR1-regulated genes confirmed previously known functions of SR1 and uncovered a potential role for this TF in salt stress. Our results showed that *SR1* mutant is more tolerant to salt stress than the wild type and complemented line. Improved tolerance of *sr1* seedlings to salt is accompanied with the induction of salt-responsive genes. Furthermore, ChIP-PCR results showed that SR1 binds to promoters of several salt-responsive genes. These results suggest that SR1 acts as a negative regulator of salt tolerance by directly repressing the expression of salt-responsive genes. Overall, this study identified SR1-regulated genes globally and uncovered a previously uncharacterized role for SR1 in salt stress response.

Introduction

As discussed above in the general introduction section, SR1 is the most-studied member of the SR family of transcription factors. Studies on this transcription factor have shown that it functions as a negative regulator of plant immunity[316,357,358], a positive regulator of insect

resistance[388,394] and cold-induced gene expression[315,341]. Also, the core DNA binding motif of SR1 is part of a rapid stress response element (RSRE - *VCGCGB*) found in the promoters of many genes that are rapidly activated in response to stress[320,321]. It has been shown that SR1 can activate reporter genes driven by RSRE in a Ca^{2+}-dependent manner[321], further suggesting that SR1 is involved in the expression of many genes in response to abiotic and biotic stresses[323]. Collectively, these findings indicate an important role of SR1 in regulating biotic and abiotic stress responses and it may be a good candidate for genetic engineering to develop crops with enhanced tolerance to one or more stresses.

For biotechnological approaches to be successful, it is important to understand the molecular mechanisms regulating SR1 functions as well as identifying the full set of genes regulated by SR1. Although SR1 has been shown to play important regulatory roles in plant immunity, herbivory and cold-induced gene expression, the full set of SR1-regulated genes is still largely unknown. So, an in-depth study of SR1-regulated genes using deep sequencing of transcriptomes will provide a global view on SR1-regulated genes. It will broaden our understanding of the roles that SR1 plays in plant stress responses, the mechanisms underlying them and potentially uncover new roles. Previously, Galon *et al.*, (2008) compared the expression of genes in WT and *SR1* knockout mutant line (*sr1-1*) using microarrays and identified only 105 differentially expressed (DE) genes. Since a complemented line was not included in that study and because of some limitations associated with microarrays, it was necessary to perform a comprehensive analysis of SR1-regulated gene expression using next generation sequencing approaches for the reasons described below.

Therefore, the main focus of our study is on the global analysis of SR1-regulated genes using RNA-seq. RNA-seq has many advantages compared to DNA microarrays. RNA-seq profiles

the transcriptome using deep-sequencing technologies that significantly increase the depth of transcriptome analysis. It has no background signal and is more sensitive in detecting genes with very low expression and more accurate in detecting expression of extremely abundant genes[395-398]. RNA-seq avoids technical issues in microarray studies related to probe performance such as cross-hybridization, the limited detection range of individual probes, as well as non-specific hybridization[396-398]. In addition, for this study, we used RNA from WT, *SR1* mutant (*sr1-1*), and a complemented line (SR1-YFP)[316], which will allow the identification of genes that are regulated specifically by SR1. Our study uncovered many more SR1-regulated genes and suggested several new roles for SR1 in other stresses. We have experimentally validated one of the predicted novel roles of SR1 in this study using two mutant alleles of *SR1* and a complemented line.

Results

Loss of SR1 Resulted in Misregulation of About 3000 Genes

Although SR1 TF is known to regulate multiple stress responses in plants, an in-depth study of SR1-regualted genes (direct or indirect) in the genome using deep sequencing of transcriptomes has not been performed. Here we performed RNA-seq analysis of gene expression with RNA from wild type, *SR1* loss-of-function mutant and a complemented line in which the mutant phenotypes are rescued[316,388]. Prior to RNA-seq, genotypes of all three lines were verified by genomic PCR and RT-qPCR (Fig. 1A). In the complemented line, the expression of *SR1* at the protein level was also confirmed (Fig. 1B). For each line, two biological replicates were sequenced using Illumina platform. About 37 to 45 million high quality reads (FastQC quality score is >36) were obtained for each replicate (Table 1). About 80 million reads for each line were used for gene expression analysis. Around 94% of reads from each sample were mapped to the Arabidopsis genome (TAIR10) (Table 1).

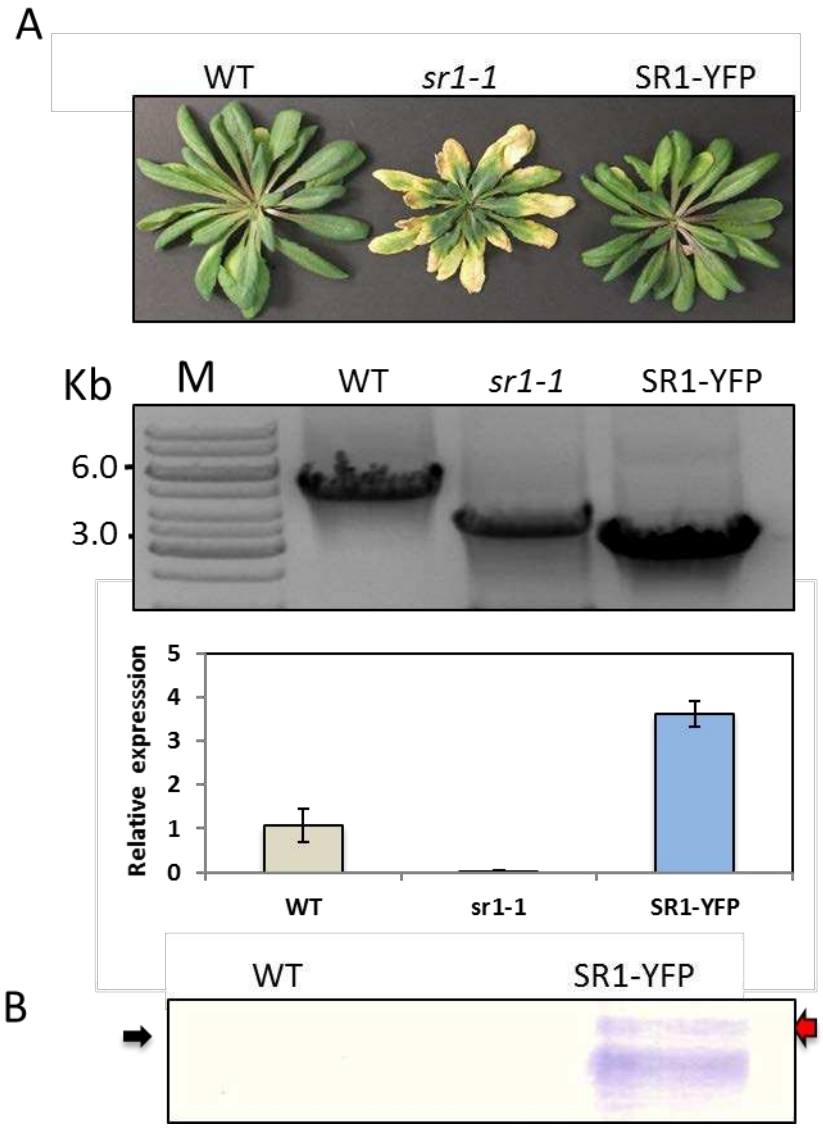


Figure 1. Verification of genotypes used for RNA-seq. A) Top panel: Phenotypes of 40-day-old-plants of wild type (WT), *SR1* mutant (*sr1-1*) and *sr1-1* complemented with *SR1* (SRI-YFP) grown as described in the Methods section. Middle panel: Genomic PCR of the three genotypes. Prior to RNA-seq, genomic PCR was performed with *SR1*-specific primers in case of wild-type and SR1-YFP whereas SR1-specific forward primer and Lba1 reverse primer (T-DNA specific primer) were used for *sr1-1*. In all three cases, the expected amplicon size was obtained. Bottom panel: Analysis of *SR1* expression using RT-qPCR in two-week-old seedlings of wild type (WT), *SR1* mutant (*sr1-1*) and complemented line (SRI-YFP). **B)** Immunodetection of SR1-YFP protein in the nuclear extracts of the transgenic line expressing SR1-YFP using anti-GFP antibody.

Table 1. Mapping statistics of RNA-seq reads

Sample	Total Reads	Reads mapped	Percent Mapped	Uniquely Mapped	Percent Uniquely Mapped	Multiple Hits
WTSR1_R1	37857222	35886091	94.8	32436144	90.4	3449947
WTSR1_R2	43257556	40792316	94.3	37269214	91.4	3523102
KOSR1_R1	45443405	42610742	93.8	39268747	92.2	3341995
KOSR1_R2	41943988	39615994	94.4	36355172	91.8	3260822
SR1YFP_R1	42479202	39987615	94.1	36689779	91.8	3297836
SR1YFP_R2	37006004	35056382	94.7	32469773	92.6	2586609

Of these, ~90 to 92% of the reads were uniquely mapped. The expression of each transcript in each sample was measured by the number of reads per kilobase per million reads (RPKM).

A very high linear correlation was observed in the expression of genes among the replicates indicating that there are no significant differences in gene expression among the biological replicates (Fig. 2). The R^2 values were between 0.87 and 0.9 for the replicates of all three lines (Fig. 2). However, there was a substantial effect of *SR1* loss on gene expression as evident from linear regression values when compared to WT (Fig. 2B). Also, expression of *SR1* in *sr1* mutant significantly restored gene expression changes observed in the mutant (Fig. 2).

Using the Cufflinks package, we identified differentially expressed (DE) genes by comparing the transcriptomes of the mutant and wild type. A total of 2973 genes (Adj. $P \leq 0.05$ and fold change > 2) were misregulated in *sr1* as compared to the WT. Expression of about ~85% of DE genes was partially or fully restored to wild type level in the complemented line (Fig. 3). These results suggest that the DE genes in the mutant are either direct or indirect targets of SR1 and that the loss of this TF has substantial effect on expression of large number of genes (Fig. 4A). Among the DE genes, 1046 were up-regulated whereas 1927 were down-regulated (Fig. 4A). Using RT-qPCR we validated the expression of 9 randomly selected DE genes. The RT-qPCR results corroborated RNA-seq data and the observed changes in the mutant were fully or partially restored in the complemented line (Fig. 4B & 4C). In addition, expression of several other DE genes involved in salt stress was also verified by RT-qPCR (see below).

GO Term Enrichment of DE Genes for Biological Processes

SR1 is known to function in plant immunity, herbivory and cold-regulated gene expression[315,316,341,357,388]. To verify if the DE genes function in these processes and to gain some

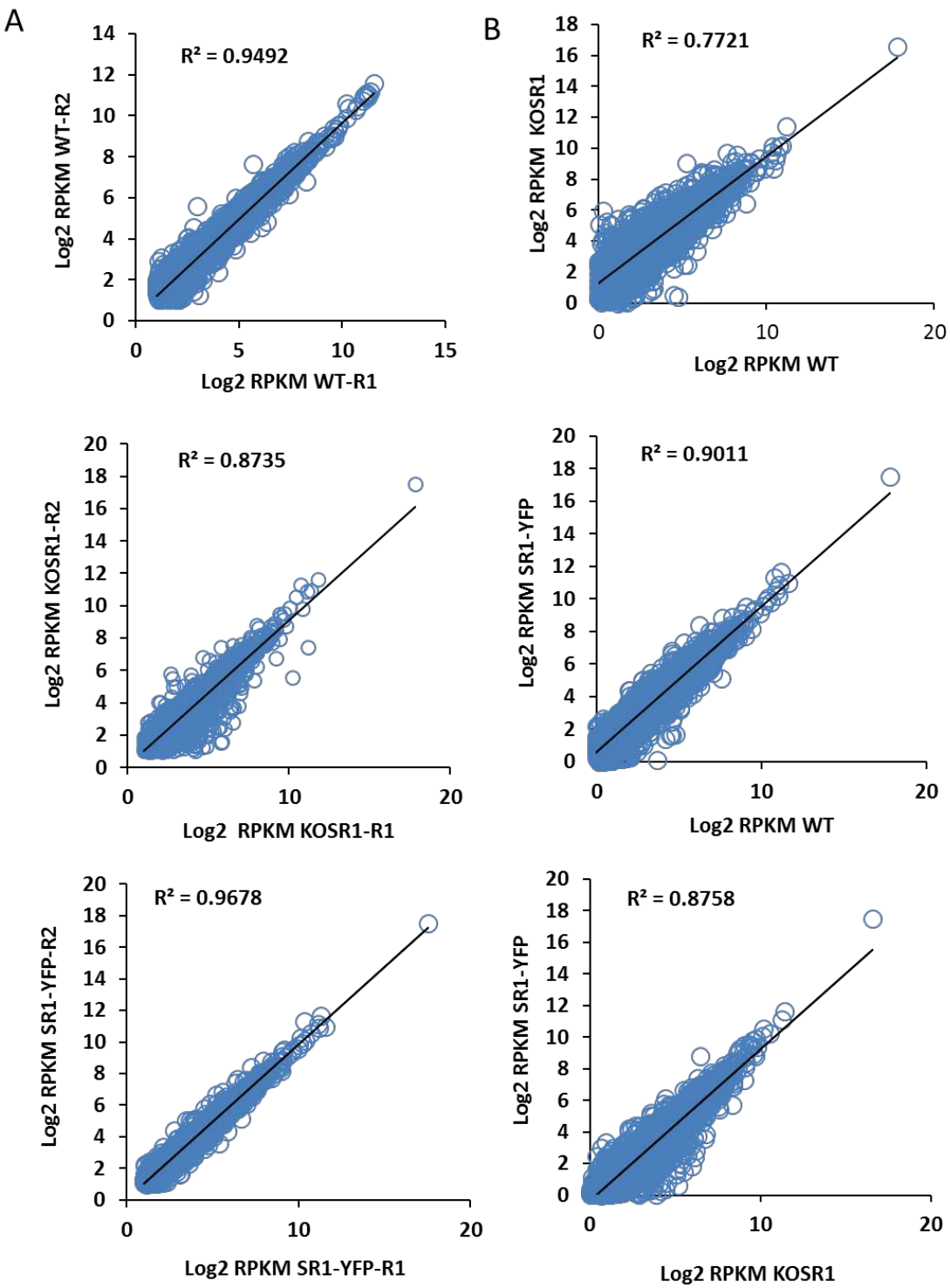


Figure 2. Scatter plot of RPKM values between replicates or genotypes. The Log2 transformed values of replicates of wild type, *sr1-1* (KOSR1*)* and SR1-YFP (panel A) and between the genotypes of wild type, *sr1-1* (KOSR1*)* and *SR1-YFP* (panel B) are plotted.

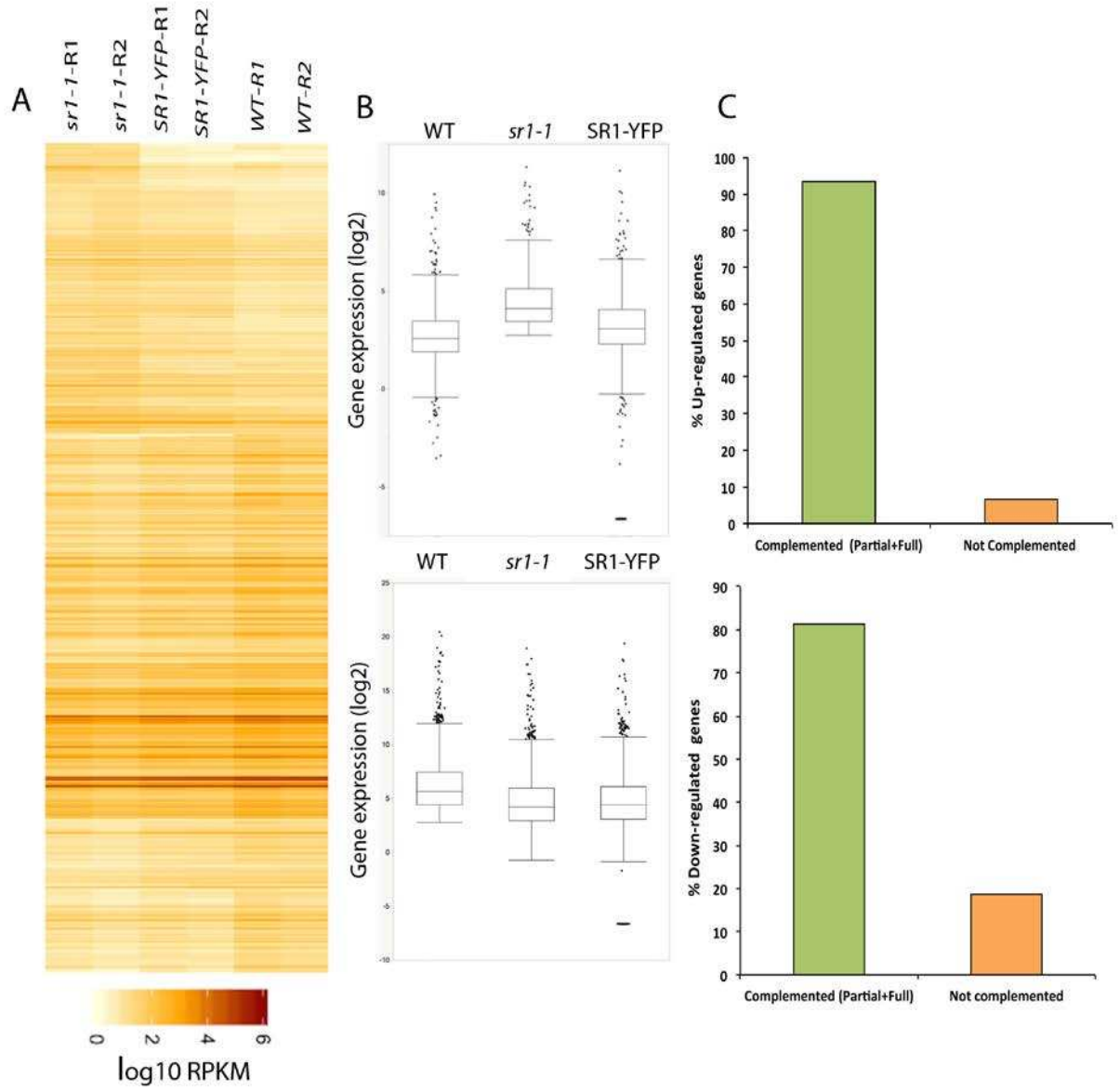


Figure 3. Differentially expressed genes in WT, *sr1-1* and SR1-YFP plants. A) Heatmap of DE genes in two biological replicates of WT, *sr1-1* and SR1-YFP plants. RPKM values were used to generate the heatmap with CummeRbund[399]. **B)** Box-and-whisker plots showing expression of up- (top panel) and down-regulated (bottom panel) DE genes in different genotypes. **C)** Percentage of up- (top panel) and down-regulated (bottom panel) DE genes that are either fully or partially complemented in SR1-YFP line.

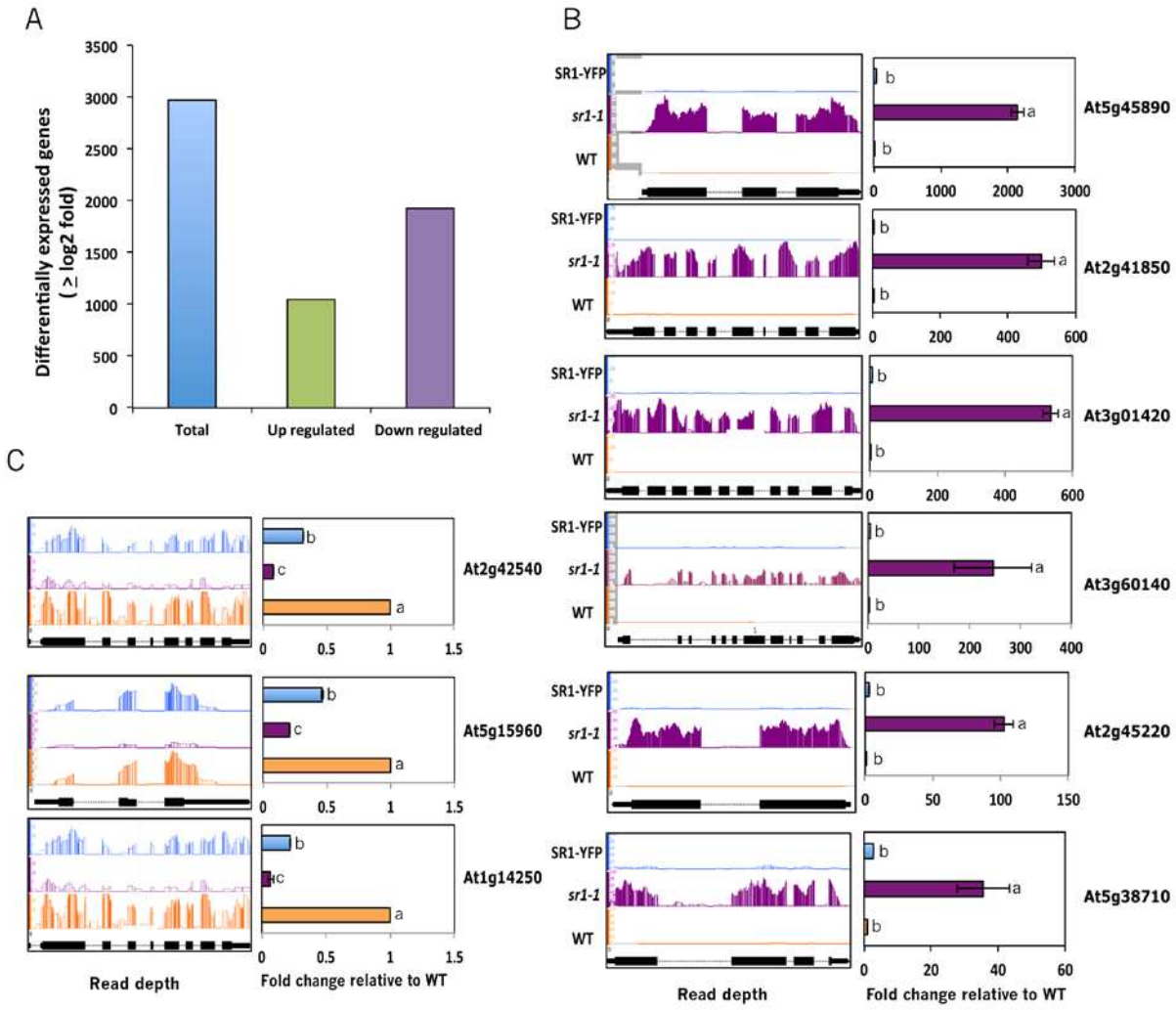


Figure 4. SR1-regulated genes in Arabidopsis. A) Total DE, up- or down-regulated genes. **B)** RT-qPCR validation of randomly selected up-regulated genes. **C)** RT-qPCR of randomly selected down-regulated genes. Left panels in B and C show relative sequence read abundance (Integrated Genome Browser view) as histograms in WT, *sr1-1* and SR1-YFP lines. The Y-axis indicates read depth with the same scale for all three lines. The gene structure is shown below the read depth profile. The lines represent introns and the boxes represent exons. The thinner boxes represent 5' and 3' UTRs. Right panels in B and C show fold change in expression level relative to WT. WT values were considered as 1. Student t-test was performed and significant differences ($P < 0.05$) among samples are labeled with different letters. The error bars represent SD.

insight into other functions of SR1, we performed Gene ontology (GO) enrichment analysis using the whole genome as background. Two methods, AgriGO and GeneCoDis, for singular GO term enrichment analysis yielded similar results with slight variation in the number of GO terms and the order of significance (data not shown). Results obtained with GeneCoDis are presented in Fig. 5. A total of 81 GO terms for biological processes were enriched (Fig. 5). Consistent with the previous known functions of SR1, GO terms related to plant response to pathogens and abiotic factors were among the enriched terms. Analysis of the up- and down-regulated genes separately resulted in enrichment of 95 and 52 GO terms, respectively (Fig. 6). Majority of the up-regulated GO terms are associated with plant defense response to biotic factors. In addition, GO terms "response to salt stress" and "response to water deprivation" are also highly enriched in the up-regulated genes. (Fig. 6A). A significant enrichment of GO terms associated with abiotic factors such as "response to cold" and "response to water deprivation" was observed in down-regulated genes (Fig. 6B).

DE Genes are Enriched for SR1 Binding Motif

Previous studies showed that SR1 binds to *VCGCGB* (where V=A, C or G; B=C, G or T) and *MCGTGT* (where M= A or C) motifs in the promoter regions of SR1-regulated genes[315,316,400-402]. The rapid activation of the general stress-responsive genes is also mediated through RSRE element (*VCGCGB)*, as promoters of these genes exhibit significant enrichment for this motif[320,321]. Here we determined whether the promoter regions of DE genes are enriched for the *VCGCGB* and *MCGTGT* motifs. As shown in Fig. 7A, both these motifs are enriched in the promoters (-1000 bp upstream of translation start site -TSS) of all DE genes ($P < 0.0001$). As significant enrichment for SR1 binding motifs was observed, we further checked for actual number of differentially up- or down-regulated genes that contained *VCGCGB* and/or *MCGTGT* in their promoters. Out of 1046

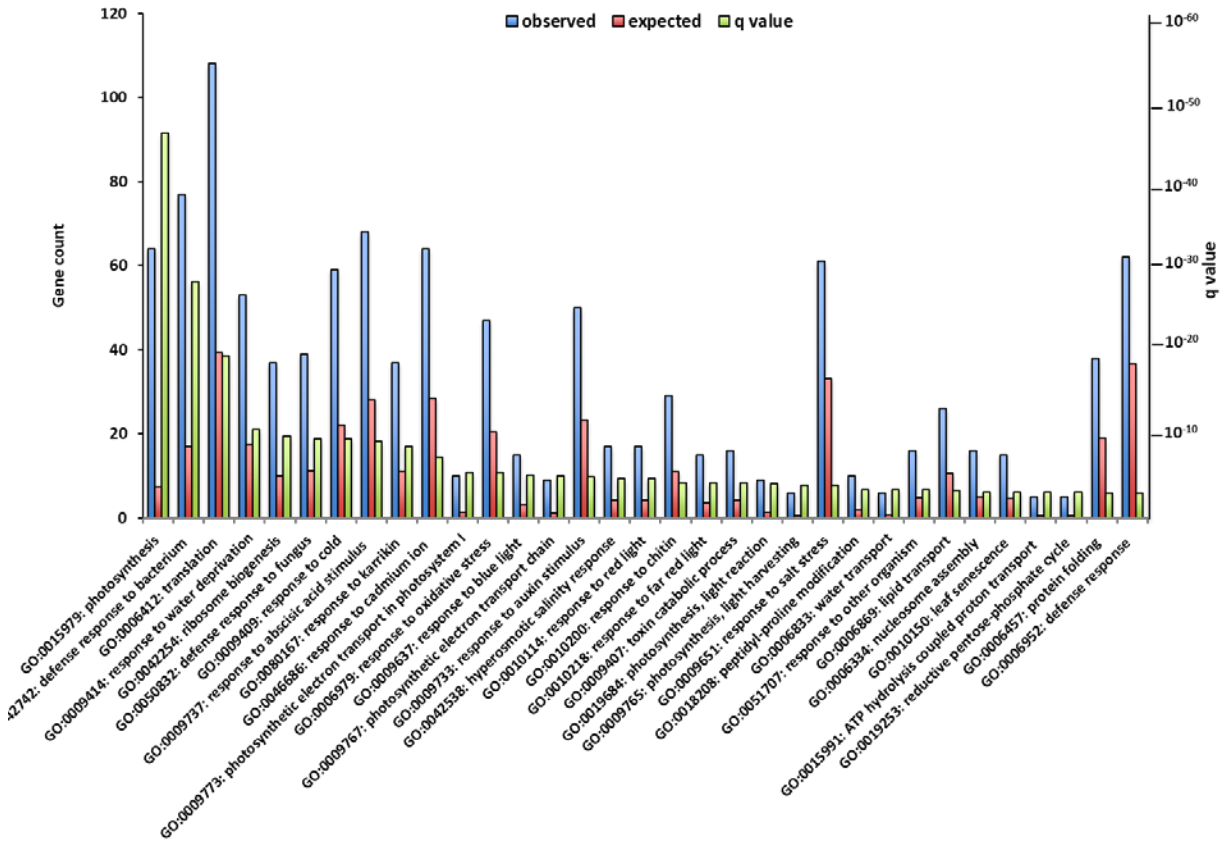


Figure 5. GO term enrichment analysis. GO term enrichment analysis for biological processes of all DE. For each GO term, the expected and observed gene numbers along with the statistical significance (q-value) for the enrichment is presented. Observed: Number of DE genes with a GO term for biological processes. Expected: Number of genes expected for each GO term in the whole genome.

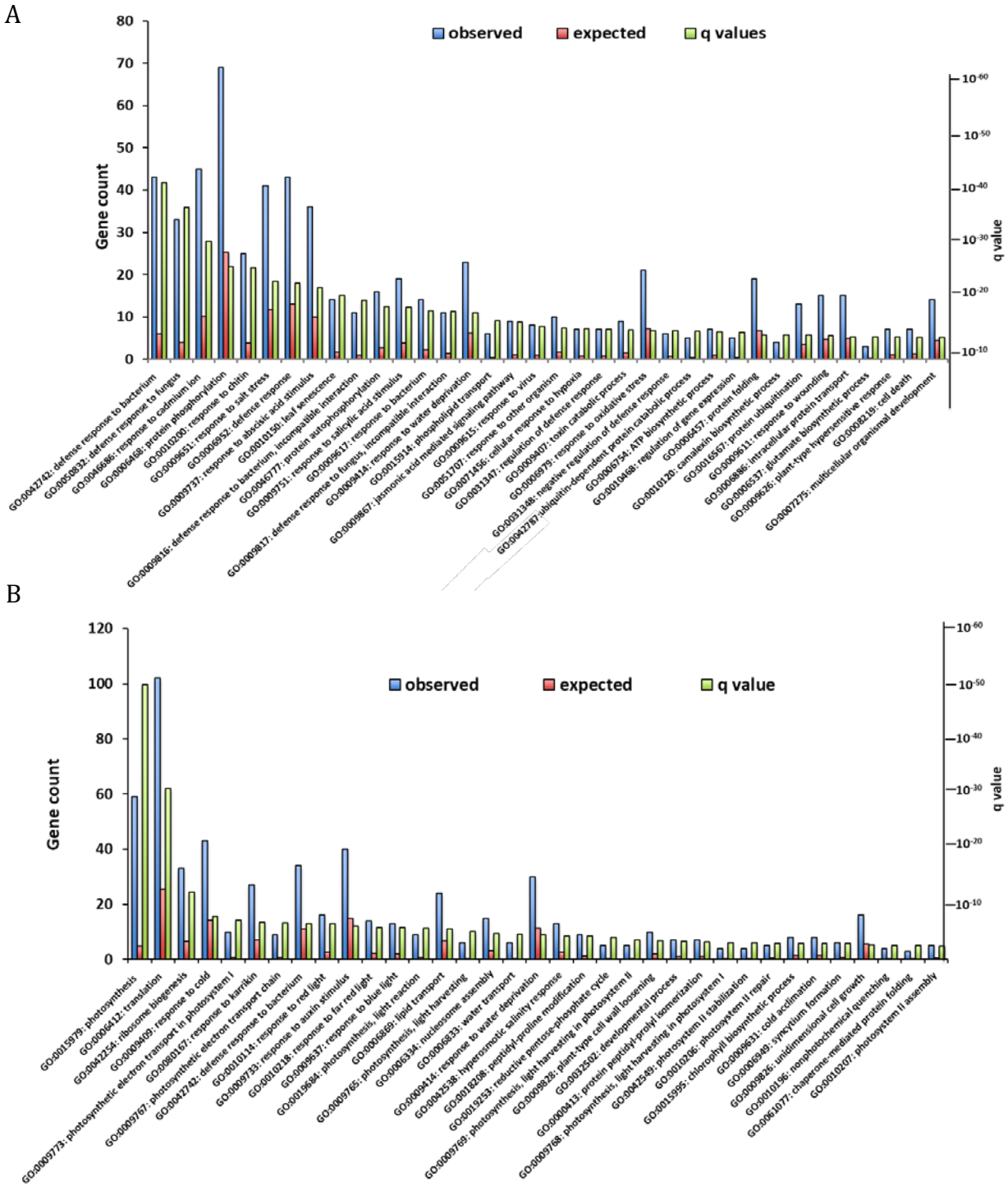


Figure 6. GO term enrichment analysis for up- and down-regulated genes. GO term enrichment analysis for biological processes of **A)** up-regulated and **B)** down-regulated genes. For each GO term, the expected and observed gene numbers along with the statistical significance (q-value) for the enrichment is presented. Observed: Number of DE genes with a GO term for biological processes. Expected: Number of genes expected for each GO term in the whole genome. "Response to salt stress" GO term is indicated with an arrow.

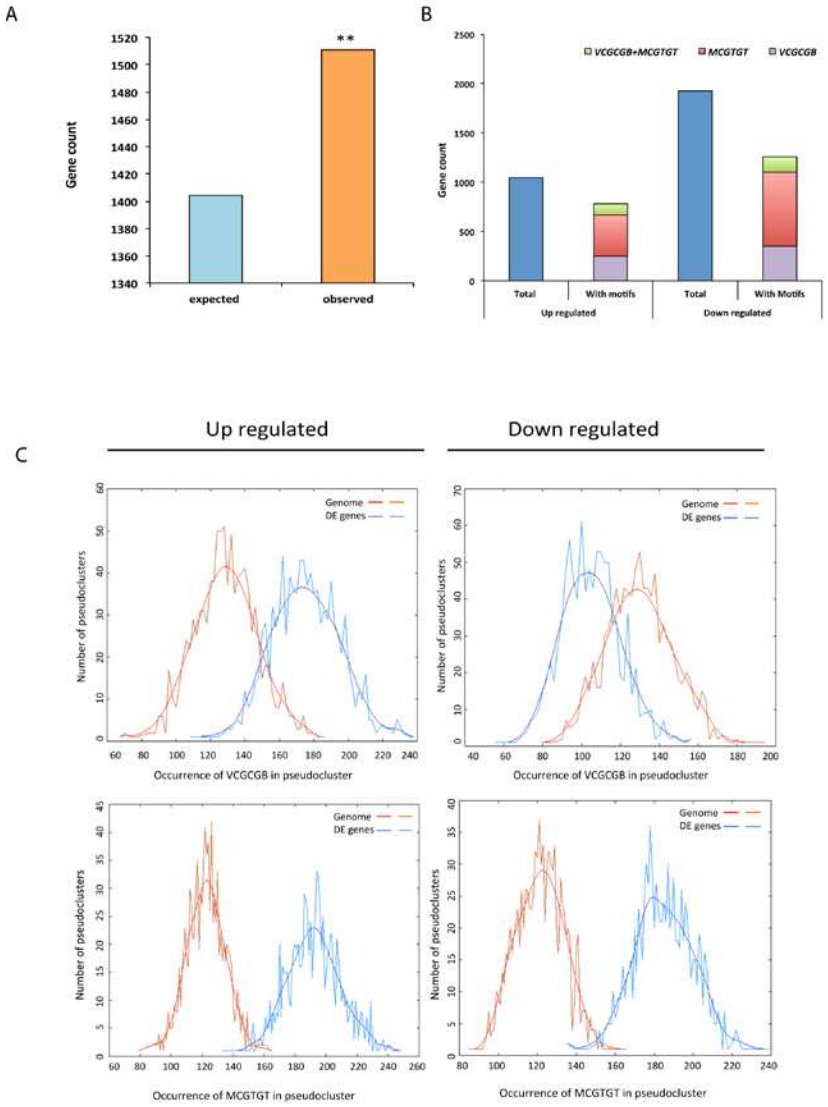


Figure 7. SR1-binding sites in the promoters of up- and down-regulated genes. A) A significant enrichment of the SR1 binding motifs (*VCGCGB+MCGTGT*) in the upstream (-1000 bp) of TSS of all DE genes. Asterisks on the bar represent significant overrepresentation of binding sites with a $P < 0.0001$ **B)** Total number of up- and down-regulated genes and the number of the SR1-regulated genes that contain SR1 binding sites *VCGCGB* or *MCGTGT* or *MCGCGT+VCGCGB* in the -1000 bp promoter region. **C**) Top panel: POBO analysis of RSRE (*VCGCGB)* motif in the -500 bp upstream of TSS. 1000 pseudoclusters were generated from top 500 genes from up- or down-regulated genes and genome background. The jagged lines show the motif frequencies from which the best-fit curve is derived. RSRE element is significantly overrepresented with a two-tailed $P < 0.0001$ in the upstream sequences of up-regulated genes but not with down-regulated genes. Bottom panel: POBO analysis of a second SR1 recognition motif (*MCGTGT*) using the -500 bp upstream of TSS in 1000 pseudo clusters of top 500 DE genes and genome background. The jagged lines show the motif frequencies from which the best-fit curve is derived. SR1 binding sites are significantly over represented (two-tailed $P < 0.0001$).

genes that are up-regulated, 665 (~64%) contained a minimum of one motif of either type (Fig.7B). Of these, 37% contain *VCGCGB*, 39% have *MCGTGT* and 16% have both *VCGCGB* and *MCGTGT* (Fig. 7B). Similarly, out of 1927 down-regulated genes, 1098 (57%) have one or more of these motifs. Of these, 32% have *VCGCGB*, 67% have *MCGTGT* element and 13% have both (Fig. 7B). Together, these results indicate that a significant number (59%) of DE genes are likely direct targets for SR1.

To identify if these motifs are enriched in the promoters of up- or down-regulated genes, we further analyzed the promoters using POBO analysis with upstream regions of top 500 up-regulated or down-regulated gens using the whole genome as background. This analysis revealed a significant enrichment ($P < 0.0001$) of both *cis*-elements (*VCGCGB* and *MCGTGT*) in the up-regulated genes whereas in the down-regulated genes only *MCGTGT* was enriched (Fig. 7C).

GO Term Enrichment of SR1 Binding Motif-Containing Genes

To understand the biological role of putative direct targets of SR1, we performed a separate GO enrichment analysis using either up- or down-regulated genes that have one or more SR1 binding motifs. In the up-regulated genes, 61 GO categories showed significant enrichment. Top 30 GO categories are represented in Fig. 8A. Consistent with known function of SR1, the genes were highly enriched for the GO terms that are predominantly associated with plants response to pathogens/pests. The other highly enriched GO terms include abiotic stress and hormonal responses. One of the GO terms that is of special interest is “response to salt stress” for the following reasons: i) it is the second most enriched GO term after “response to bacterium” ii) this GO term comprises 27 genes (second most of all other categories), iii) expression of the majority of these genes is altered in opposite direction in the mutant and complemented plants and iv) SR1 was not previously known to be involved in salt stress.

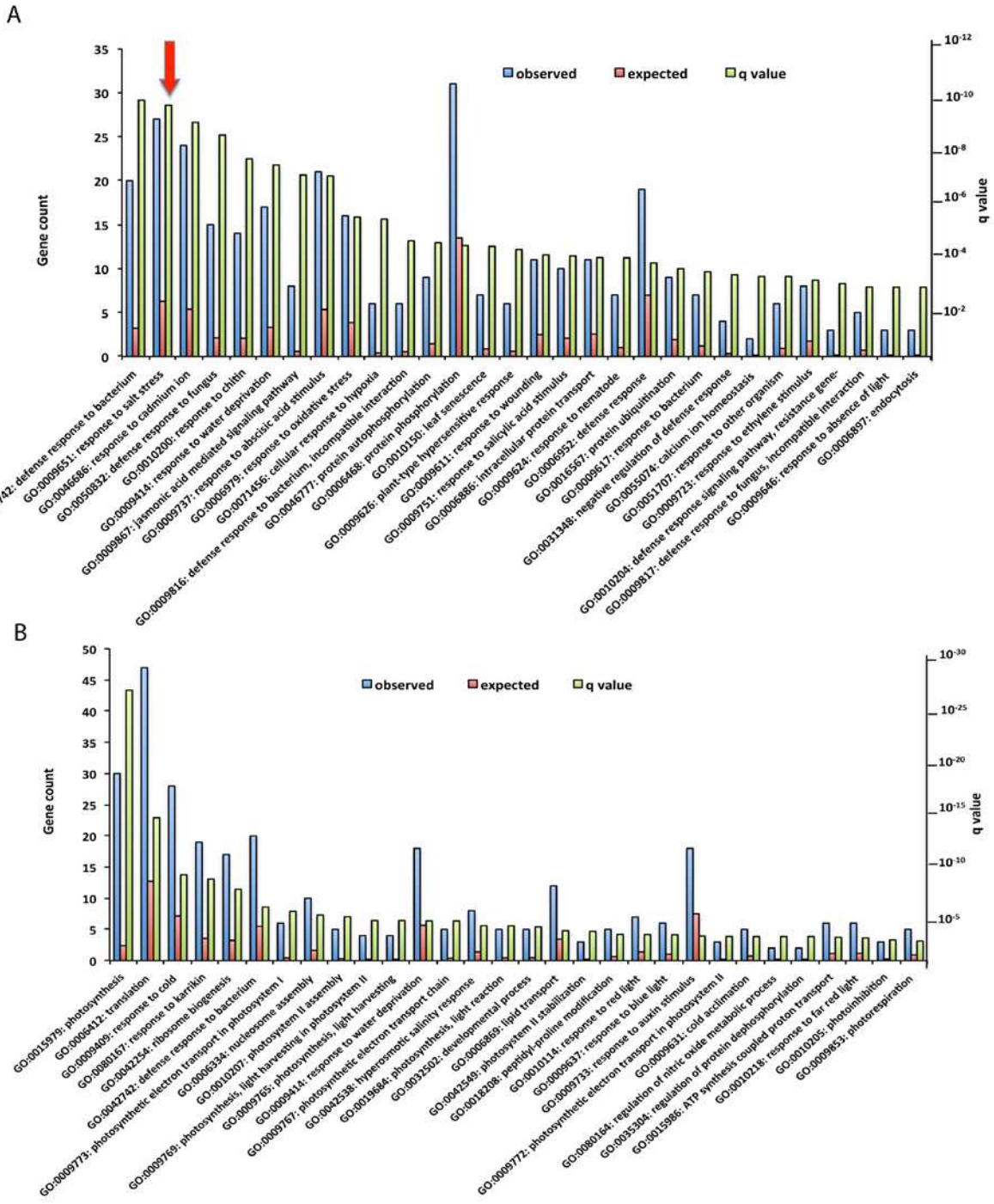


Figure 8. GO term enrichment analysis for potential direct targets of SR1. GO term enrichment analysis for biological processes of **A)** up- and **B)** down-regulated genes containing SR1 binding motif. For each GO term, the expected and observed gene numbers along with the statistical significance (q-value) for the enrichment is presented. Observed: Number of DE genes associated with a GO term for biological processes. Expected: Number of genes expected for each GO term in the genome. "Response to salt stress" GO term is indicated with an arrow.

GO analysis with the down-regulated genes revealed enrichment for only 37 GO terms. The highest enrichment for biological processes is associated with photosynthesis (Fig. 8B). Importantly, unlike the GO terms observed in up-regulated DE genes, there was a significant enrichment for GO term associated with only cold stress. Interestingly, down-regulated DE genes with SR1 binding motif also contributed towards the process of "response to bacterium" (Fig. 8B). These results indicated that genes involved in a biological process can be either up- or down-regulated by SR1 depending on the gene.

SR1 Regulates the Expression of Other SRs

Analysis of promoters of six Arabidopsis *SR*s (*SR1 to SR6*) for the presence of SR1 binding motifs revealed that *SR3, SR4, SR5* and *SR6* contain one or more of these motifs (Table 2), suggesting that their expression could be regulated by SR1. To test if any of these *SRs* are mis-regulated in *SR1* mutant, we checked RNA-seq data for their expression. Interestingly, the expression of all five *SR*s (*SR2* to *SR6)* is significantly elevated in the mutant and fully or partially suppressed in the complemented line (Fig. 9, left panel). To validate these RNA-seq results, RT-qPCR was performed, and the results were in agreement with RNA-seq data (Fig. 9, right panel), indicating that SR1 suppresses the expression of other *SR*s.

SR1 Regulates Expression of Many Transcription Factors

The observed DE genes are likely due to direct and indirect effects of SR1; i.e., SR1 may directly bind to the promoters of these genes and regulate their expression or regulate other TFs, which in turn regulate expression of down-stream genes. In Arabidopsis, there are over 1716 genes encoding TFs, which are grouped into 58 families[403]. Among the DE genes, we found 179 TFs belonging to 40 families (Fig. 10). Of these families, WRKY ($P < 0.0006$), S1Fa like < P, 0.0007), GATA ($P < 0.01$), ERF ($P < 0.03$), EIL ($P < 0.04$) and ZF-HD ($P < 0.04$) are highly enriched (Fig.

Table 2. SR1 binding motifs in other *SRs* promoters

Promoter	Motif	Location
SR1/*CAMTA3*	*ACGTGA*	-365
	TCGTGT	-967
SR2/*CAMTA1*	------	-----
SR3/*CAMTA6*	CCGCGG	-748
	CCGCGA	-1348
SR4/*CAMTA2*	*ACGCGC*	-616
	CCGCGG	-1951
	ACGTGT	-302, -310
	ACGTGG	-593
	ACGTGA	-479
SR5/*CAMTA4*	*CCGCGG*	-1872
	ACGTGG	-35
	ACGTGC	-208
SR6/*CAMTA5*	*CCGCGG*	-1054, -1197
	ACGCGG	-541
	ACGTGT	-3391

Motif highlighted is consensus-binding motif of SR1. The motifs that are not highlighted contain a part of consensus motif.

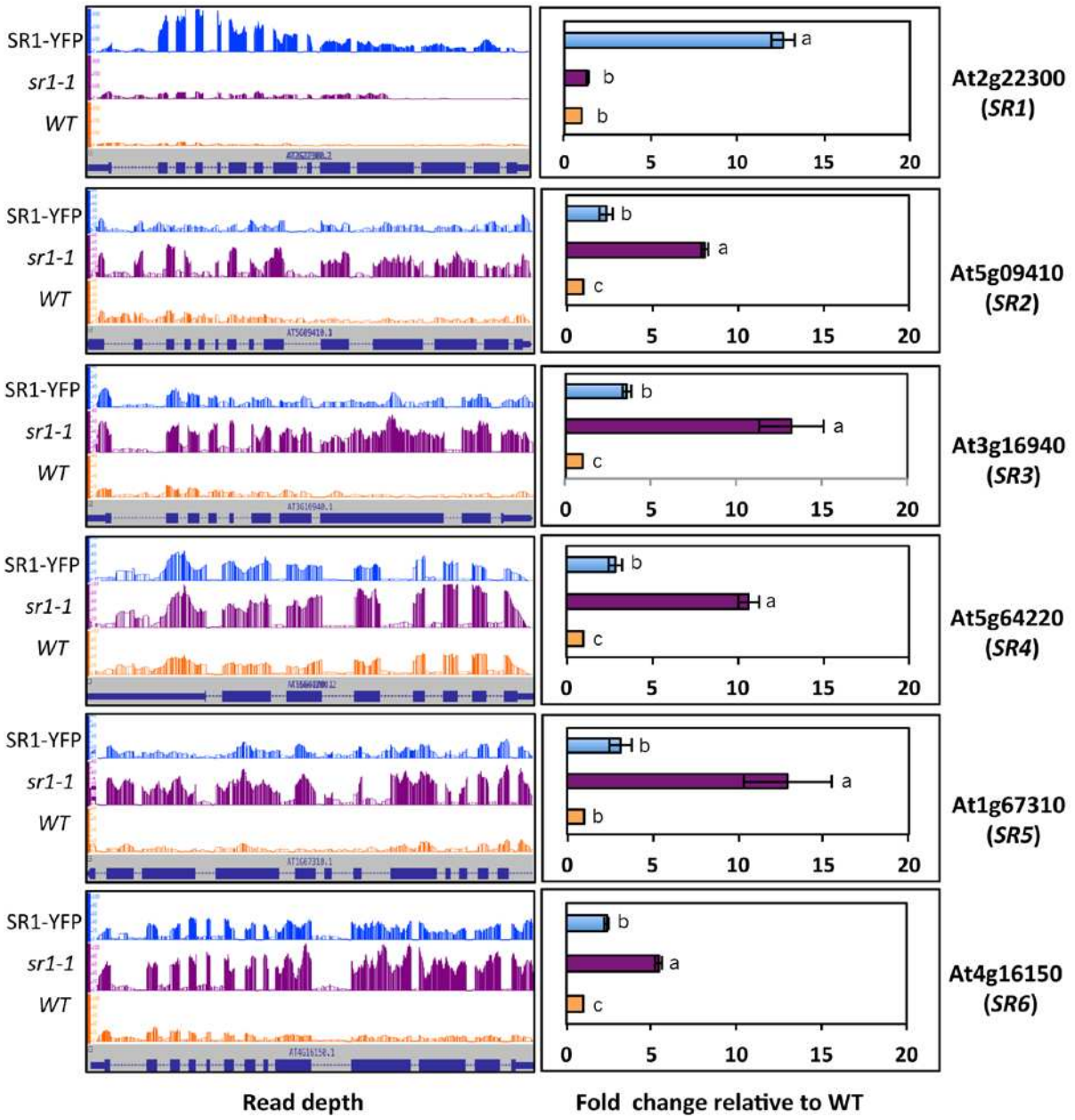


Figure 9. SR1 represses the expression of other members of the SR family. Expression profiles of *SRs* in WT, *sr1-1* and SR1-YFP lines. Panels on left show relative sequence read abundance as histograms (IGB view) in WT, *sr1-1* mutant and SR1-YFP. The Y-axis indicates read depth with the same scale for all three lines. The gene structure is shown below the read depth profile. The lines represent introns and the boxes represent exons. The thinner boxes represent 5' and 3' UTRs. Right panels show fold change in expression level relative to WT based on RT-qPCR analysis. WT values were considered as 1. Student t-test was performed and significant differences ($P < 0.05$) among samples are labeled with different letters. The error bars represent SD.

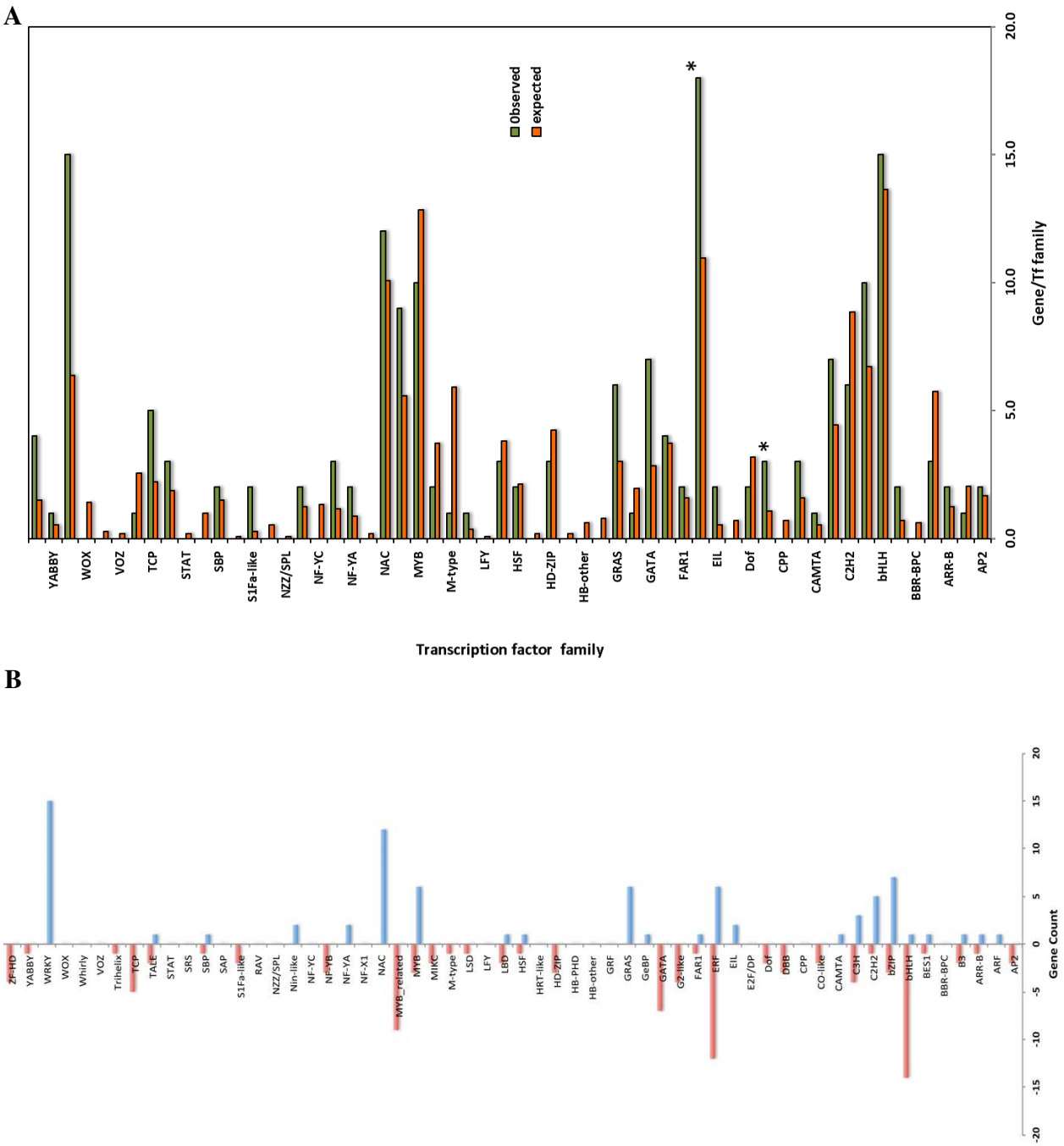


Figure 10. TF gene families in DE genes A) Enrichment of TF families in all DE genes. DE genes are enriched ($P < 0.05$) for specific TF families, which are indicated with an asterisk. Observed: Number of genes associated with particular TF family in DE genes. Expected: Number of genes expected in each individual TF family in the genome. **B)** Up- and down-regulated DE genes in each TF family.

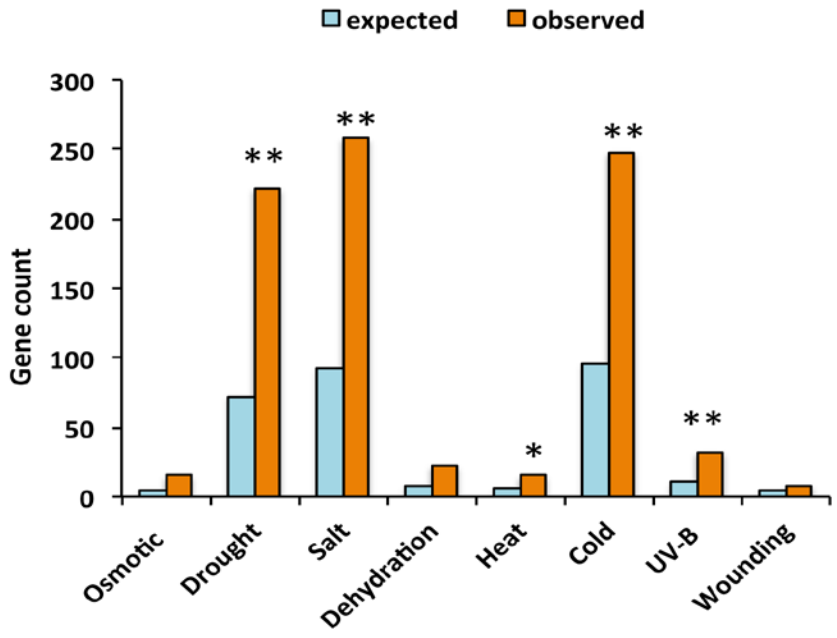
10A). Further examination of the TF families revealed that the genes of 33 of them contain SR1 binding sites (*VCGCGB* and *MCGTGT*) in their upstream region (-1000bp of TSS), suggesting that they are likely direct targets of SR1. The number of TFs in each family that are affected and the direction of their expression change (up or down) in the mutant are shown in Fig. 10B. Interestingly, expression of all TFs in certain families (e.g. WRKYs, NAC and GRAS) is up-regulated whereas all members in some other families are suppressed (e.g. ZF-HD, NF-Y3, Tri-helix and TALE) (see Fig. 10B). The fact that expression of about 10% of all TFs is altered in the mutant suggests that many of the SR1-regulated genes in our DE list, especially those that do not contain SR1 binding motif, are likely indirect targets of SR1.

SR1 Negatively Regulates Salt Stress Tolerance

Since the promoters of a large number of DE genes contained RSRE, we performed enrichment analysis to determine if particular stress responsive genes contributed maximally to the DE list. This analysis revealed a substantial enrichment ($P < 0.001$) of different abiotic stress responsive genes with large number of them implicated in salt stress (Fig. 11A). Interestingly, 27 salt-responsive genes are up-regulated in the mutant. Furthermore, in the complemented line expression of these genes was either restored to the wild type level or repressed (Fig. 11B). GO term enrichment analysis of SR1-binding motif containing up-regulated genes also showed strong enrichment of a term associated with salt stress (Fig. 8A). SR1 is known to regulate cold-induced gene expression[315], but its function in salt stress is not known. We, therefore, investigated the role of SR1 in salt stress tolerance.

Wild type, two loss-of-function mutants of *SR1* (*sr1-1* and *sr1-2)* and the complemented line[316] were tested for salt tolerance. Root growth of all four genotypes was scored for salt tolerance by growing them on different concentrations (0, 100, 150 mM) of NaCl (Fig. 12A). Interestingly, .

A



B

Gene ID	#VCGCGB motif	#MCGTGT motif	log2fold change *sr1-1*	log2fold change *SR1-YFP*	Gene Description
AT1G02500	0	1	1.10778	*	AtSAM1, MAT1, SAM-1, SAM1, S-adenosylmethionine synthetase 1
AT1G02930	2	0	1.42063	-1.00704	ATGST1, ATGSTF3, ATGSTF6, ERD11, GST1, GSTF6, glutathione S-transferase 6
AT1G04120	0	2	1.47448	*	ABCC5, ATABCC5, ATMRP5, MRP5, MRP5, multidrug resistance-associated protein 5
AT1G08930	2	1	1.65228	*	ERD6, Major facilitator superfamily protein
AT1G09210	0	3	1.02848	*	AtCRT1b, CRT1b, calreticulin 1b
AT1G18570	0	1	1.98654	*	AtMYB51, BW51A, BW51B, HIG1, MYB51, myb domain protein 51
AT1G25220	0	1	1.16071	*	ASB1, TRP4, WEI7, anthranilate synthase beta subunit 1
AT1G27730	0	3	1.14815	*	STZ, ZAT10, salt tolerance zinc finger
AT1G32230	2	0	1.33721	*	ATP8, CEO, CEO1, RCD1, WWE protein-protein interaction domain protein family
AT1G54100	0	1	1.23998	*	ALDH7B4, aldehyde dehydrogenase 7B4
AT1G56340	2	0	1.15667	*	AtCRT1a, CRT1, CRT1a, calreticulin 1a
AT1G73260	0	3	4.39129	-4.26972	ATKTI1, KTI1, kunitz trypsin inhibitor 1
AT1G77510	0	1	1.28246	-1.41193	ATPDI6, ATPDIL1-2, PDI6, PDIL1-2, PDI-like 1-2
AT2G05710	0	3	1.30274	*	ACO3, aconitase 3
AT2G38470	0	1	1.62435		ATWRKY33, WRKY33, WRKY DNA-binding protein 33
AT2G47190	0	1	3.01748	-3.16814	ATMYB2, MYB2, myb domain protein 2
AT2G47510	0	2	1.16648	*	FUM1, fumarase 1
AT3G08720	2	1	1.83333	*	ATPK19, ATPK2, ATS6K2, S6K2, serine/threonine protein kinase 2
AT3G09940	0	1	2.40955	-2.20745	ATMDAR3, MDAR2, MDAR3, MDHAR, monodehydroascorbate reductase
AT3G12360	0	1	1.07218	*	ITN1, Ankyrin repeat family protein
AT3G55270	2	0	1.32637	*	ATMKP1, MKP1, mitogen-activated protein kinase phosphatase 1
AT4G14630	0	3	4.50278	-2.96507	GLP9, germin-like protein 9
AT4G16260	0	3	1.07374	-1.1585	Glycosyl hydrolase superfamily protein
AT4G24190	2	2	1.18094	-1.45529	AtHsp90-7, AtHsp90.7, HSP90.7, SHD, Chaperone protein htpG family protein
AT4G37910	2	0	1.18832	*	mtHsc70-1, mitochondrial heat shock protein 70-1
AT5G15650	2	0	1.0795	*	ATRGP2, RGP2, reversibly glycosylated polypeptide 2
AT5G56030	0	1	1.2793	*	AtHsp90.2, ERD8, HSP81-2, HSP90.2, heat shock protein 81-2

Figure 11. Abiotic stress responsive genes are over-represented in DE genes. A) A significant number of DE genes are associated with abiotic stress response in comparison with genome background with a $P < 0.0001$ (**) and $P < 0.05$(*). **B)** SR1 regulates the expression of salt-responsive genes. List of salt-responsive genes that are enriched in the GO term "response to salt stress" is presented. Transcript levels of these genes in the mutant and complemented line and the number of SR1 binding motifs in the upstream 1000 bp of the TSS are presented. Asterisks in the table indicate that the expression level in the complemented line is restored to wild type. In case of eight other genes that are highlighted, their expression is repressed in SR1-YFP as compared to the mutant.

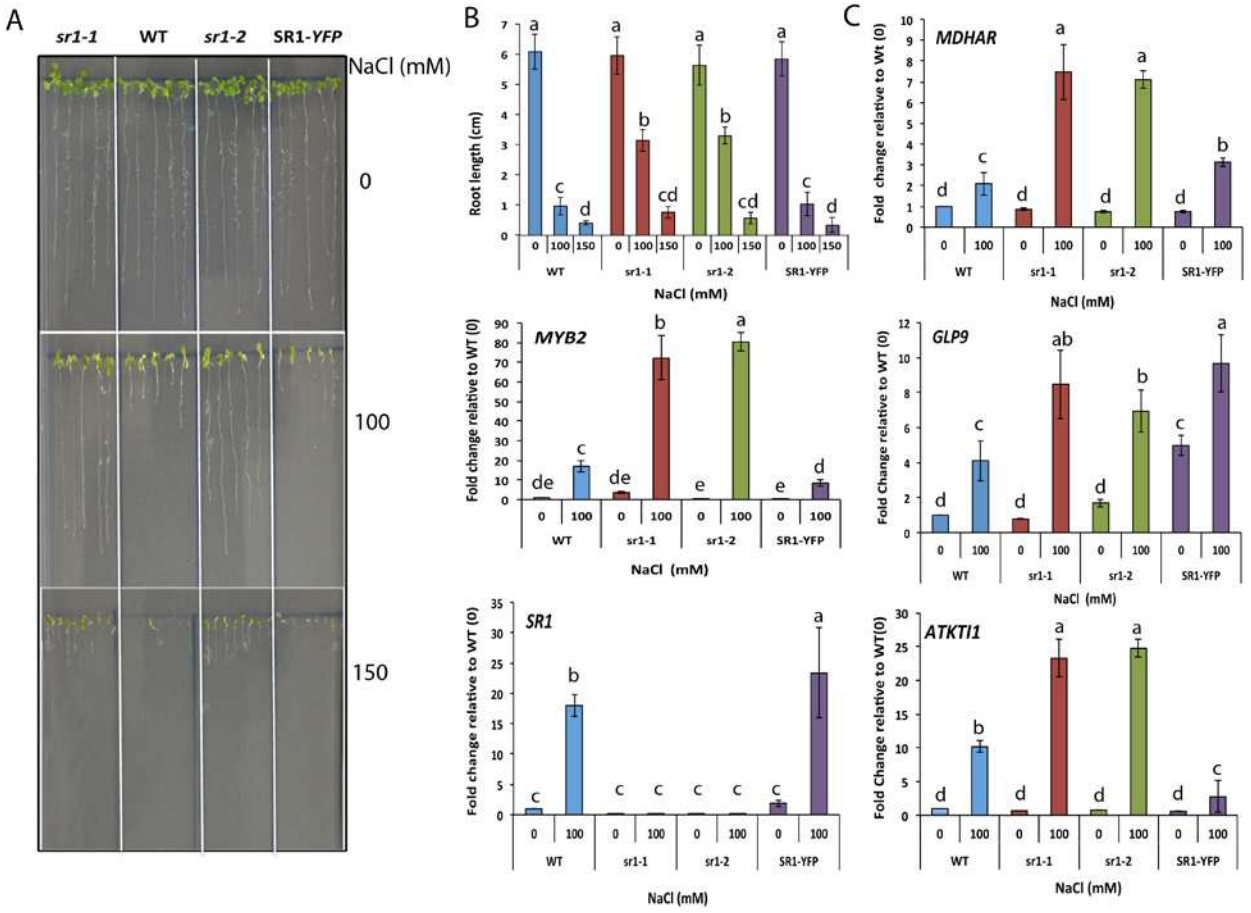


Figure 12. SR1 is a negative regulator of salt tolerance. A) Growth of seedlings of WT, *sr1-1, sr1-2* and SR1-YFP on MS plates containing different concentrations of salt. Seeds were plated on ½ strength MS medium supplemented with 0, 100 and 150 mM of NaCl and were allowed to germinate and grow for two weeks. The photographs were taken after two weeks. **B)** Top panel: root length was measured for each seedling for all three genotypes and plotted against the concentration of NaCl. Three biological replicates were used. Eight seedlings for each genotype per treatment for each biological replicate were included. Middle and Bottom panels: Expression levels of *MYB2* and *SR1* TFs under salt stress in different genotypes. Two-week-old seedlings grown on MS medium supplemented with 0 and 100 mM NaCl concentrations were used. A significant increase in the expression of these two TFs was observed. Salt-induced enhancement of *MYB2* expression level was significantly higher in *sr1-1* and *sr1-2* lines. **C)** SR1 regulates the expression of other salt-responsive genes. Expression levels of *MDHAR*, *GLP9* and *ATKTI1* in two-weeks-old seedlings exposed to 0 and 100 mM NaCl are determined by RT-qPCR. The expression levels of salt-responsive genes were normalized with *ACTIN2*. Fold change in expression level relative to WT controls (WT-0) is presented. WT-0 values were considered as 1. Student t-test was performed and significant differences ($P < 0.05$) among samples are labeled with different letters. The error bars represent SD.

a significant difference in the primary root length in a NaCl concentration dependent manner was observed (Fig. 12A). At 100 mM NaCl, a significant difference in root length was observed among the genotypes (Fig. 12A). A significant suppression in the primary root growth was noted in WT and SR1-YFP lines as compared to mutant lines (*sr1-1* or *sr1-2*), indicating decreased sensitivity of mutants to salt stress (Fig. 12A, middle panel) as compared to WT and SR1-YFP. Even at 150 mM NaCl, mutants were found to be more tolerant to salt stress. These results suggest that SR1 negatively regulates salt tolerance.

SR1 Suppresses the Expression of Salt-Responsive Genes

To gain further insights into the role of SR1 in salt stress, the expression level of 27 salt-responsive genes under the GO category of "response to salt stimulus" was compared in *sr1-1* and SR1-YFP lines. Nineteen out of 27 salt-responsive genes were represented in both *sr1-1* and SR1-YFP data sets and their expression profiles were opposite to each other (Fig. 11B). Motif analysis of upstream regions of these genes indicated that a number of them contain SR1 binding motif (Fig. 13). Orthologs of four Arabidopsis genes (At1g73260, At2g47190, At3g09940 and At4g14630) that were previously reported to be involved in salt tolerance[404-407] and contain an SR1 binding motif in their promoter were selected as representatives to analyze their expression under control and salt stress conditions. The expression of these four genes was verified by RT-qPCR analysis. Expression levels of all four genes were significantly higher in both *sr1* knockout mutants as compared to WT or SR1-YFP in the presence of salt (Fig. 12B, middle panel and Fig. 12C), suggesting that SR1 represses the expression of these salt-responsive genes. Analysis of RNA-seq data for expression of these four genes also showed increased expression in the mutant and their expression was restored to the wild type in the complemented line (Fig. 14, left panel). The expression pattern of these four genes was confirmed by RT-qPCR analysis (Fig. 14, right panel).

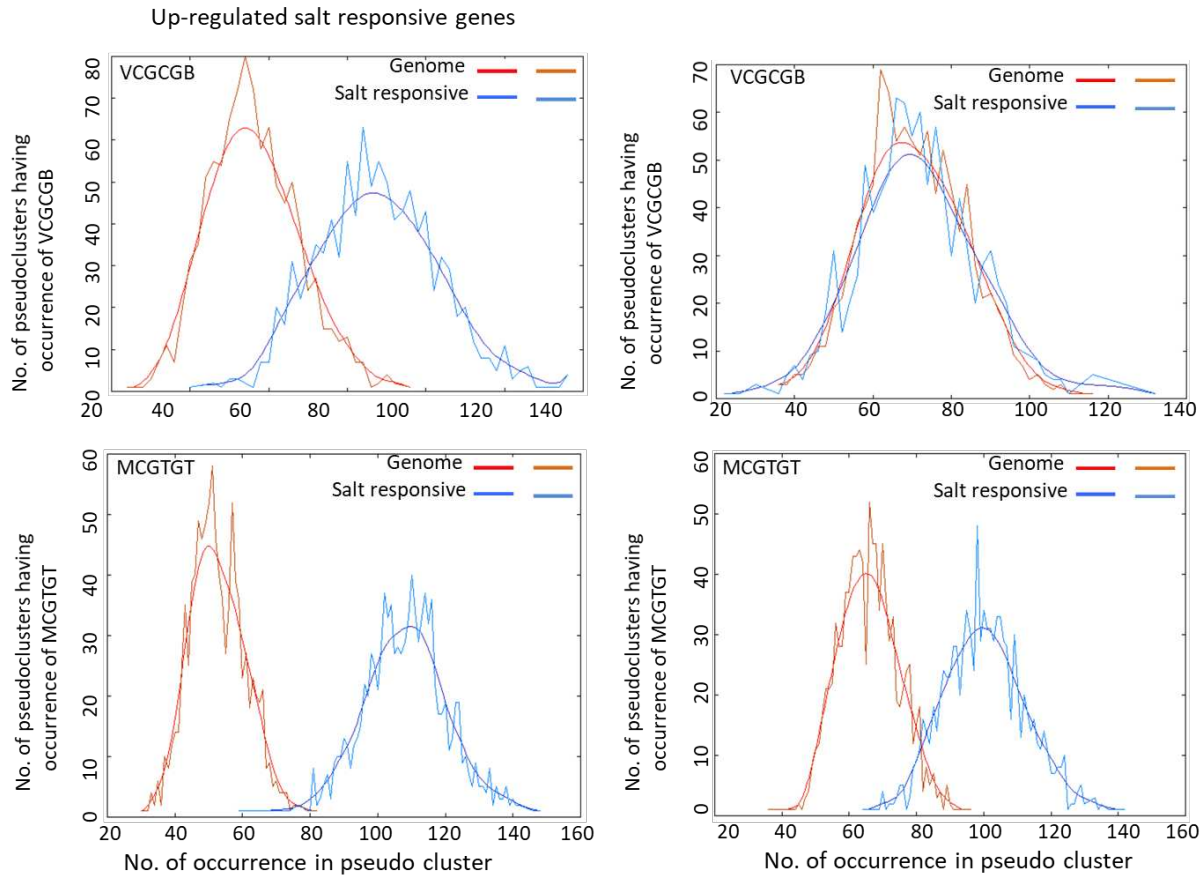


Figure 13. Promoter analysis of differentially regulated salt-responsive genes. POBO analysis indicating the occurrence of the *RSRE* element (*VCGCGB*) and *MCGTGT* in the upstream (-1000 bp from TSS) of salt-responsive DE genes. Data pertaining to 114 up-regulated and 144 down-regulated genes were plotted. A significant (two tailed $P < 0.0001$) enrichment of *VCGCGB* motif was found only in the promoter regions of up-regulated genes

.

Majority of the salt-responsive genes are known to contain *cis*-elements in their promoter regions to which known TFs bind. These include G box (*CACGTG*), N box *CACG[G/A]C and NAC (CATGTG)* that bind G_box bHLH, N_box_bHLH and Nac_box_NAC TFs, respectively. To understand the regulation of these salt-responsive DE genes by SR1, POBO analysis was performed for the enrichment of these *cis*-elements as well as RSRE (*VCGCGB)* element in the upstream regions of all salt-responsive genes. A significant enrichment ($P < 0.0001$) for *VCGCGB* and *MCGTGT* was observed in the upstream region (-1000bp) of the salt-responsive genes that were up-regulated (Fig. 13). In contrast, no enrichment for *MCGTGT* motif was noted in the upstream regions of down-regulated genes (Fig. 13).

Further, significant enrichment for the G box (*CACGTG*), N box (*CACGGC*) and no enrichment for *NAC (CATGTG)* element in the promoter regions of the up-regulated salt stress-responsive genes were observed (Fig. 15). Significantly, enrichment of specific sequences (*ACGTGT*, *CCGTGT*, *ACGCGT*, and *ACGCGC*) within the SR1 binding consensus motif was also observed (Fig. 15). In contrast to the up-regulated salt-responsive genes, a significant enrichment for only G box (*CACGTG*) element and the SR1 binding motif *ACGTGT* was found in down-regulated salt-responsive genes (Fig. 16). These results clearly suggest dual regulation of salt responsive genes by different TFs and preferential usage of certain *cis*-elements (*ACGTGT*, *CCGTGT*, *ACGCGT*, and *ACGCGC*) within the consensus motif of these TFs.

SR1 Binds to the Promoter Regions of Salt-Responsive Genes

Earlier studies have shown that SR1 binds to the promoter regions of *EDS1*, *NDR1* and *EIN3* that are involved in plant defense and ethylene signaling[316,358]. As shown in Fig. 13, there is a significant enrichment of SR1 binding sites in the DE genes that are responsive to salt stress. Consistent with this, expression levels of salt-responsive genes were significantly up-regulated in

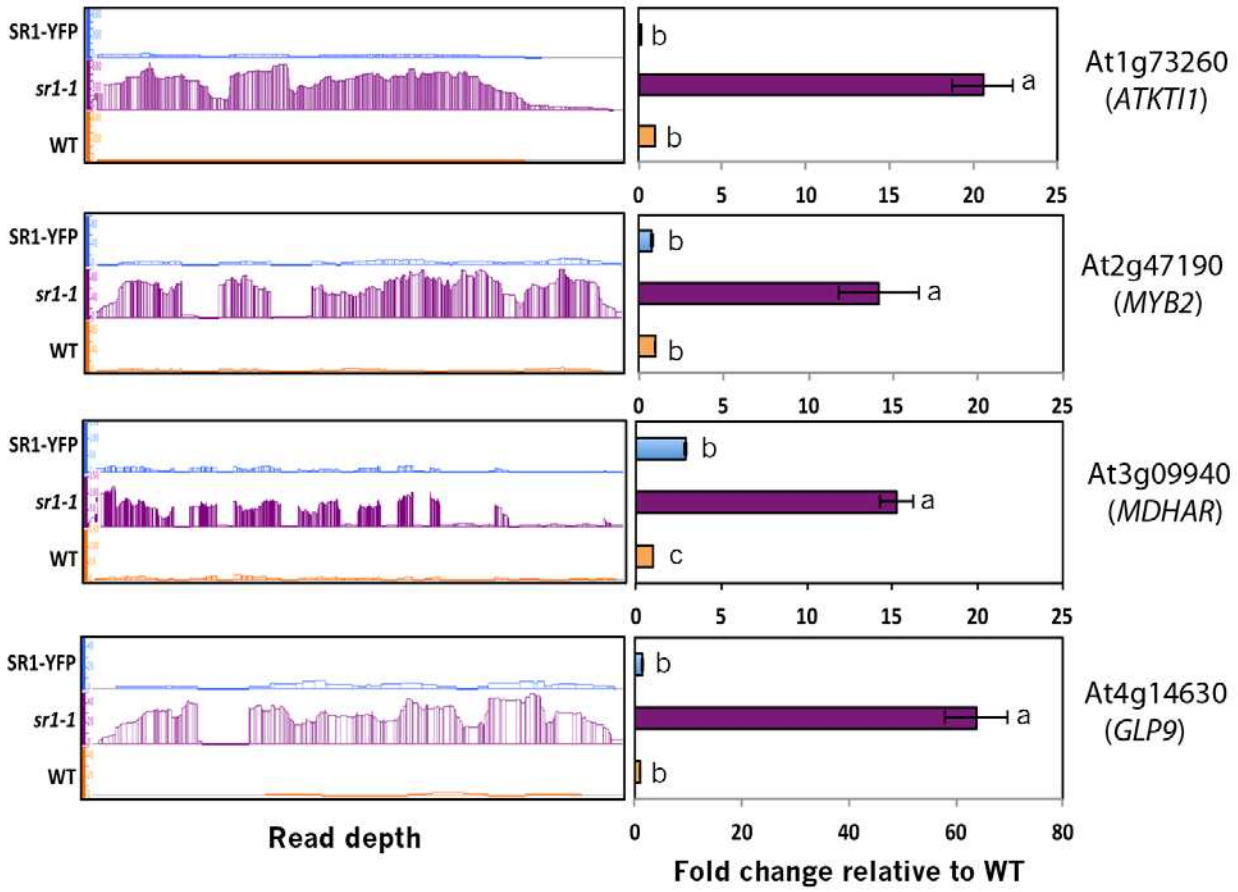


Figure 14. SR1 regulation of salt-responsive genes. A) Expression levels of a few representative salt stress-responsive genes in WT, *sr1-1* and SR1-YFP. Left Panels: relative sequence read abundance (IGB view) as histograms in wild type (WT), *SR1* mutant (*sr1-1)* mutant and the complemented line (SR1-YFP). The Y-axis indicates read depth with the same scale for all three lines. Right panels: Expression analysis of salt-responsive genes using RT-qPCR. Panels on right show fold change in expression level relative to WT. WT values were considered as 1. Student t-test was performed and significant differences ($P < 0.05$) among samples are labeled with different letters. The error bars represent SD.

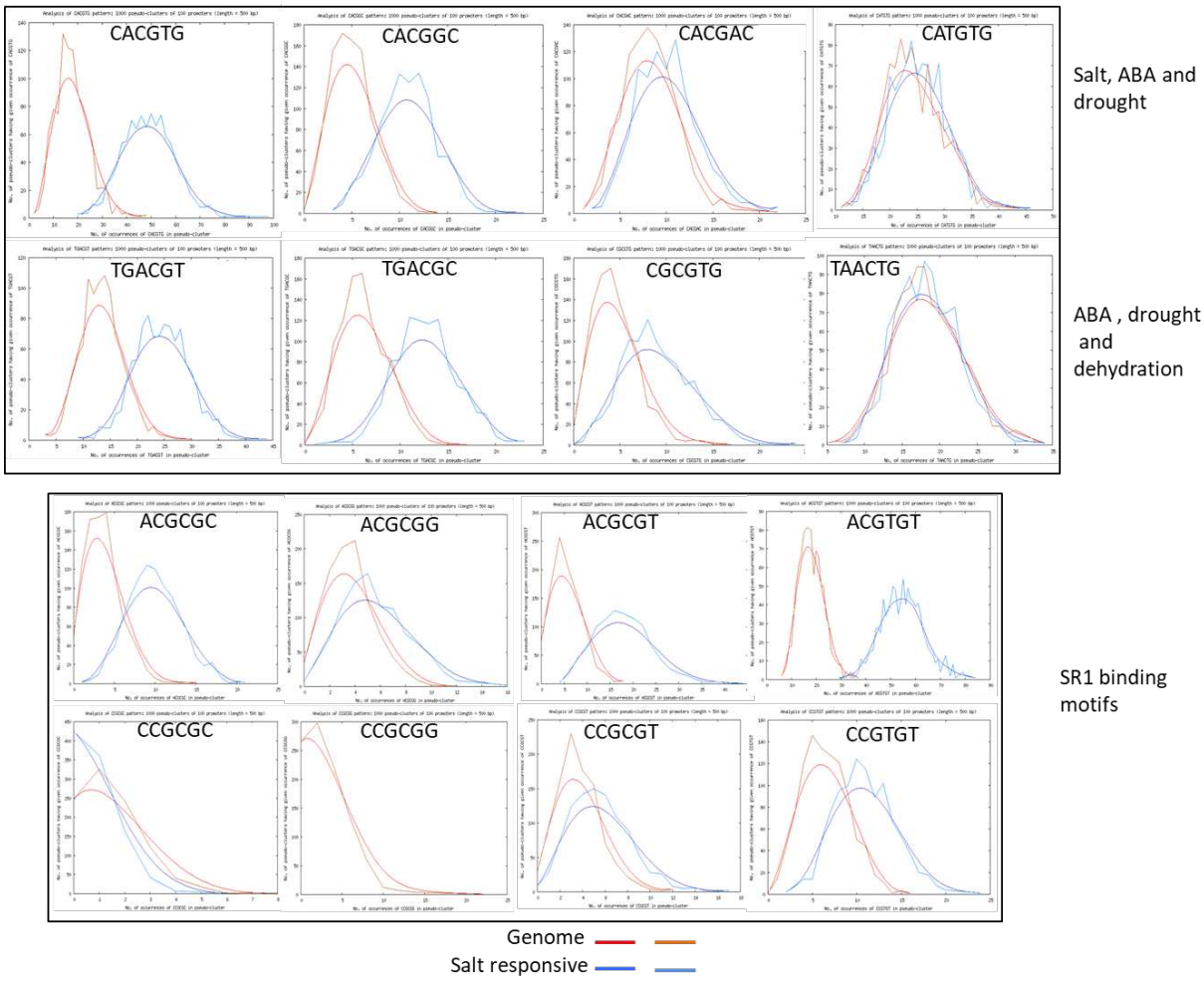


Figure 15. Promoter analysis of up-regulated regulated salt-responsive genes. POBO analysis calculating the occurrence of salt-specific *cis*-elements in the upstream (500 bp of TSS) of salt-responsive 114 up-regulated genes (Top panel). Occurrence of SR1 binding motifs was also plotted (Bottom panel). A significant (two tailed $P < 0.0001$) enrichment of *CACGTG (G-Box)* and *CACGGC (N-box)* motifs was observed in the promoter regions of up-regulated genes.

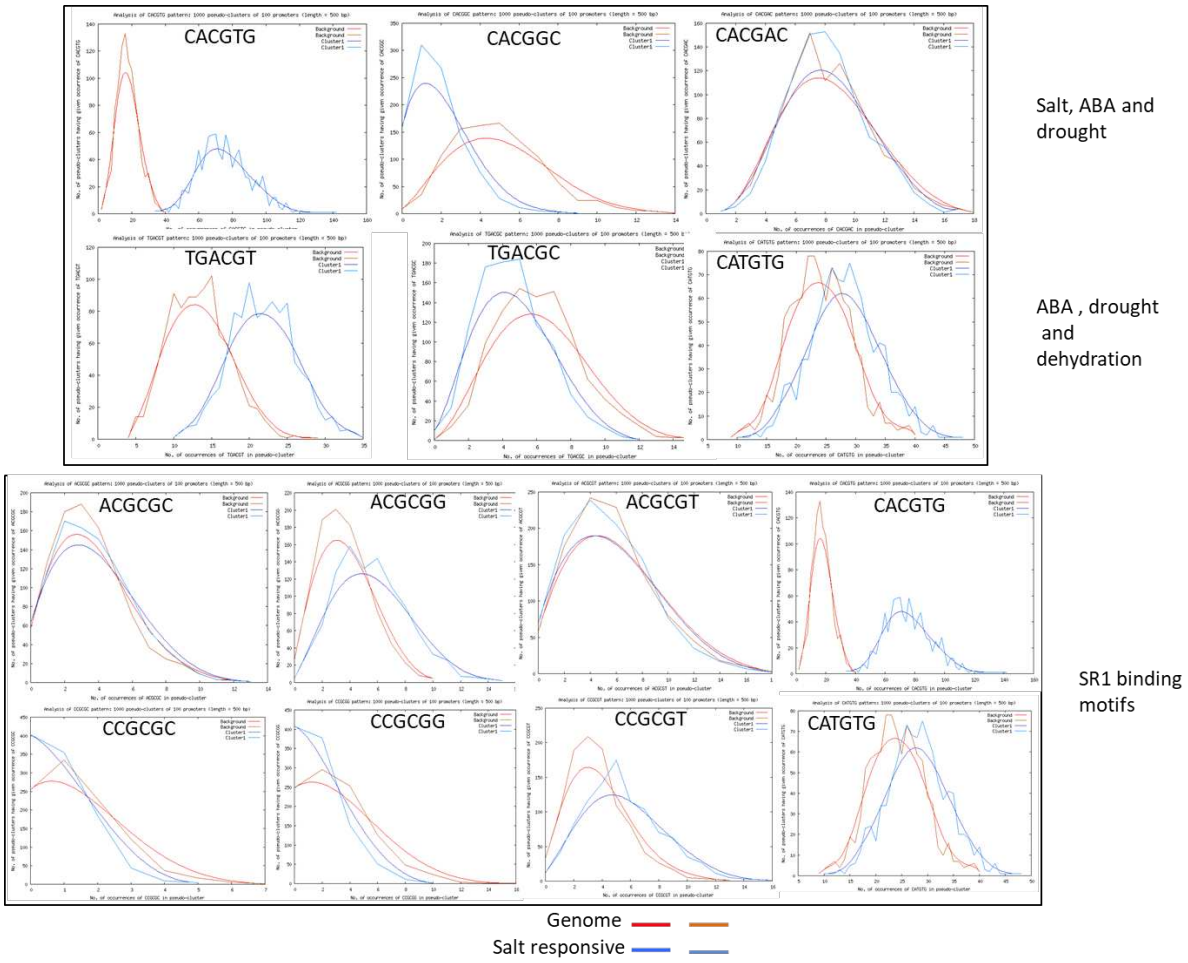


Figure 16. Promoter analysis of down-regulated salt-responsive genes. POBO analysis calculating the occurrence of salt specific *cis*-elements in the upstream (500 bp of TSS) of salt-responsive down-regulated genes (Top panel). Occurrence *SR1* recognition motifs was also plotted (Bottom panel). Data pertaining to 144 down-regulated genes were plotted. A significant (two tailed $P < 0.0001$) enrichment of only *CACGTG (G-Box)* motif was found in the promoter regions of down-regulated genes.

sr1-1 (Fig. 11B). Restoration of transcript levels of salt-responsive genes in the SR1-YFP line to wild type level and the presence of SR1 binding sites in their promoter regions suggest that these are potential direct targets of SR1. We determined the expression levels of four of these genes in WT, *sr1-1* and SR1-YFP using RT-qPCR. This analysis indicated significantly higher transcript levels of the salt-responsive genes in *sr1-1* (Fig. 14, right panel).

To confirm that SR1 binds to the promoter region of these genes, we performed ChIP-PCR assays using the complemented line expressing SR1-YFP. First, the ChIP'ed DNA obtained with anti-GFP antibody showed a significant enrichment for previously known targets of SR1, the *EDS1* and *NDR1* promoters thus validating earlier reports (Fig. 17). We then performed enrichment analysis for promoters of several salt-responsive genes (*ATKTI1*, *MDAR3*, *HSP90*-7, *GST1*, *Glycosyl hydrolase GLP9* and *MYB*2) whose expression is increased in the mutant and contained one or more SR1 binding sites. Interestingly, a significant enrichment for promoters of these genes was noted in immunoprecipitated DNA (Fig. 17), suggesting *in vivo* binding of SR1-YFP to these promoters and direct regulation of these genes by SR1. To address the specificity of SR1 binding to these promoters, we performed ChIP-PCR with primers corresponding to the promoter of *ACTIN2,* whose expression is not affected in the mutant (Fig. 18) and also to two other genes [*GRAS2* (*At1g07530)* and *At1g15790*] that are misregulated in *sr1,* but do not contain SR1 binding motifs. For all three genes, there was no enrichment of promoters in the ChIP'ed DNA (Fig. 18), indicating that binding of SR1 salt-responsive genes is specific.

Discussion

SR1 Regulates Expression of Genes Involved in Multiple Stress Responses

Recent studies using *SR1* loss-of-function mutants have shown that it regulates biotic and cold stress responses[315,316,341,357,388]. Despite its important role in multiple stress responses, a

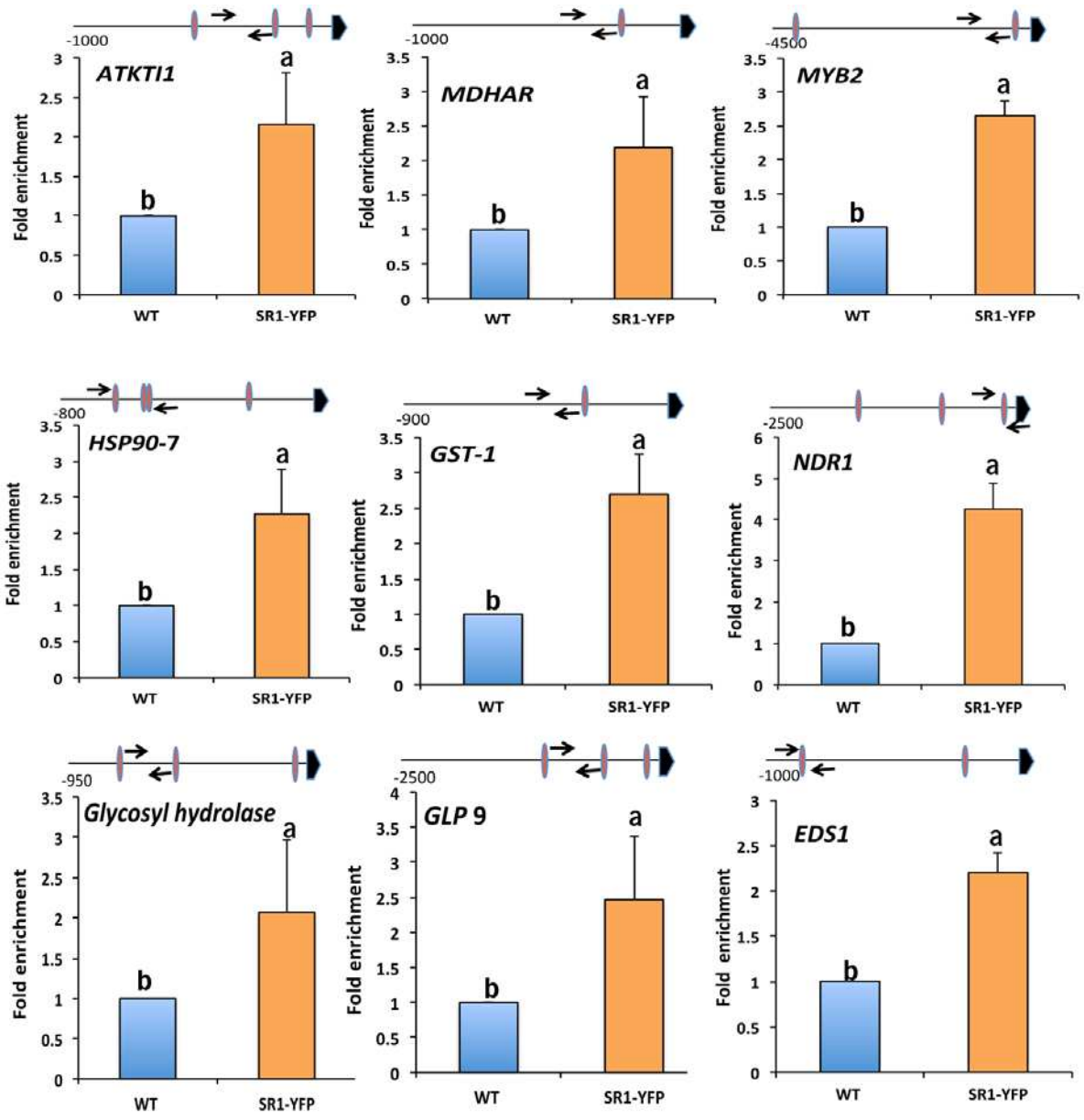


Figure 17. SR1 binds to the promoters of salt responsive genes. ChIP-PCR of upstream regions of salt-responsive genes containing *VCGCGB* or *MCGTGT* or *MCGCGT+VCGCGB*. Chromatin from 15-day-old seedlings from WT and SR1-YFP was immunoprecipitated with anti-GFP antibody and used in PCR with primers flanking the putative SR1 binding sites. The results obtained from four independent ChIP experiments were used to calculate fold enrichment. Data was normalized to DNA input levels as well as *ACTIN2*. The values of WT were considered as 1. Student t-test was performed and significant differences ($P < 0.05$) among samples are labeled with different letters. Schematic diagram over each panel shows SR1 binding sites (as oval shape) and the location of primers used in ChIP-PCR are indicated with arrows. Bold arrowhead indicates TSS.

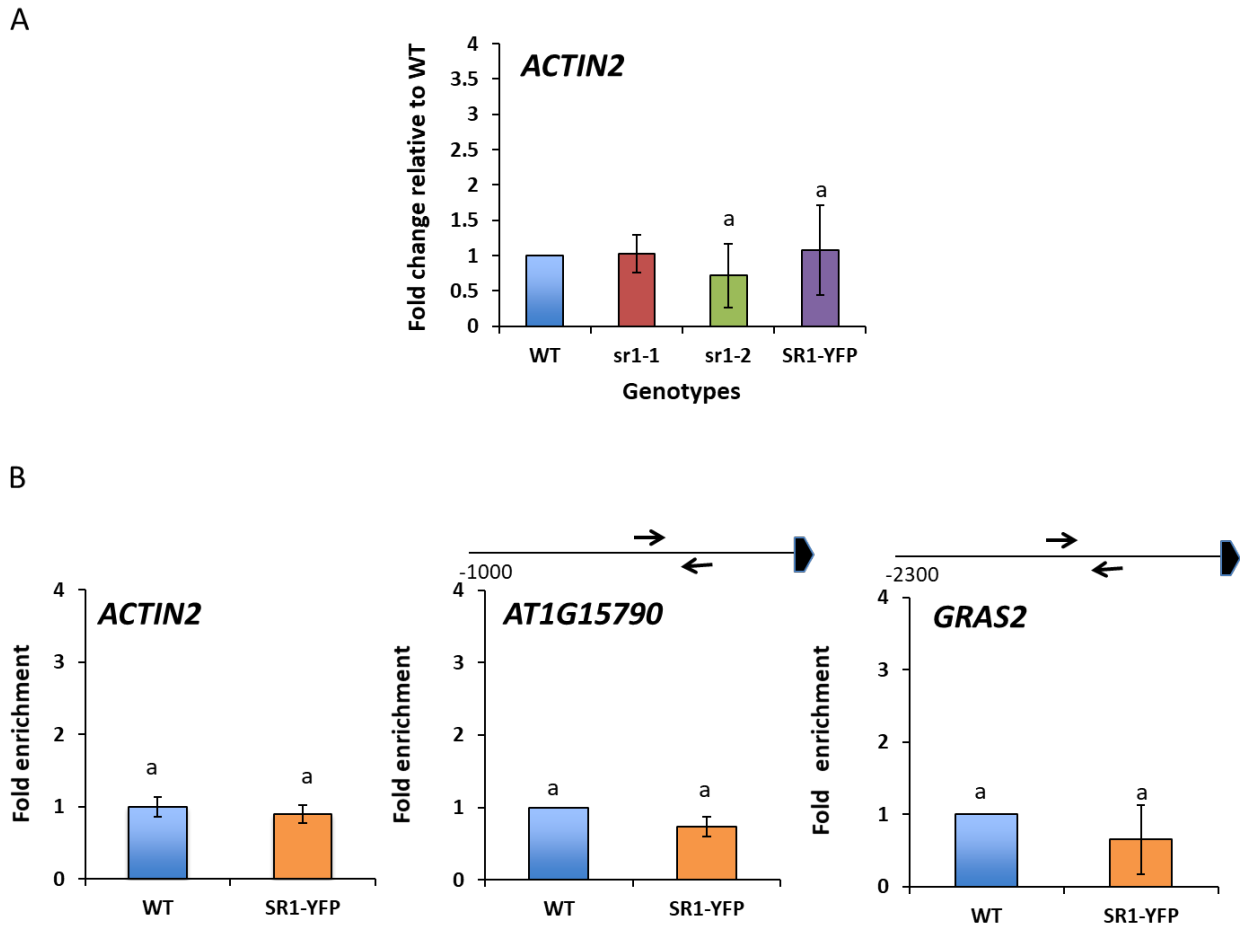


Figure 18. A) Expression of *ACTIN2* in all genotypes**. B)** ChIP-PCR of *ACTIN2* and two other genes that do not contain SR1 binding motifs in their promoters. Chromatin from 15-day-old seedlings from WT and SR1-YFP was immunoprecipitated with anti-GFP antibody and used in PCR. The results obtained from four independent ChIP experiments were used to calculate fold enrichment. In case of *ACTIN2* that data were normalized to DNA input levels. In case of *At1g15790* and *GRAS2* promoters, the data were normalized with DNA input and *ACTIN2*. The values of WT were considered as 1. Student t-test was performed and significant differences ($P<0.05$) among samples are labeled with different letters. Schematic diagram over the panel shows the location of primers (indicated by arrows) used in ChIP-PCR. Bold arrowhead indicates TSS.

comprehensive analysis on SR1-regulated genes is lacking. Our global transcriptome analysis using RNA-seq revealed that a large number of genes involved in diverse stress responses are regulated either directly or indirectly by SR1 (Fig. 4 and Fig. 10). Previously Galon *et al.*, (2008) compared the expression of genes in WT and *sr1-1* using microarrays and identified only 105 DE genes (99 up-regulated and 6 down-regulated genes)[357]. In that study, a complemented line was not included, hence it was difficult to ascertain that these DE genes are SR1-regulated. Our study significantly differs from the former study in a number of ways. Here we used next generation sequencing that significantly increased the depth of transcriptome analysis. More importantly, the use of a complemented line in which mutant phenotypes are rescued allowed us to identify the genes that are regulated specifically by SR1 (Fig. 1).

Our study revealed thirty times more DE genes as compared to the previous study[357]. This huge difference in the number of DE genes is likely due to the technology used here and the depth of RNA-seq. Over half of the DE genes reported in the previous study were found in our analysis. The absence of some DE genes from a previous study in our list could be due to limitations associated with different methodologies such as probe cross hybridization in microarray or more likely due to the tissues used for DE analysis as the age of the plants used in these two studies is different. In fact, developmental regulation of expression levels of *SRs* has been previously reported[326,400,408]. Reproducibility among replicates (Fig. 2), full or partial restoration of expression of ~85% of DE genes in our complemented line to wild type level (Fig. 2 and Fig. 3) and RT-qPCR validation of expression of a number of randomly selected DE genes indicates that the DE genes are bona fide SR1 targets. Enrichment of DE genes in multiple abiotic stress-responses indicates that SR1 plays a major role in cross-talk between multiple stress signal transduction pathways (Fig. 11).

Earlier, SRs were shown to differentially respond to various stresses such as heat, cold, salinity, drought, UV and stress hormones such as ethylene and ABA[409]. Further, many of the SRs have been implicated for their regulatory role in abiotic stress responses[321,323,325,327,341]. GO analysis of the DE genes indicated high enrichment of GO terms associated with diverse cellular processes that are critical for plant responses to biotic stresses such as bacteria and fungi, and abiotic stresses including drought, cold, salt and oxidative stress. These results suggest that SR1 could function as an important integrator of variety of stress responses. Consistent with these results, SR1 is already known to play an important role in at least four different stress responses[315,316,341,357,388].

SR1 Binding Motifs Containing Genes are Both Up- and Down-Regulated

Earlier studies identified *CGCG* and *CGTG* as core sequences to which SR1 binds through its CG1 DNA binding domain[400]. Furthermore, several studies identified *VCGCGB* and *MCGTGT* as consensus element, through which the SR1 regulates the expression of target genes[315,316,341,402]. Analysis of DE genes showed that >59% of SR1-regulated genes contain *VCGCGB and MCGTGT* elements and these motifs are significantly enriched in their promoter regions (Fig. 7). Among the genes that contain SR1 binding motif, in up-regulated genes both elements contributed towards the enrichment whereas highest representation of *MCGTGT* motif was observed in the down-regulated genes. Further, POBO analysis using the whole genome as a background also confirmed this observation (Fig. 7). The up-regulated genes containing SR1 binding sites, not only highly enriched for GO terms related to defense response to bacterium and fungi, but also for response to salt stress, water deprivation, and response to some hormones. In contrast, GO term enrichment of down-regulated genes that contain SR1 binding motifs exhibited significant enrichment for "response to cold" and "cold acclimation" apart from other cellular processes. This is consistent

with the previous reports where SR1 was shown to function as a positive regulator of genes involved in the cold response[315,341]. Indeed, a preferential enrichment of either up- or down-regulated SR1 binding motif-containing genes for a biological process indicates that SR1 binds different *cis*-elements for regulation of different biological processes.

Previous studies have shown that SR1 acts as a critical regulator of both basal and systemic acquired resistance[316,341,361]. A significant increase in the levels of SA in the loss-of-function mutants of *SR1* has been reported[315,316,341]. Our gene expression analysis also indicated that 66% of the SA responsive genes have *VCGCGB or MCGCG* elements in their promoters indicating that they are potential direct targets of SR1. Some of these include *TGA3*, *NAC0062*, *CBP60G*, *EDS5*, *WRKY8,* and *MPK1*. Earlier *CBP60g* along with *SARD1* had been described as key regulators of *ICS1* induction and SA synthesis[410,411]. Du *et al*., (2009) have shown direct binding of SR1 to the *EDS1* promoter and repression of its expression, indicating repressive activity of this TF in regulating these genes[316].

SR1 Suppresses the Expression of Other Members of SR Family

Loss-of-function of *SR1* significantly relieved the suppressive effect of SR1 on other *SRs* expression. Furthermore, expression of other *SRs* is significantly reduced in the complemented line (Fig. 9), indicating that SR1 controls the expression of other *SR* genes. Regulation of expression of some of these *SR* genes is likely through direct binding of SR1 to *cis* elements (*VCGCGB* or *MCGTGT)* in their promoter. With the exception of *SR2,* promoters of the rest of the *SRs* (*SR3, 4, 5* and *6*) do contain the *cis*-elements variation of the *CGCG* box, which could be involved in regulation of these genes by SR1 (Table 2). We analyzed the promoter sequence of *SRs* for non-canonical binding motifs (i.e. with core sequence being similar and nucleotide at 5' and 3' end of the element being different) and found them to contain motifs related to SR1 binding

sites (Table 2). Interestingly, elevated expression level of *SR2* in *sr1-1* and its down-regulation in the complemented line, even in the absence of SR1 binding motifs in its promoter region, indicate the existence of an alternate mechanism by which SR1 regulates *SR2* expression. Previously, the *VSP1* promoter, which does not contain a canonical SR1 binding motif, was shown to be regulated directly by SR1[394], thus indicating the existence of alternate regulatory pathways. Thus, it is possible that SR1 also regulates *SRs* through non-canonical *cis*-elements in their promoters.

Indirect Regulation of SR1-Regulated Genes

Enrichment analysis for TF families in DE genes indicated highest enrichment for genes in the WRKY, EIL, ERF, ZF-HD and S1Fa TF families. The WRKY TFs, which were all up-regulated (Fig. 10A), bind W-box in the defense genes that are primarily implicated in regulation of defense responses against pathogen infection. However, these TFs are also implicated in other cellular processes such as abiotic stresses[412]. Some members of this family (*WRKY18 WRKY33, WRKY40, WRKY46, WRKY70, WRKY53, WRKY70* and *WRKY75*) have SR1 binding sites in their promoter, indicating that they are likely direct targets of SR1. Given that GO terms enrichment for the "response to bacterium/pathogen" and "response to abiotic stresses" was observed in DE genes, it is possible that these TFs may regulate the expression of DE genes that do not contain an SR1 binding motif. In the down-regulated genes, the highest representation of ZF-HD, ERF, AP2, bHLH, and TCP TF families was observed, indicating that the members of these families are positively regulated in SR1. Together, these data indicate a complex network of regulation of expression of TFs by SR1.

RSRE is Enriched Only in Up-Regulated Genes

Recent studies identified *VCGCGB* as the core element that is enriched in a majority of early-activated genes that are also regulated by Ca^{2+} under stress conditions[320]. As the RSRE

element *VCGCGB* is identical to the binding site of SRs (*VCGCGB*), many studies implicated SRs in general and SR1 in particular in regulation of general stress responses. Our analysis of the promoter region of all the DE genes indicated that a significant percentage of the genes contain this element, thus establishing their role in general stress response (Fig. 7). Further, the fact that the majority of these genes are misregulated in *sr1-1* and are implicated in various stress signaling pathways, confirmed the significant role played by SR1 in their regulation. Interestingly, POBO analysis indicated the enrichment of RSRE motif only in the promoter regions of the DE genes that were up-regulated, but not in genes that were down-regulated (Fig.7C). This might be due to increased occurrence of abiotic stress responsive (with exception of cold responsive) genes in up-regulated genes and/or that the negative regulation by SR1 is not mediated through the RSRE element. As evident from Fig. 11A, significant enrichment of GO terms for the abiotic stresses such as "responses to salt stress" and "water deprivation" was observed only in the up-regulated DE genes. Furthermore, enrichment of *VCGCGB* motif was significantly higher in the up-regulated DE genes. Absence of enrichment for *VCGCGB* in the down-regulated DE genes and enrichment for GO terms "response to cold" and "cold acclimation" clearly suggest that SR1 positively regulates cold responsive genes through utilization of *VCGTGT* rather than *VCGCGB* (Fig. 7C, Fig. 8, and Fig. 13). In fact, a significant enrichment of the SR1 binding sites, *VCGTGT* and *VCGCGB,* was noted in the early cold-responsive genes[341].

SR1 Confers Salt Sensitivity by Repressing the Expression of Salt-Responsive Genes

GO analysis of the up-regulated genes that contain SR1 binding sites in their promoters exhibited significant enrichment of a GO term associated with "response to salt stress" (Fig. 11A), suggesting a new role for SR1 in salt tolerance. Interestingly, both mutant lines of *SR1* performed better in terms of root growth under increasing concentrations of NaCl when compared with the

WT and SR1-YFP seedlings. Thus, our results suggest that SR1 acts as a negative regulator of seedling growth under salt stress. This negative regulation of salt stress by SR1 is similar to that observed under biotic stress[316,357] and differs from that of the cold stress response[315], where it functions as a positive regulator. Previously, Galon *et al*., (2010)[327] and Pandey *et al*., (2013) [325] identified SR2/CAMTA1, another member of SR family TF, to be a positive regulator of salt stress. Mutants lacking this TF exhibited increased sensitivity to salt and drought stresses, suggesting that SR1 and SR2 have opposing functions in salt stress[325,327].

In order to resolve the regulation (direct versus indirect) by SR1, salt-responsive genes were identified and subjected to POBO analysis for enrichment of *VCGCGB* in their upstream region. Analysis of the promoters of the salt-responsive DE genes revealed significant enrichment for RSRE (*VCGCGB*) in up-regulated genes (Fig. 13). Hence, it is possible that some of these genes could be direct targets of SR1. Similar analysis of promoters of down-regulated salt-responsive genes did not show enrichment of RSRE, suggesting that i) SR1 utilizes different motifs to regulate expression of these genes and/or ii) other proteins (including other SRs) might activate these genes, whose expression may be regulated by SR1.

As our data showed a negative regulatory role for SR1 in salt stress, we determined the effect of SR1 mutation on the expression levels of the genes associated with the biological process "response to salt stress". Twenty-seven genes associated with this GO term were screened for the presence of SR1 binding sites in their promoters, their expression levels and ability of SR1 to complement their expression in SR1-YFP line. Although several genes fit these criteria (Fig. 11B), *KTI1*, *MYB2*, *MDAR3*, *GLP9* were selected along with *SR1* and their expression levels were determined in different genotypes in response to salt stress. Earlier reports have shown that overexpression of *MYB2* and orthologs of other three genes confer salt tolerance[405,406,413,414].

Exposure to salt stress significantly enhanced their expression levels by two-fold in both WT and SR1-YFP seedlings. In contrast, >12 to 15 fold higher induction of these genes was observed in both mutant alleles of *SR1*. Interestingly, *SR1* expression in WT and SR1-YFP was about 12 to 16 fold higher in salt-treated seedlings as compared to their respective controls (Fig. 12). Since the 35S promoter driving *SR1-YFP* is known to be non-responsive to salt stress, the observed increase in *SR1-YFP* transcript may be due to its increased stability in the presence of salt[415].

Many members of different TF families are known to regulate expression of genes involved in salt stress by binding to the various *cis*-elements in the promoters of salt-responsive genes[404,405]. In response to salt stress, TFs such as G_box_BHLH and N_box_bHLH bind to the *cis*-element *CACGTG* and *CACG[G/A]C,* respectively, and regulate their expression[416,417]. In this study, we analyzed the enrichment for *cis*-elements to which various TFs bind in the upstream regions of salt-responsive DE genes. We compared if there are any differences in the enrichment pattern among the salt-responsive up- and down-regulated DE genes using POBO analysis. We found enrichment ($P < 0.0001$) for G Box (*CACGTG*), N box (*CACGGC*) but not NAC (*CATGTG*) in up-regulated salt-responsive genes (Fig. 15). Only G Box (*CACGTG*) enrichment was noted in the down-regulated genes (Fig. 16). Analysis for co-enrichment of SR1 binding motifs showed enrichment for *ACGTGT, CCGTGT, ACGCGT* and *ACGCGC* in the promoter regions of both up- and down-regulated salt-responsive genes. The observations that i) promoter regions of these genes have 1 to 3 SR1 binding sites, and ii) SR1 binds to promoters of some of these genes (Fig. 17) provide evidence that SR1 directly regulates their expression.

Based on our work we propose a model (Fig. 19) to explain the role of SR1 in salt stress response. Previous studies have demonstrated that exposure of plants to salt stress changes cytosolic Ca^{2+} levels[418]. In addition, Ca^{2+} through CAM has been shown to regulate SR1 activity[316].

Our work showed that AtSR1 either directly and/or indirectly suppresses the expression of salt-responsive genes that are necessary for salt tolerance thereby conferring salt sensitivity. In summary, our results showed that a large number of genes that are associated with biotic and abiotic stress responses are regulated by SR1. A large fraction of these genes (~59%) contain one or more binding sites of SR1 in their promoter region, suggesting that they may be regulated directly by this TF. Our transcriptome analysis revealed a novel role for SR1 in salt stress. By analyzing growth phenotypes and salt-responsive genes we confirmed that SR1 functions in salt stress response. Further, our results showed that SR1 functions as a negative regulator of salt tolerance. These results provide novel insights into the role of SR1 in abiotic stress tolerance in general and salt stress in particular. Future studies using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) should allow identification of direct targets of SR1[419].

Materials and Methods

Plant Materials and Growth Conditions

Three Arabidopsis genotypes were used in this study; WT (Columbia-0), two alleles of *SR1* mutant (*sr1-1*, *sr1-2)* in Col-0 background, and a complemented line (SR1-YFP); and were developed earlier[316]. Surface sterilized seeds were sown in sterilized soil, allowed to germinate and grown for 40 days in a growth chamber at 21$\pm$1ºC with 60% humidity, 200 µmoles/m^2/sec light under day neutral condition. To test salt stress tolerance in these genotypes, surface sterilized seeds were plated on ~70ml of ½ strength MS medium supplemented with 1% sucrose, 0.5 g/L of MES along with 0, 100 or 150 mM NaCl and 0.8% (w/v) Phytoblend in square sterile Petri dishes. The seeds were germinated, and seedlings were grown vertically for two weeks to score for the seedling growth and root length. All genotypes were grown on the same plate to minimize the differences due to any changes in microenvironment. After 14 days, root length was measured and

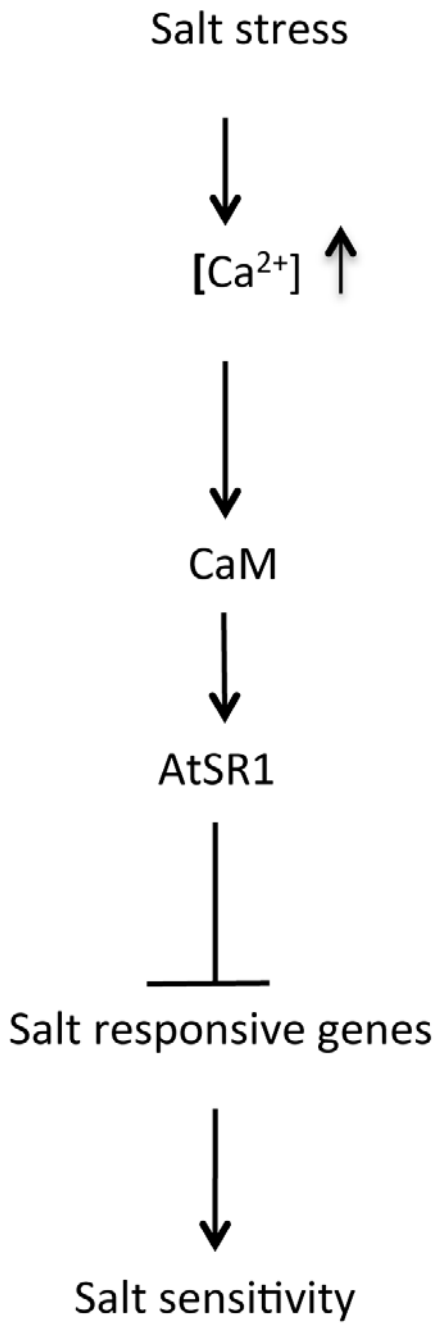


Figure 19. Proposed model for the role of SR1 in salt stress response. (see text for details).

seedlings were photographed. All experiments were performed three times with a minimum of three replicates.

Western Blot Analysis

Leaf material was flash frozen, ground in liquid nitrogen and nuclear extracts were prepared from nuclei preparation essentially as described in Xing *et al*., (2015)[420] with slight modifications. The pellet containing nuclei was resuspended in nuclear lysis buffer and sonicated using Covaris M220 Focused –ultrasonicator for 8 min at 7ºC with settings of peak power 75, duty factor 5 and 200 cycles/burst. The extract was clarified by centrifugation for 10 min at 16,000g at 4ºC. Immunoprecipitation was performed essentially as described in Xing *et al.* , (2015)[420] using Chromotek GFP-TRAP_A beads. Immunoprecipitated protein was separated from beads by boiling at 95ºC for 10 min in 60 µl of 1x SDS loading buffer. Thirty µl of extract was resolved in 12% SDS gels and blotted on to a PVDF membrane. The blot was probed with anti-GFP antibody (sc-8334, Santa Cruz Biotechnology) and detected with secondary antibody conjugated with alkaline phosphatase detection system.

RNA–Seq

Total RNA from leaves (collected at 4 p.m.) of 40-day-old plants of three genotypes was isolated using miRNAeasy kit (Qiagen, USA#217004). Any contaminating genomic DNA was removed using on column DNAse digestion. Ribosomal RNA was removed using a Ribozero Plant kit and the sequencing libraries were prepared from rRNA-depleted samples using TruSeq stranded RNA-seq kit (Illumina) as per manufacturer instructions and single-end sequencing of the library was done at the Genome Sequencing & Analysis Core Resource, Duke University using Illumina Hi seq 2000. All RNA-seq reads were deposited at NCBI in the GenBank sequence read archive (SRA) under the accession number SRP073518.

Mapping of the Reads and Identification of DE Genes

The reads were aligned to the TAIR 10 version of the Arabidopsis genome using TopHat[399] using default settings. The read alignments were assembled into transcriptome assembly using Cufflinks. The assemblies for each replicate were merged together using Cuffmerge utility[399]. Using Cuffdiff tool[399] the aligned reads and merged assembly for each genotype were utilized for calculating the expression level differences of various genes. The DE genes list was computed using Cuffdiff[399]. Those genes that met the following criteria were considered as DE genes: i) The q-value ≤ 0.05, ii) the fold change > 2, and iii) The sum of the RPKM from the comparing genotypes > 10. The common genes that are represented in one or more data sets were identified using the VENNY (http://bioinfogp.cnb.csic.es/tools/venny/) a web-based tool. Heat map of differentially expressed genes was generated using CummeRbund[399]. Box-and-whisker plots of DE genes were generated using the log2 transformed expression values in WT, *sr1-1* and SR1-YFP with JMP Pro12 statistical software. For scatterplot analysis, the FPKM values were log2 transformed and genes with ≥ 1 value were used.

Bioinformatics Analysis to Identify SR1 Binding Motif-Containing Genes

To identify the number of DE genes with SR1 binding motifs *VCGCGB* and *MCGTGT* in their promoter, "Patmatch" (Version 1.1) utility tool (www. arabidopsis.org) was used. This tool identifies the motif on both the strands from the dataset of "*TAIR10 Loci Upstream sequences-1000bp*". 1000bp sequence preceding the TSS was used for this analysis. Up- and down-regulated genes were included as input for scoring both type and number of SR1 binding motifs.

GO Enrichment Analysis

GO analysis was performed for term enrichment using GeneCodis[421]. Single enrichment analysis with TAIR GO annotations was performed using the hyper geometric test with Benjamin-

Hochberg FDR correction with a significance of $P < 0.05$. The genes that are up- or down-regulated for each data set were analyzed separately.

To identify various TFs in the DE genes, a list of all TFs was obtained from Plant TF Database (version 3.0)[403] and all DE genes were queried against the total TF list. TAIR 10 ID of all TF genes was used as input for identifying the genes encoding the TF and classifying them based on the similarity with Total TF family list. The TFs and the genes responsive to various abiotic stress conditions were obtained from STIFB (Stress Responsive TF Database) (http://caps.ncbs.res.in/stifdb2/). Promoters of the genes that contained *cis*-element for binding of the TFs that are implicated in abiotic stress response were retrieved for the analysis. DE genes were queried against the list of the genes for a specific abiotic stress. Further, on the basis of overlap of locus ID (TAIR ID) between the lists of genes, they were further categorized into different subsets.

For the promoter analysis either 500 or 1000 bp upstream of the start codon was extracted from TAIR using an online tool for bulk sequence retrieval. For the estimation of the enrichment for a particular *cis*-elements in the set of promoter sequences (-500 or -1000 bp) were used as input for POBO analysis (http://ekhidna.biocenter.helsinki.fi/poxo/pobo)[422].

Validation of DE Genes Using RT-qPCR Analysis

Primers for validation of DE genes using Real time qPCR (RT-qPCR) were designed using Primer Quest web tool (http://www.idtdna.com/Primerquest/Home/Index) from IDT (USA) (Table 3). Nine DE genes were randomly selected and analyzed for their expression levels using RT-qPCR. cDNA from 40-day-old plants was prepared with SuperScript III first Strand Synthesis kit (Invitrogen), and diluted to 1:5 ratio with sterile nuclease free water, 1.5 µl of the diluted cDNA was used for each reaction. For every qPCR reaction, 5 µl of 2X LightCycler 480 SYBR Green I

Master mix (Roche) was used along with 1µl of 5 µM of each primer in a final reaction volume of 10 µl. For each genotype, cDNA from two independent biological replicates was used. Three technical replicates were used for each sample. RT-qPCR was performed in a Roche LC480 machine (Roche) using the preprogramed "SYBR green-I 96 well program". *ACTIN2* was used as a reference gene as this gene does not exhibit any difference in its expression levels among the various genotypes (Fig. 18). Fold change in expression was calculated and plotted with respect to WT. The expression level in WT for each gene is considered as 1.

RT-qPCR Analysis of Salt-Responsive Genes

Fourteen-day-old control and salt-treated seedlings of different genotypes were collected and flash frozen in liquid nitrogen. The frozen tissues were ground to fine powder in 2 ml microfuge tubes with metal ball bearings. Total RNA was isolated using Trizol and then subjected to DNAse (Promega) treatment to remove any genomic DNA. Two µg of total RNA was used for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) as per manufacturer instructions. The cDNA was diluted 5 times and 2.5µl/reaction was used as a template. Expression analysis was performed using RT-qPCR as described above. The data obtained was normalized with *ACTIN2* and fold change in the expression level was calculated relative to WT control i.e, 0 mM NaCl. The expression level in WT control was considered as 1. A minimum of three technical replicates and three biological replicates were used for each experiment.

RNA isolated from three genotypes was used for cDNA synthesis to analyze the expression of other members of SR family (*SR2-SR6)*. cDNA synthesis, primer design and RT-qPCR analysis were done as described above. The expression levels of the SR genes were normalized with *ACTIN2* and fold change in the expression was calculated relative to WT. The values of WT were considered as 1.

ChIP-PCR

For chromatin immunoprecipitation (ChIP) assays, 15day-old seedlings of WT and SR1-YFP were grown on ½ MS medium with 1% sucrose under 16/8 h day/night cycle at 21°C. ChIP assay was performed as described by Werner Aufsatz with modifications using GFP-Trap_A beads (http://www.abcam.com/protocols/chip-using-plant-samples---arabidopsis). Briefly, nuclear extract was prepared from formaldehyde cross-linked (1%) seedlings of WT and SR1-YFP as above and diluted with ChIP dilution buffer and pre-cleared with bab-20 agarose beads. The pre-cleared nuclear extract was further incubated with GFP–Trap_A beads for 15 h at 4°C on rotatory wheel. The beads were collected by centrifugation and washed sequentially with 1 ml of low salt wash buffer, 1ml of high salt wash buffer, 1 ml LiCl wash buffer and 1ml TE buffer. Each wash was carried out by resuspending the beads in wash buffer and rotating on a wheel at 4°C for 5 min and centrifuging at 2500g for 2 min, and the supernatant was discarded. The protein-DNA complex was eluted twice with 250 µl of elution buffer (1%SDS and 0.1 M $NaHCO_3$) and reverse cross-linked by incubating the eluate at 65°C for 6-8 h followed by 3h of proteinase K treatment at 45°C with gentle shaking. The DNA was purified using phenol:chloroform/isoamyl alcohol and precipitated using absolute ethanol followed by washing with 75% ethanol. Air-dried DNA pellet was resuspended in 70 µl of TE buffer with RNAse A (10 µg/ml). The precipitated DNA was used for qPCR with the primers specific to a region of promoter in the target genes. Data was normalized to DNA input levels as well as *ACTIN2*. The results obtained from four independent ChIP experiments were used to calculate fold enrichment. The values of WT were considered as 1.

Table 3. List of primers used in chapter 2.

S.No.	Name	Sequence
1	AtSR1-FW	CCATTTAAATATGGCGGAAGCAAGA CGATTCAGCCCA
2	AtSR1-RW	CGCGGATCCTTAACTGGTCCACAAAG ATGAGGACATA
3	Q-SR1-FW	CTCGGGAGGAGACTGAAATTG
4	Q-SR1-RW	AGGAGCAACACATTGGAGAATA
5	Q-SR2-FW	GGG TAT GAC TGG GCC ATT AAA
6	Q-SR2-RW	TTT CCT CCC TGC CAC TAA AC
7	Q-SR3-FW	CTC TGT GCC AGT CTT GGA TAC
8	Q-SR3-RW	GAG CAG TCC ACC CTT GTT TAT
9	Q-SR4-FW	CCA ATC TTA GCA GCA GGA GTT A
10	Q-SR4-RW	GAC AAG TAC AGC GAC AGT ATC C
11	Q-SR5-FW	TGG ATT GCA GGA AGA CTC AAA
12	Q-SR5-RW	GGA GCT ACC AGT GCA GAA TAA G
13	Q-SR6-FW	GGG ACC ATC TCT TTG AGC TTA C
14	Q-SR6-RW	CTC CAA GCC CTT TAG AGT CAT ATT
15	AT5G45890-FW	ATGAGGATGTCCCGGTTAATG
16	AT5G45890-RW	GTGAACACACCAGACGAATAGA
17	AT2G41850-FW	CCGGTACAGACAATGGAGTAAG
18	AT2G41850-RW	TTGCTCTTGTCGCAGTAGTC
19	AT3G01420-FW	ACGTCGACTTAGCTGCTTTAG
20	AT3G01420-RW	CTCCGTTAGATCTTCCCACTTG
21	AT3G60140-FW	GACAACGACGACGGTACAAA
22	AT3G60140-RW	CTCTTACGTCACACCCATCTTC
23	AT2G45220-FW	GAAGATCCGACCCGAATCAAA
24	AT2G45220-RW	GTCTCCAAGGTCTACCCAAATAAG
25	AT5G38710-FW	GCCTCAAATCCGTGTGTCTTAG
26	AT5G38710-RW	CCGACCAATACGCCATGTAATC
27	AT2G42540-FW	CTCAGTTCGTCGTCGTTTCT
28	AT2G42540-RW	GTTGAGGTCATCGAGGATGTT
29	AT5G15960-FW	GCTGAGGAGAAGAGCAATGT
30	AT5G15960-RW	CCGCATCCGATACACTCTTT
31	AT1G14250-FW	CAGTCACAGTTTCCTCGACTT
32	AT1G14250-RW	GGGTCTTCAACTATTCCATCCC
33	NDR1-CHIP-FW**	TTGGTTCTTTTTGATAACCCAAAGT
34	NDR1-CHIP-RW**	TTTGGTTTGCTGATTGGTTGATATT
35	EDS1-CHIP-FW**	TGGTTATGCAATTTGGTTTAGCCAA
36	EDS1-CHIP-RW**	ACCGAATTAACTAACTACACCTTCTT
37	ACTIN2CHIP-FW**	GATCCTAGTCTTTTAGTGTGCATTC
38	ACTIN2CHIP-RW**	ATTAAATGATTGATCGGTTTTCGTG
39	ATKTI1-CHIP FW	TTGTAATTTGTCAGGAACGGAGA

40	ATKTI1-CHIP RW	GTGTCCTGACGTGTGGATTT
41	MDAHAR-CHIP FW	TCACGAACGTTATCCCACTAAA
42	MDAHAR-CHIP RW	CATTGGCATTATTCACTCGAATCT
43	HSP90-7-CHIP-FW	TCTCTGGTGAGGAAGGAAGT
44	HSP90-7-CHIP-RW	CACTCATCCGTAGTAGCAATATGT
45	GST1-CHIP FW	TGATCTAACTCGAGCATCCAAC
46	GST1-CHIP RW	CCACAAGAATAGTCCTTCATCTACTA
47	Glycos transf-CHIP FW	ATACGGCTGCTCTTGTTAAGT
48	Glycos transf-CHIP RW	CCACTCATGAATTGGTTACTGATTT
49	GLP9-CHIP FW	AAGTAGTAACAGCCTCTCTCTTTC
50	GLP9-CHIP RW	TTGGGTTGCTTGATTCGTTAAG
51	MYB2-CHIP FW	CGTGATTGCACACAACAAGAAG
52	MYB2-CHIP RW	CACACAGTATCGCAGACGTAAG
53	AT1G15790 FW	TGTTCCAAATCTTGGGCTACAA
54	AT1G15790 RW	CCTCTTCCACAGTCAACAACTC
55	AT1G07530 FW	GGA CCT TACTGGCTTCGTTATG
56	AT1G07530 RW	GGGAGAGATGGTTTGGACTTTG
57	ACTIN2 FW	GGCAAGTCATCACGATTGG
58	ACTIN2 RW	CAGCTTCCATTCCCACAAAC
59	LBa1	TGGTTCACGTAGTGGGCCATCG

** Primers are adopted from Nie *et al*., 2012.

CHAPTER 3

A 500 NUCLEOTIDES REGION IN THE 3' END OF *SR1* OPEN READING FRAME IS REQUIRED FOR ITS ACCUMULATION IN RESPONSE TO CYCLOHEXIMIDE AND SALT STRESS

Summary

Soil salinity, one of the most prevalent environmental stresses, causes enormous losses in global crop yields every year. Therefore, it is imperative to generate salt tolerant cultivars. To achieve this goal, it is essential to understand the mechanisms by which plants respond to and cope with salt stress. Stress-induced reprogramming of gene expression at multiple levels contributes to the survival of plants under adverse environmental conditions. The control of mRNA stability is one of the post-transcriptional mechanisms that is highly regulated under stress conditions leading to changes in expression pattern of many genes. In this study, we show that salt stress increases the level of *SR1* mRNA, which encodes a transcription factor, by enhancing its stability. Multiple lines of evidence indicate that ROS generated by NADPH oxidase activity mediate *SR1* transcript stability. Furthermore, cycloheximide (CHX), a protein synthesis inhibitor, also increased *SR1* mRNA stability, albeit to a higher level than in the presence of salt, suggesting a role for one or more labile proteins in *SR1* mRNA turnover. Similar to salt, ROS generated by NADPH oxidase is also involved in CHX induced *SR1* mRNA accumulation. To gain further insights into mechanisms involved in salt-induced *SR1* stability, the roles of different mRNA degradation pathways were examined in mutants that are impaired in either nonsense-mediated decay (NMD) or mRNA decapping pathway. These studies have revealed that neither the NMD pathway nor the decapping of *SR1* mRNA is required for its decay. However, decapping activity is required for

salt- and CHX- accumulation of *SR1* mRNA. To identify any specific regions within the open reading frame of the *SR1* transcript (~3 kb) that are responsible for the salt-induced accumulation of *SR1* level, we generated transgenic lines expressing several truncated versions of the *SR1* coding region in the *sr1* mutant background. Then, we analyzed accumulation of each version in response to salt stress and CHX. Interestingly, we identified a 500 nts region in the 3' end of the *SR1* coding sequence to be required for both salt- and CHX-induced stability of *SR1* mRNA. Potential mechanisms by which this region confers *SR1* transcript stability in response to salt and CHX are discussed.

Introduction

Soil salinity is one of the most prevalent environmental stresses affecting agriculture and hampers crop productivity in many areas of the world[423,424]. It affects more than 23% of the cultivated land worldwide. Moreover, soil salinity results in 12 billion dollar loss in global agricultural production every year as most of our crop plants are glycophytes, which are highly sensitive to salinity[424-427]. Salinity adversely affects plants by causing osmotic stress, ion toxicity, oxidative stress, altering metabolic processes and membrane disorganization[428-435]. Cell division and cell expansion are also adversely impacted by salt stress. Together, these effects reduce plant growth, development and survival. In addition, soil salinization is predicted to increase in the coming few decades with the expected global climate change[5]. Therefore, to ensure future food security, there is a great need for developing salt tolerant cultivars that can perform well in salinized lands. To achieve this objective, it is vital to understand the mechanisms by which plants respond to salt stress. For this reason, this has been an active area of research during the last several decades[436-440].In order to survive in soils with high salinity, plants have developed various physiological and biochemical mechanisms to exclude salt from their cells or to tolerate its

presence in the cells. These mechanisms include ion transport and uptake[441], ion homeostasis and compartmentalization[442,443], biosynthesis of osmoprotectants and compatible solutes[444-446]. Also, activation of antioxidant enzymes and synthesis of antioxidant compounds[447], as well as hormone modulation[448,449] are involved. Many of these physiological changes result from changes in gene expression guided by salt-induced signal transduction pathways such as Ca^{+2}, ROS, abscisic acid (ABA), and mitogen-activated protein kinases (MAPKs)[450-452]. During the last few decades, a large amount of research has been done to understand the mechanisms of salt tolerance in plants[453]. These studies have identified physiological mechanisms as well as sets of genes and transcription factors that are involved in adaptive responses to salinity stress[454-462]. These data enabled plant biologists to enhance salt tolerance in economically important plants using traditional plant breeding as well as biotechnological approaches. However, the mechanisms underlying salinity tolerance are still far from being completely understood[426].

Genetic engineering has been proved to be an efficient approach to develop salinity-tolerant plants. Overexpression of genes that have been shown to be induced by salt stress or required for stress adaptation is one of the fundamental methods used to improve plant salt tolerance. For example, overexpression of the vacuolar Na^+/H^+ antiporter or genes involved in the synthesis or accumulation of osmoprotectants such as proline[463] or glycinebetaine[464] improves salt tolerance in several plants[21]. Furthermore, manipulation of the genes encoding antioxidant enzymes, such as peroxidase, superoxide dismutase, ascorbate peroxidase and glutathione reductases can also enhance plant salt tolerance[465]. In addition, overexpression of regulatory genes in signaling pathways, such as protein kinases (MAPK, CDPK) also increases plant salt tolerance[466]. From these studies, it is evident that genetic engineering approach will become more

powerful as more candidate genes associated with salinity tolerance are identified and widely utilized[467].

Plants use a wide array of mechanisms to regulate gene expression in response to stresses, including transcriptional, post-transcriptional and translational mechanisms[468,469]. In addition to transcriptional regulation, post-transcriptional mechanisms such as pre-mRNA processing and editing, nuclear export, mRNA localization and stability are also critical for fine-tuning gene expression in eukaryotes[468-472]. Among these post-transcriptional control mechanisms, the control of mRNA stability is a fundamental process that is highly regulated and can be modulated by extracellular and intracellular stimuli changing the expression pattern of many genes[473-476]. Differential control of mRNA stability was found to be critical for the control of gene expression during development and in response to chemical and environmental stimuli[472,477-482]. For example, mRNA stability of the pea photosynthetic electron carrier *ferredoxin1* (*Fed-1*) gene is increased in response to light[483-485]. Also, the mRNA decay rates of α-amylase 3 (*αAmy3*) transcripts, an enzyme that catalyzes the hydrolysis of α-1,4-linked glucose polymers, significantly increases in sucrose-starved cells[486,487]. Furthermore, the osmotic stress-responsive genes in human cells[488], yeast[489-492] and plants[480,493,494] have been demonstrated to be regulated by their mRNA stability.

In mammalian cells, several signal transduction pathways that are involved in regulation of mRNA stability in response to environmental stimuli have been identified including Ca^{2+}-signaling, MAP kinase, c-Jun amino-terminal kinase (JNK), and calcineurin pathways[482]. However, in plants, the mechanisms and signaling pathways regulating mRNA turnover during stress conditions are still not well understood in many cases. Further in-depth studies on elucidating the determinants of mRNA stability in response to stresses will pave the way to engineer the stability of desired transcripts to fine-tune gene expression at the post-transcriptional level. Identification

of sequence elements in mRNA (*cis*-elements) as well as proteins (*trans*-factors) that interact with these *cis*-elements to modulate mRNA stability will open new avenues to develop stress tolerant crops. Consequently, biotechnological approaches can be used to introduce mRNA stabilizing elements in the desired gene and optimize gene expression at the level of mRNA and enhance stress tolerance[495-500].

Recently, the sucrose-non-fermenting 1-related protein kinase 2 (SnRK2) and MAP kinases activated by salt stress were found to be involved in regulation of mRNA stability pathways[488,501-503]. Moreover, transcripts of the majority of osmotic stress-responsive genes are known to have short half lifes[504]. It has been also reported that mutations in the mRNA decay machinery alter plant sensitivity to salinity stress[480]. In addition, osmotic stress alters the activity of the mRNA decapping machinery which subsequently modulates transcript levels of different genes involved in salt stress response[505]. Interestingly, the induced changes in transcript abundance in response to salt stress are not always reflected in the proteome[506,507]. Together, these studies suggest a new layer of regulation in salt-induced gene expression at the posttranscriptional level, especially at the mRNA decay level. However, our understanding of the mechanisms regulating gene expression in response to stress at the posttranscriptional level is still fragmentary.

In our previous study, while investigating the role of SR1 in salt stress response we found that *SR1* transcript level is significantly increased in WT and SR1-YFP complemented Arabidopsis seedlings in response to salt stress. Since the 35S promoter driving *SR1-YFP* is known to be non-responsive to salt stress[508], the observed increase in *SR1-YFP* transcript may be due to its enhanced mRNA stability in the presence of salt. This finding suggests that post-transcriptional control of *SR1* mRNA stability may have a role in plant response to salt stress. In our ongoing efforts to identify the mechanisms that regulate *SR1* expression and functions, the present study is focused

on studying the mechanism(s) that regulates *SR1* mRNA and protein at the posttranscriptional level in the presence of salt stress.

Results

NaCl Treatment Increases SR1 mRNA Level

We have reinvestigated the effect of salt stress on *SR1* mRNA accumulation. As shown in Fig. 20A, treatment of two-week-old seedlings of WT Arabidopsis as well as SR1-YFP complemented line with NaCl (150 mM) for 3h, increased *SR1* transcript levels about 12 times in both lines as compared to untreated seedlings. These results suggest that accumulation of *SR1* transcripts in the presence of salt is likely due to post-transcriptional regulation of mRNA level, possibly mRNA stability, rather than enhanced transcription, as the 35S promoter driving the transcription of *SR1-YFP* has been demonstrated to be non-responsive to salt stress[508]. Furthermore, our results showed that the increase in *SR1* transcript by NaCl is concentration- and time-dependent (Fig. 20B & 20C). A dose-response experiment demonstrated a gradual accumulation of *SR1* mRNA by increasing the concentration of NaCl from 100 to 200 mM. There was no significant increase in *SR1* mRNA accumulation below 100 mM NaCl. The gradual increase in *SR1* transcript level was observed between 100-200 mM NaCl, and reached the maximum at 200 mM (10.89±1.95 fold), then started to decrease at higher concentrations (300 mM), possibly due to cell death (Fig. 20B). Analysis of *SR1* mRNA level at different time points after NaCl treatment showed significant accumulation of *SR1* transcript (5.76±1.45 fold) in 1h and its level continued to increase gradually with time and reached the maximum at 6h (23.54±2.93 fold). Treatment times longer than 6h also showed a significant increase in mRNA level (14.39±1.72 fold), but the fold increase is much less as compared to 6h treatment (Fig. 20C).

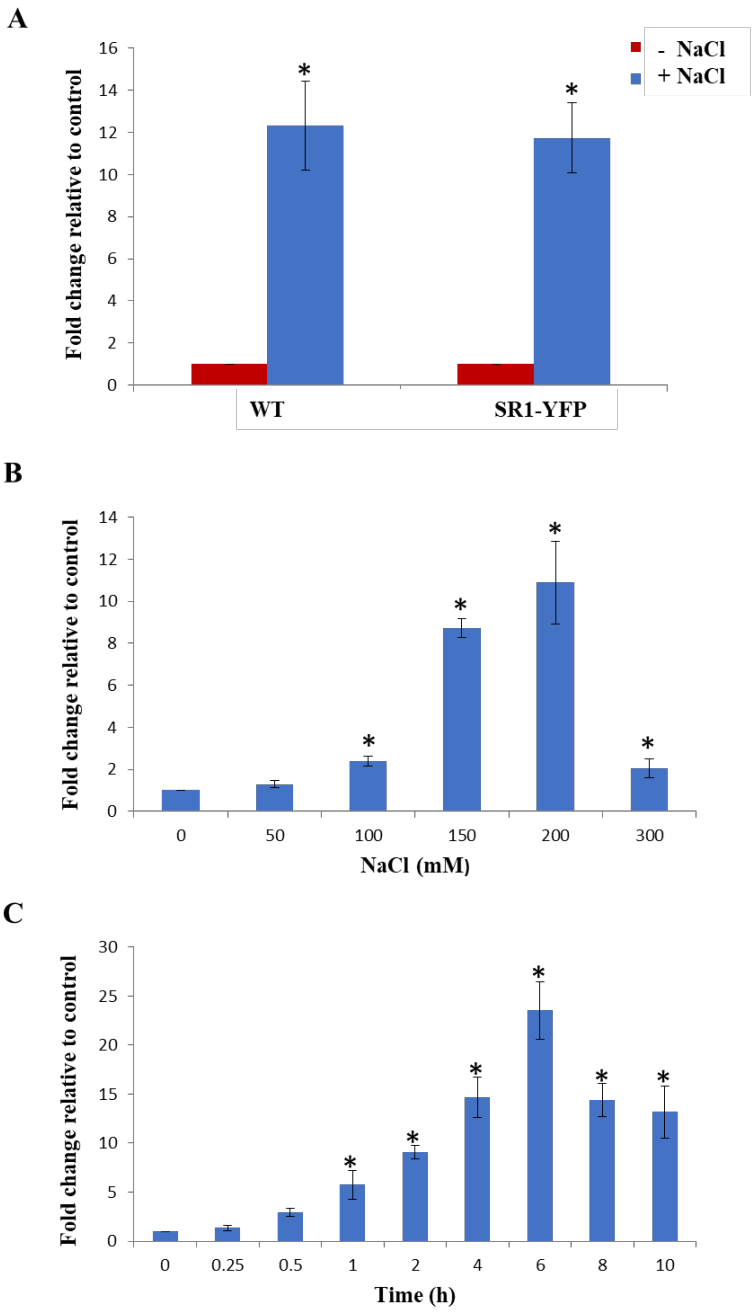


Figure 20. NaCl treatment increases *SR1* mRNA level. **A)** Two-week-old seedlings of WT and SR1-YFP transgenic Arabidopsis lines were treated with NaCl (150 mM) for 3h. **B)** Two-week-old WT Arabidopsis seedlings were treated with different concentrations of NaCl (0-300 mM) for 3h. **C)** Two-week-old WT Arabidopsis seedlings were treated with NaCl (150 mM) for different time periods (0-10h). All figures show fold change in *SR1* mRNA level relative to untreated control based on the RT-qPCR analysis. Untreated control values were set to 1. Three biological replicates were used for each experiment. Student t-test was performed, and the asterisks indicate significant difference ($p < 0.05$) compared with the untreated control. The error bars represent SD

H_2O_2 and paraquat Treatments Increased the Level of SR1 mRNA

Enhancement of *SR1* transcript level by NaCl treatment suggests that common signal molecules elicited by salt stress may mediate the effect of salt on *SR1* mRNA. In particular, it is well established that salt stress induces production of reactive oxygen species (ROS) in plant tissues, which mediate salt stress responses and ultimately allow plants cope with salt stress[509-513]. Hence, it is likely that salt through ROS regulates *SR1* transcript level. To test this hypothesis, we examined the effects of two different ROS-inducing reagents, H_2O_2 and paraquat (PQ), on the accumulation of *SR1* transcript (Fig. 21). Paraquat (methyl viologen) is an herbicide that generated ROS by catalyzing the reduction of O_2 to superoxide anion radicals (O_2^-), which can subsequently form H_2O_2 and hydroxyl radical (OH^-)[514-517]. As shown in Fig. 21, treatments of WT seedlings with either H_2O_2 or PQ enhanced the accumulation of *SR1* mRNA in a dose- and time-dependent manner. H_2O_2 treatment significantly enhanced *SR1* transcript level at concentrations from 2 to 50 mM. The highest increase in transcript level (13.22±1.65 fold) was observed at 10 mM H_2O_2 treatment, then dropped at higher concentrations (Fig 21A). On the other hand, PQ was effective inducer for *SR1* mRNA accumulation at concentrations higher than 2 µM and peaked (9.92±1.18 fold) at 5 µM (Fig. 21B). At higher concentrations, the extent of increase in the transcript was lower than at 5 µM. Although the maximum *SR1* transcript level in H_2O_2 treated seedlings was higher than in PQ treatment, the induction profiles under both treatments were similar, and *SR1* transcript accumulation was detected in both treatments as early as 5min after treatment, peaked at 10-15min, then slowly declined till reached control level by 6h (Fig 21C & 21D). The decrease of *SR1* transcript level at high concentrations and long treatment periods of H_2O_2 or PQ may be attributed to cell death. ROS are highly reactive molecules that can interact with essential cellular molecules such as proteins, DNA, pigments and lipids leading to cell damage and death[518-524].

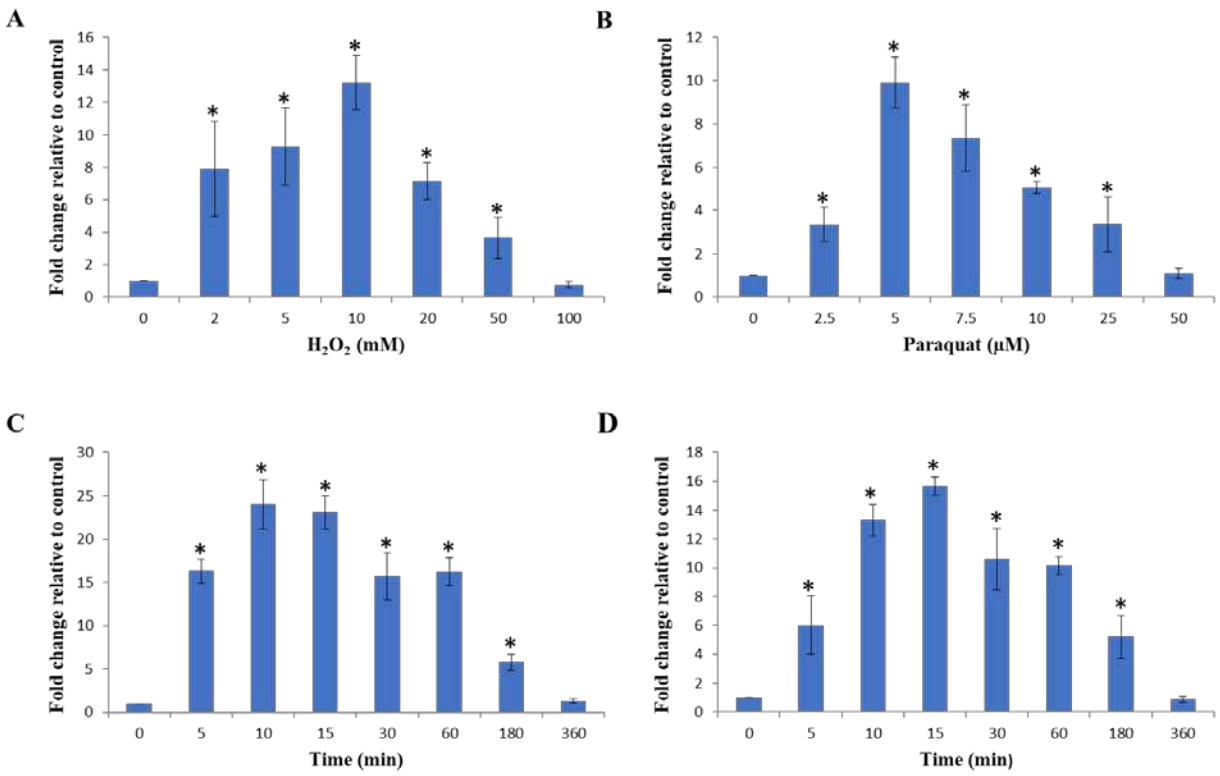


Figure 21. H_2O_2 and paraquat treatment enhanced the level of *SR1* mRNA. Two-week-old WT Arabidopsis seedlings were treated with different concentrations of **A)** H_2O_2 (0-100 mM) or **B)** PQ (0-50 µM) for 1h. **C)** Two-week-old WT Arabidopsis seedlings were treated with 10 mM H_2O_2 or 5 µM PQ (**D**) for different time periods (0-6h). Transcript accumulation was analyzed by RT-qPCR. Fold change in transcript level relative to the untreated control is presented. Untreated control values were considered as 1. Three biological replicates were averaged for each experiment. Student t-test was performed, and the asterisks indicate significant difference ($p < 0.05$) compared with the untreated control. The error bars represent SD.

As in WT plants, H_2O_2 and PQ treatments also enhanced accumulation of *SR1-YFP* transcript (16.41±1.48 fold & 10.22±1.43 fold, respectively) in transgenic lines in which *SR1-YFP* expression is driven by 35S promoter (Fig. 22A). H_2O_2 was previously demonstrated to not affect the expression of genes driven by the 35S promoter[508], which implies that H_2O_2-induced accumulation of *SR1* transcripts is likely due to enhanced mRNA stability rather than enhanced transcription. Collectively, these data suggest that salt-induced stabilization of *SR1* mRNA is mediated by ROS.

ROS Produced by NADPH Oxidase Mediate NaCl-Induced Accumulation of SR1 mRNA

To further confirm our results that salt-induced stabilization of *SR1* mRNA may be mediated by ROS, we tested the effect of the synthetic antioxidant dimethylthiourea (DMTU) on the salt-induced accumulation of *SR1* mRNA. Pre-treatment of WT seedlings with the hydroxyl radical scavenger, DMTU (20 μM) significantly attenuated NaCl-induced *SR1* mRNA accumulation (Fig. 22B), indicating that ROS mediate salt-induced accumulation of *SR1* mRNA. Also, we have examined the involvement of NADPH oxidases in the NaCl-induced accumulation of *SR1* transcript. The plasma membrane-bound NADPH oxidases are the major sources of ROS production during biotic and abiotic stresses[525]. We used diphenylene iodonium (DPI)[526,527], a potent NADPH oxidase inhibitor, to test if ROS generated by NADPH oxidases mediate salt effect on *SR1* mRNA. Pretreatment of seedlings with DPI, significantly reduced NaCl-induced accumulation of *SR1* mRNA (Fig. 22B). These results suggest that ROS generated by NADPH oxidase activity under salt stress likely mediate salt-induced accumulation of *SR1* mRNA.

NaCl Treatment Enhances SR1 mRNA Stability

The salt-induced accumulation of *SR1* mRNA may be attributed to increased transcription, increased mRNA stability, or a combination of both. As mentioned above, the salt-induced

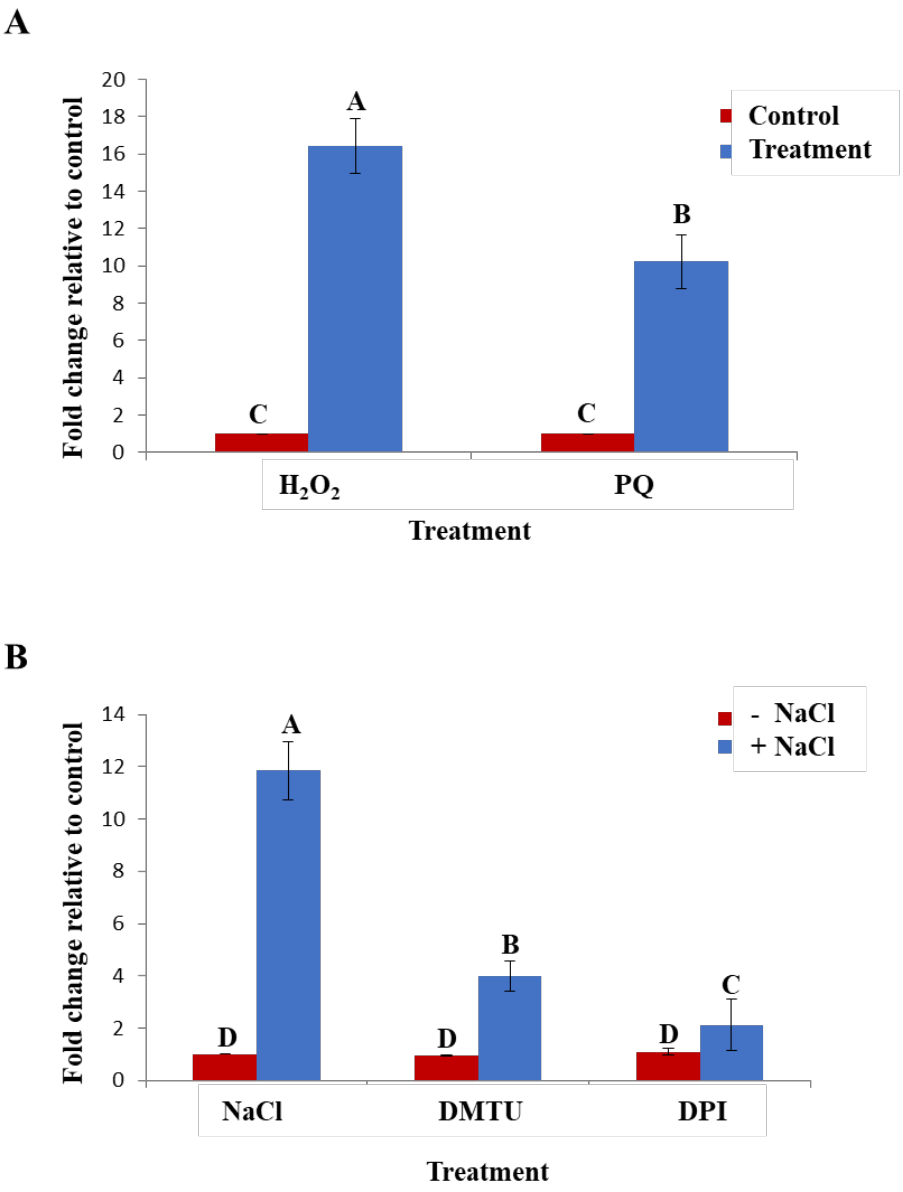


Figure 22. ROS produced by NADPH oxidase mediate NaCl-induced accumulation of *SR1* mRNA. A) Two-week-old seedlings of SR1-YFP complemented line seedlings were treated with H_2O_2 (10 mM) or PQ (5 µM) for 1h. **B)** Two-week-old WT Arabidopsis seedlings were pretreated with DMTU (20 µM) or DPI (100 µM) for 2h followed by incubation for additional 2h with DMTU (20 µM) plus NaCl (150 mM) or DPI (100 µM) plus NaCl (150 mM), respectively. Sole treatments with DPI (100 µM) and DMTU (20 µM) for 4h as well as NaCl (150 mM) for 2h were also performed. The transcript levels of *SR1* were measured by RT-qPCR. Presented is the fold change in *SR1* transcript level relative to its level in untreated control seedlings. *SR1* transcript level in untreated control seedlings was set to 1. The presented values represent the average of three biological replicates, and the error bars represent the SD. Student t-test was performed and significant differences ($P < 0.05$) between treatments and the untreated control are labeled with asterisk.

accumulation of *SR1-YFP* transcript in the complemented line suggests that the salt-enhanced accumulation of *SR1* transcripts is likely due to enhanced mRNA stability rather than enhanced transcription. The 35S promoter driving *SR1-YFP* transcription was previously demonstrated to be non-responsive to salt stress[508]. To further test this hypothesis, we monitored the decline in *SR1* transcript level both in untreated as well as NaCl-treated seedlings in the presence of mRNA synthesis inhibitors, actinomycin D (Act D) and cordycepin (CP). First, two-week-old WT Arabidopsis seedlings were pretreated with NaCl (150 mM) for 2h to induce high levels of *SR1* mRNA and that level was considered as 1. Then, the seedlings were extensively washed three times and incubated for an additional 2h in the presence of NaCl (150 mM), Act D (100 µg/ml) and CP (200 µg/ml), alone or NaCl plus either Act D or CP.

The results in Figure 23 show that NaCl treatment significantly enhanced the accumulation of *SR1* transcript. However, the degradation rate of *SR1* mRNA was rapid as accumulated mRNA degraded and reached background level within 2h after seedlings were removed from NaCl treatment (Fig. 23A & 23B). Moreover, a significant decline in *SR1* transcript level was observed with the sole treatment of Act D or CP. However, when NaCl was combined to Act D or CP, no decline in *SR1* transcript level was observed (Fig. 23A & 23B), suggesting that the degradation of *SR1* mRNA was inhibited by NaCl treatment. Thus, upregulating *SR1* mRNA level by NaCl is attributed to stabilization of *SR1* mRNA rather than enhancing transcription.

New Potein Synthesis is not Required for NaCl-Induced SR1 Transcript Accumulation

Degradation of mRNA is mediated by a large number of proteins including exonucleases, endonucleases, RNA-associated proteins, and sequence-specific RNA-binding proteins[528,529]. Therefore, to elucidate the molecular mechanism controlling the salt-induced stabilization of *SR1* mRNA, we tested the effect of cycloheximide (CHX), a protein synthesis inhibitor, on the salt-

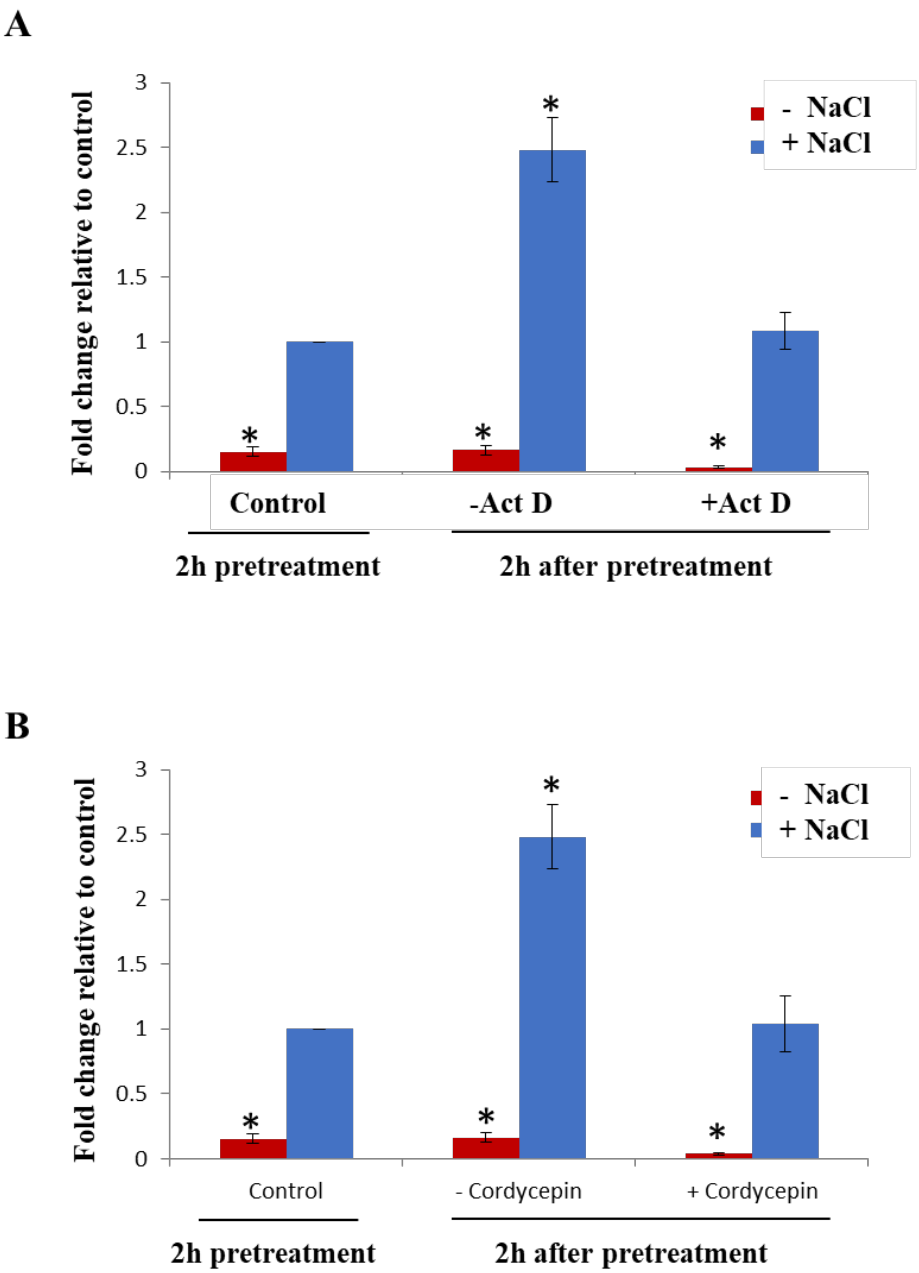


Figure 23. NaCl treatment enhances *SR1* mRNA stability. Two-week-old seedlings of WT Arabidopsis were pretreated with NaCl (150 mM) for 2h. Then, the seedlings were extensively washed three times and incubated for an additional 2h in the presence of NaCl (150 mM) and **A)** Act D (100 µg/ml) or **B)** CP (200 µg/ml), alone or with NaCl. The transcript level of *SR1* was measured by RT-qPCR. Presented is the fold change in *SR1* transcript level relative to its level after 2h of NaCl pretreatment. *SR1* transcript level after 2h of NaCl pretreatment was considered as control and was set to 1. The presented values represent the average of three biological replicates, and the error bars represent the SD. Student t-test was performed and significant differences ($P < 0.05$) between treatments and the control are labeled with asterisks.

induced accumulation of *SR1* transcript to investigate whether new protein synthesis is required for that induction (Fig. 24A). Surprisingly, the effect of salt on *SR1* mRNA accumulation was substantially potentiated by CHX (100 µM) treatment resulting in about 16-fold higher level of *SR1* mRNA than salt treatment alone. Moreover, treatment with CHX alone resulted in high accumulation of *SR1* transcript (25.77±2.49 fold) as compared to untreated seedlings. The level of *SR1* mRNA in response to CHX treatment (25.77±2.49 fold) was significantly higher than that in response to NaCl treatment (13.54±2.19 fold) (Fig. 24A). Interestingly, cotreatment with salt and CHX significantly increased *SR1* mRNA to a level equal to its accumulation level observed with CHX alone and it did not reach a level equivalent to the sum of the levels induced by separate salt and CHX treatments. These results suggest that salt and CHX induce accumulation of *SR1* mRNA by the same mechanism, which is further activated by CHX. Another possibility is that CHX induces *SR1* transcript accumulation by two different mechanisms, and one of them is shared with salt.

Additionally, CHX treatment induced accumulation of the *SR1-YFP* transcript in the SR1-YFP complemented line (Fig. 24B). However, the accumulation level of *SR1* transcript in the transgenic seedlings (34.64±3.61 fold) was significantly higher than that in WT seedlings (28.25±3.62 fold) (Fig. 24B). Together, these results suggest that the salt-inducible accumulation of *SR1* mRNA does not require new protein synthesis and that *SR1* mRNA may be negatively regulated by a labile repressor that is susceptible to inhibition of protein synthesis.

CHX-Induced Accumulation of SR1 Transcript is Dose- and Time-Dependent

As shown in Fig. 25, CHX induces *SR1* transcript level in a dose- and time-dependent manner. *SR1* mRNA was gradually elevated when the concentration of CHX increased from 10µM to 500 µM (Fig. 25A). Induction of *SR1* mRNA was clearly detectable (7.94±1.16 fold) at 10 µM,

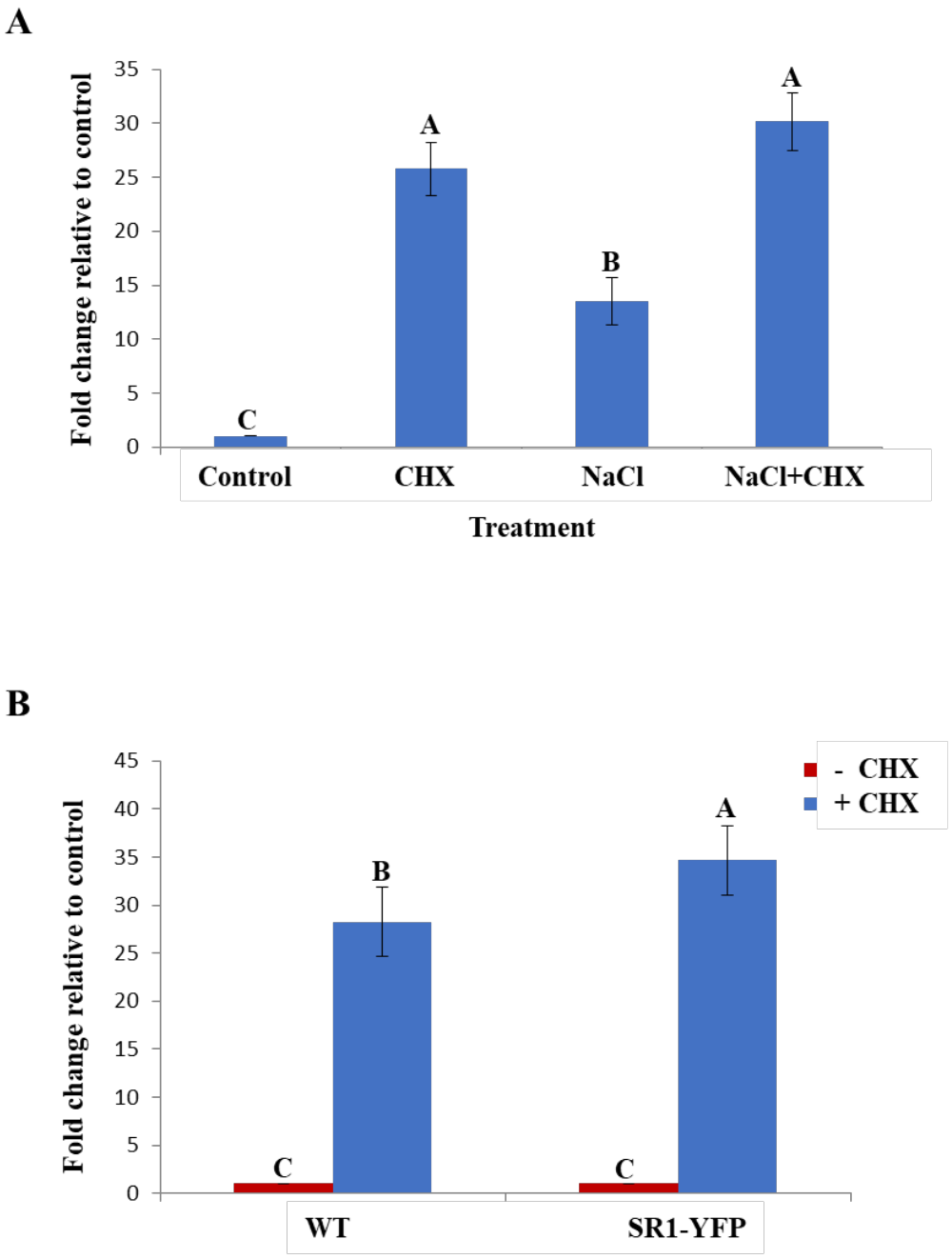


Figure 24. Induction of *SR1* mRNA by CHX. A) Two-week-old WT Arabidopsis seedlings were treated with NaCl (150 mM) and CHX (100 µM) for 3h, alone or combined **B)** Two-week-old Arabidopsis seedlings of WT and SR1-YFP complemented line were treated with CHX (100 µM) for 3h. The transcript level of *SR1* was measured by RT-qPCR. All figures show fold change in transcript level relative to untreated control based on the RT-qPCR analysis. Untreated control values were set to 1. Three biological replicates were used for each experiment. Student t-test was performed, and the different letters indicate significant difference ($p < 0.05$) compared with the untreated control. The error bars represent SD.

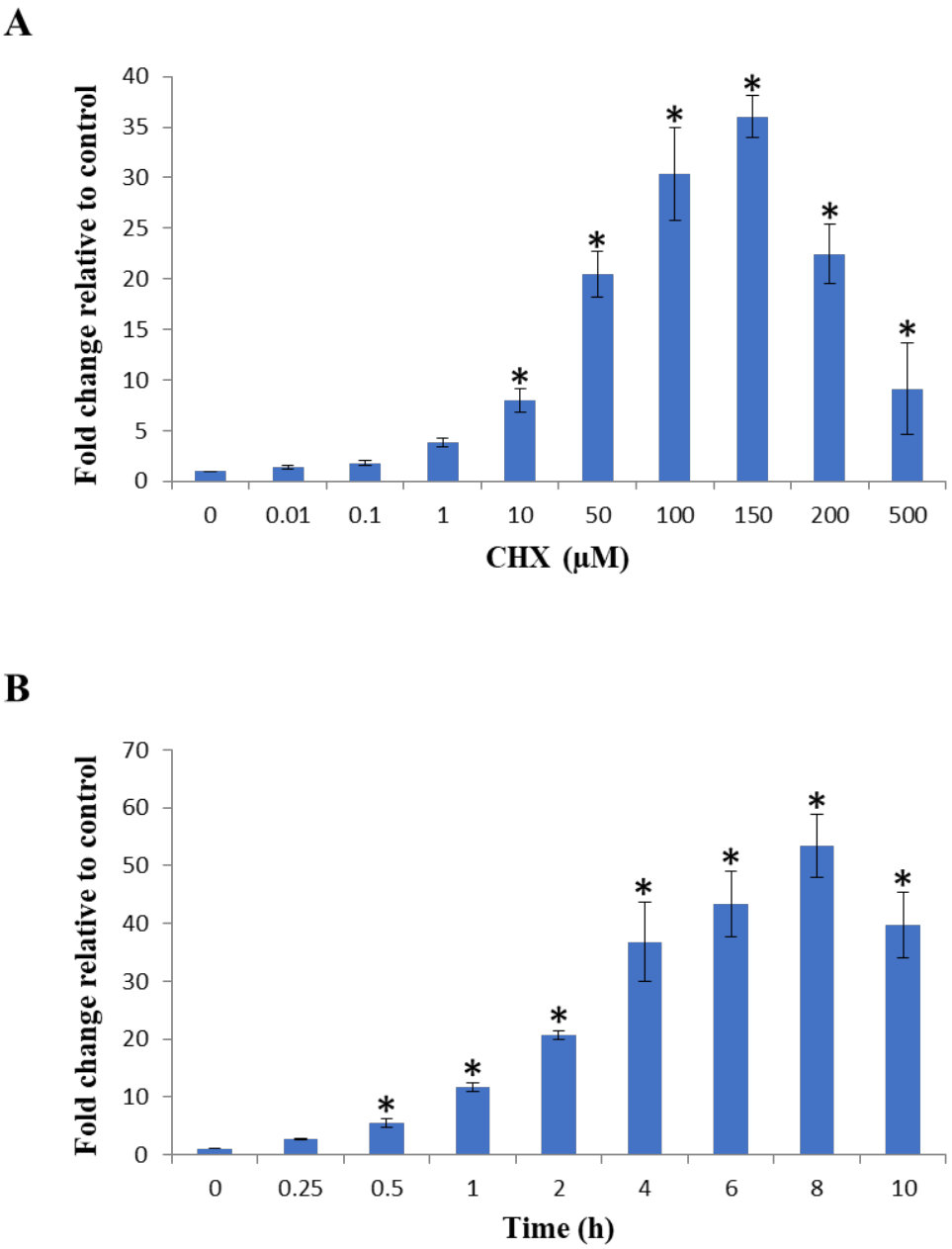


Figure 25. CHX-induced accumulation of *SR1* transcript is dose- and time-dependent. **A)** Two-week-old WT Arabidopsis seedlings were treated with different concentrations of CHX (0-500 µM) for 3h. **B)** Two-week-old WT Arabidopsis seedlings were treated with CHX (100 µM) for different time periods (0-10h). Transcript accumulation was analyzed by RT-qPCR. Fold change in transcript level relative to the untreated control is presented. Untreated control transcript levels were considered as 1. Three biological replicates were averaged for each experiment. Student t-test was performed, and the asterisks indicate significant difference ($p < 0.05$) compared with the untreated control. The error bars represent SD.

and reached the highest level (36.04±2.02 fold) at 150 µM, but was reduced at concentrations larger than that, possibly due to toxicity to the cell. Furthermore, a time-course study revealed that induction of *SR1* transcript could be detected as early as 30min (5.53±0.73 fold) after CHX treatment and was maximally induced at 8h (53.43±5.46 fold), then declined at longer treatment periods, possibly due to toxicity and cell death (Fig. 25B). For further experiments, we used 100 µM CHX and 3h treatment period to reduce the risk of cell toxicity.

CHX Treatment Enhances SR1 mRNA Stability

To get further insight into the mechanism by which CHX-induced accumulation of *SR1* mRNA, we asked whether this accumulation is also due to enhanced mRNA stability or it is attributed to enhanced transcription. To address this question, we preincubated two-week-old WT Arabidopsis seedlings for 2h with CHX (100 µM) to induce high levels of *SR1* mRNA. The *SR1* transcript level at the end of 2h of CHX pretreatment was set as 1. Then the seedlings were extensively washed three times and incubated for an additional 2h in the presence of CHX (100 µM), Act D (100 µg/ml) and CP (200 µg/ml), alone or in combinations. As shown in Fig 26, a significant decline in *SR1* transcript level was observed with the sole treatment of Act D or CP. However, when CHX was combined with Act D or CP, no decline in *SR1* transcript levels was observed (Fig. 26A & 26B). Thus, like NaCl treatment results, upregulating *SR1* mRNA level by CHX is attributed to stabilization of *SR1* mRNA rather than enhancing transcription.

Puromycin Treatment Induces Accumulation of SR1 mRNA

Similar to our results, transcripts of other stress-inducible genes were found to be also potentiated by treatments with different protein synthesis inhibitors (PSI)[530-535]. Several studies have been performed to elucidate the mechanisms regulating induction of those transcripts by PSI. These studies revealed that induction of transcripts of many of those genes was attributed to protein

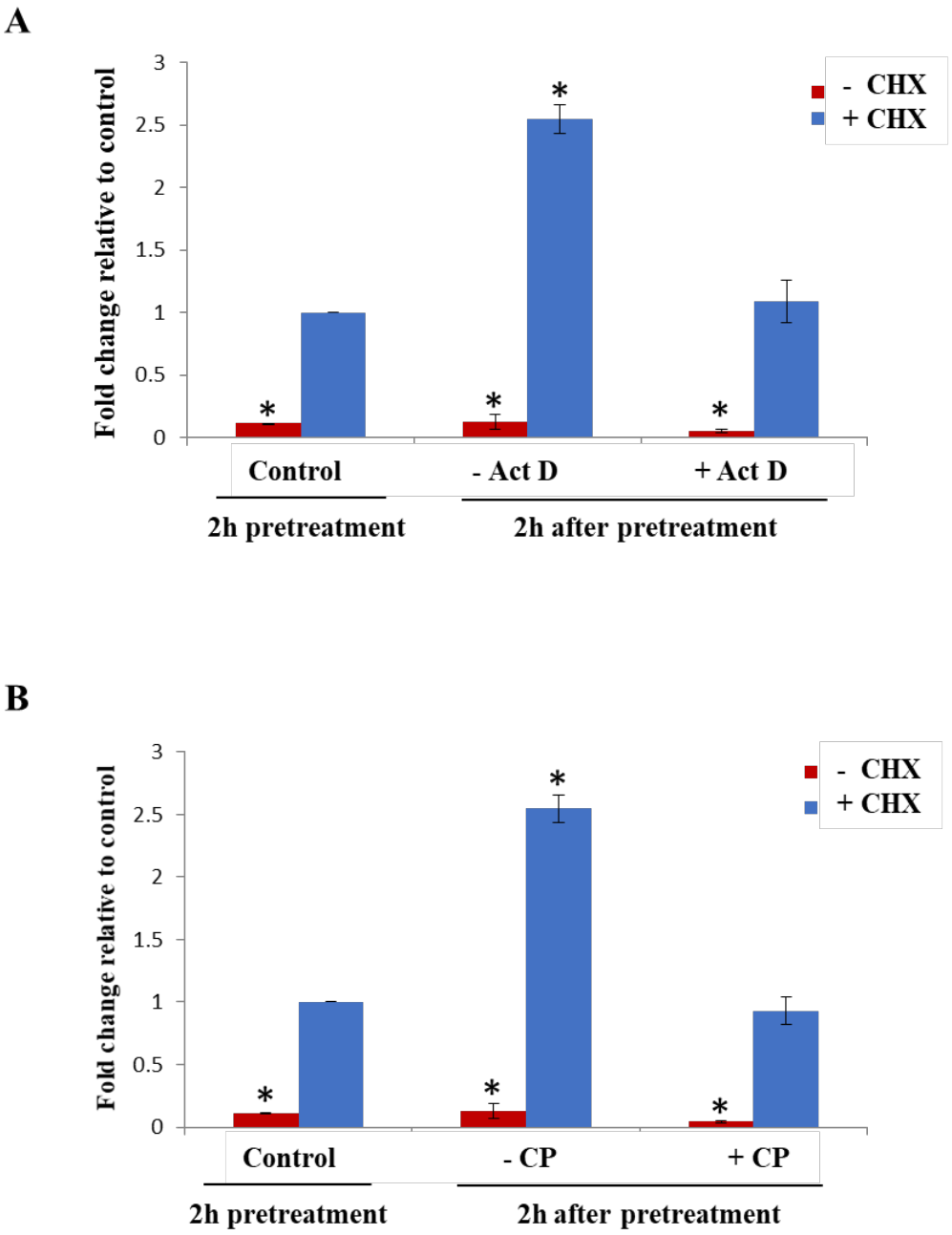


Figure 26. CHX treatment enhances *SR1* mRNA stability. Two-week-old seedlings of WT Arabidopsis were pretreated with CHX (100 µM) for 2h. Then, the seedlings were extensively washed three times and incubated for an additional 2h in the presence of CHX (100 µM) and Act D (100 µg/ml) (**A**) or CP (200 µg/ml) (**B**), alone or in combinations. The transcript level of *SR1* was measured by RT-qPCR. Fold change in *SR1* transcript level relative to its level after 2h of CHX pretreatment is presented. *SR1* transcript level after 2h of CHX pretreatment was considered as control and was set to 1. The presented values represent the average of three biological replicates, and the error bars represent the SD. Student t-test was performed and significant differences ($P < 0.05$) between treatments and the control are labeled with asterisks.

synthesis inhibition[536,537]. The induction of many other genes was related to the ability of PSI to interact and regulate the activities of molecules involved in different signaling pathways[533,538,539]. For example, besides being a cytoplasmic protein synthesis inhibitor, CHX also functions as an agonist activating signaling pathways, which in turn regulates the expression of genes responsive to extracellular stimuli such as wounding and low temperature[539-542].

To test whether CHX here acts as a protein synthesis inhibitor or as a signal for the induction of *SR1* mRNA, we examined the effect of another cytoplasmic protein synthesis inhibitor, puromycin (PUR), on the accumulation of *SR1* transcript. Our results from this experiment showed that PUR (100 µg/ml) treatment induces *SR1* transcript accumulation only 27-40% of the CHX-induced mRNA levels (Fig 27). Because puromycin inhibits protein synthesis with a similar potency to CHX[537], this result suggests that protein synthesis inhibition contributes to only small part of *SR1* mRNA induction by CHX and another mechanism is involved. In support of this notion, our previous results (Fig. 25A) showed induction of *SR1* mRNA by concentration of 10 µM CHX which was previously demonstrated not to be inhibitory to protein synthesis depending on [35S] methionine incorporation into proteins[539,543,544]. The partial induction of *SR1* mRNA level by protein synthesis inhibition suggests that CHX treatment might inhibit the synthesis of a ribonuclease or other labile proteins involved in *SR1* mRNA decay.

NMD Pathway is not Involved in SR1 mRNA Degradation

It has been previously demonstrated that inhibition of protein synthesis by CHX blocks one of the major mRNA surveillance mechanisms, nonsense-mediated mRNA decay (NMD), in plants and animals[545-548]. In addition, NMD pathway has been widely linked to plant-stress responses as several biotic as well as abiotic stresses inhibit NMD pathway in plants[549-555]. Interestingly, salt stress was one of the abiotic stresses demonstrated to inhibit NMD pathway[555]. These data

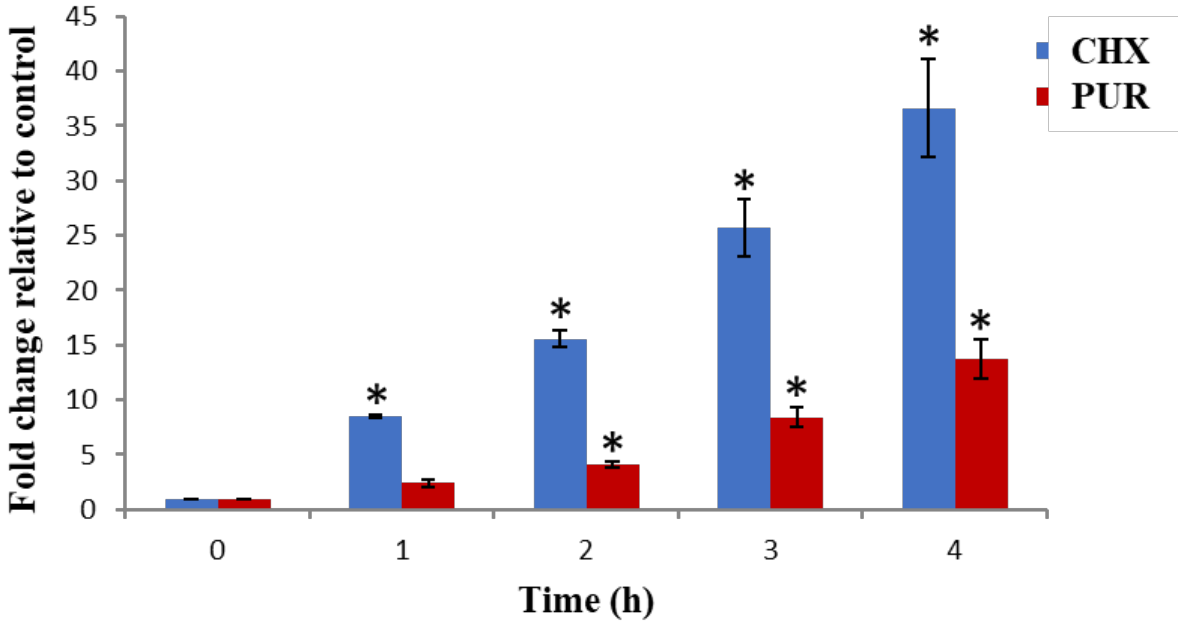


Figure 27. Puromycin treatment induces accumulation of SR1 mRNA. Two-week-old WT Arabidopsis seedlings were treated with CHX (100 µM) or PUR (100 µg/ml) for different time periods (0-3h). All figures show fold change in transcript level relative to untreated control based on the RT-qPCR analysis. Untreated control transcript levels were considered as 1. Three biological replicates were used for each experiment. Student t-test was performed and significant differences ($P < 0.05$) between treatments and the control are labeled with asterisks. The error bars represent SD.

prompted us to examine if NMD pathway is involved in the degradation of *SR1* mRNA and its inhibition by salt and CHX results in *SR1* transcript stabilization by these two factors. NMD is a translation-dependent mRNA degradation pathway that recognizes and degrades aberrant transcripts with premature translation termination codon, thereby preventing accumulation of harmful truncated proteins[556,557]. Besides functioning as a quality control mechanism, NMD can also regulate gene expression by degrading specific normal transcripts, which have sequence features that induce premature translation termination and activates mRNA decay by NMD[558-560]. Those NMD-inducing features include premature termination codon (PTC)[561-563], long (≥300–350 nts) 3′ untranslated regions (UTRs)[564-567], introns ≥ 50–55 nts downstream of termination codon[564,566,568], or upstream open reading frames (uORFs)[550,567,569,570].

We analyzed *SR1* gene sequence for the presence of any of the NMD-inducing features, but didn't find any, suggesting that *SR1* transcript may not be a direct target for NMD degradation. However, not all mRNAs which have been identified to be direct targets for NMD pathway have these features, there are additional features still to be identified[571]. Also, there is a possibility that NMD indirectly regulates *SR1* mRNA decay, i.e. NMD might be involved in the degradation of the mRNA encoding a protein that stabilizes *SR1* mRNA. To determine if NMD pathway is involved in the degradation of *SR1* mRNA directly or indirectly, we utilized a loss-of-function mutant of *UPF3*[547,572]. A functional NMD pathway requires the presence of three core factors, the up-frameshift proteins UPF1, UPF2, and UPF3 that form the core NMD machinery[562,573-576]. Silencing of any one of these proteins leads to stabilization of mRNAs that are directly or indirectly regulated by NMD[547,577]. We analyzed the abundance of *SR1* mRNA in two-week-old seedlings of *upf3* mutant and WT untreated controls as well as *upf3* mutant and WT treated with salt or CHX. If NMD pathway is involved in *SR1* mRNA degradation, one would expect an accumulation of

SR1 transcript in the untreated *upf3* mutant to a level equivalent to those in salt or CHX treated seedlings. However, our results showed that *SR1* transcript accumulated only in salt and CHX treated WT and *upf3* seedlings but not in the untreated *upf3* mutant, indicating that NMD pathway is not involved in *SR1* mRNA degradation. It should be noted that *SR1* induction levels were similar in *upf3* mutant and WT seedlings in response to salt or CHX treatments indicating that NMD pathway is not required for salt- or CHX- induction of *SR1* transcript (Fig. 28A).

Decapping is not Involved in SR1 mRNA Decay

The mRNA is usually protected from exonucleases by the poly-adenosine [poly(A)] tail and the methyl-7-guanosine cap at its 3'- and 5'- ends, respectively[578-582]. Generally, the major mRNA degradation pathway in plants initiates by shortening of the 3' poly(A) tail followed by degradation of the transcript 3' to 5' direction by the exosome complex. Alternatively, and more predominant, deadenylation activates the removal of the 5' cap and the transcript is degraded 5' to3' direction by the exonuclease XRN4[583]. It has been previously demonstrated that salt stress alters the activity of the mRNA decapping machinery, which subsequently modulates transcript levels of different genes involved in salt stress response[505]. Additionally, it has been reported that blocking translation elongation by CHX stabilizes mRNA by preventing decapping. In this regard, inhibition of translation by CHX treatment stabilizes Expansin-Like1 (*EXPL1*) and *SEN1* transcripts in Arabidopsis and the accumulated transcripts were found in the capped form. Therefore, we examined if decapping is required for *SR1* mRNA degradation or not. We have analyzed *SR1* transcript levels in NaCl and CHX treated and untreated Arabidopsis seedlings of WT and a decapping mutant, *vcs-6*[478] (Fig. 28B).

In Arabidopsis, removal of the 5'cap is catalyzed by the activity of DECAPPING 2 (DCP2) enzyme. DCP2 is only active when it binds to its activator DECAPPING1 (DCP1) while

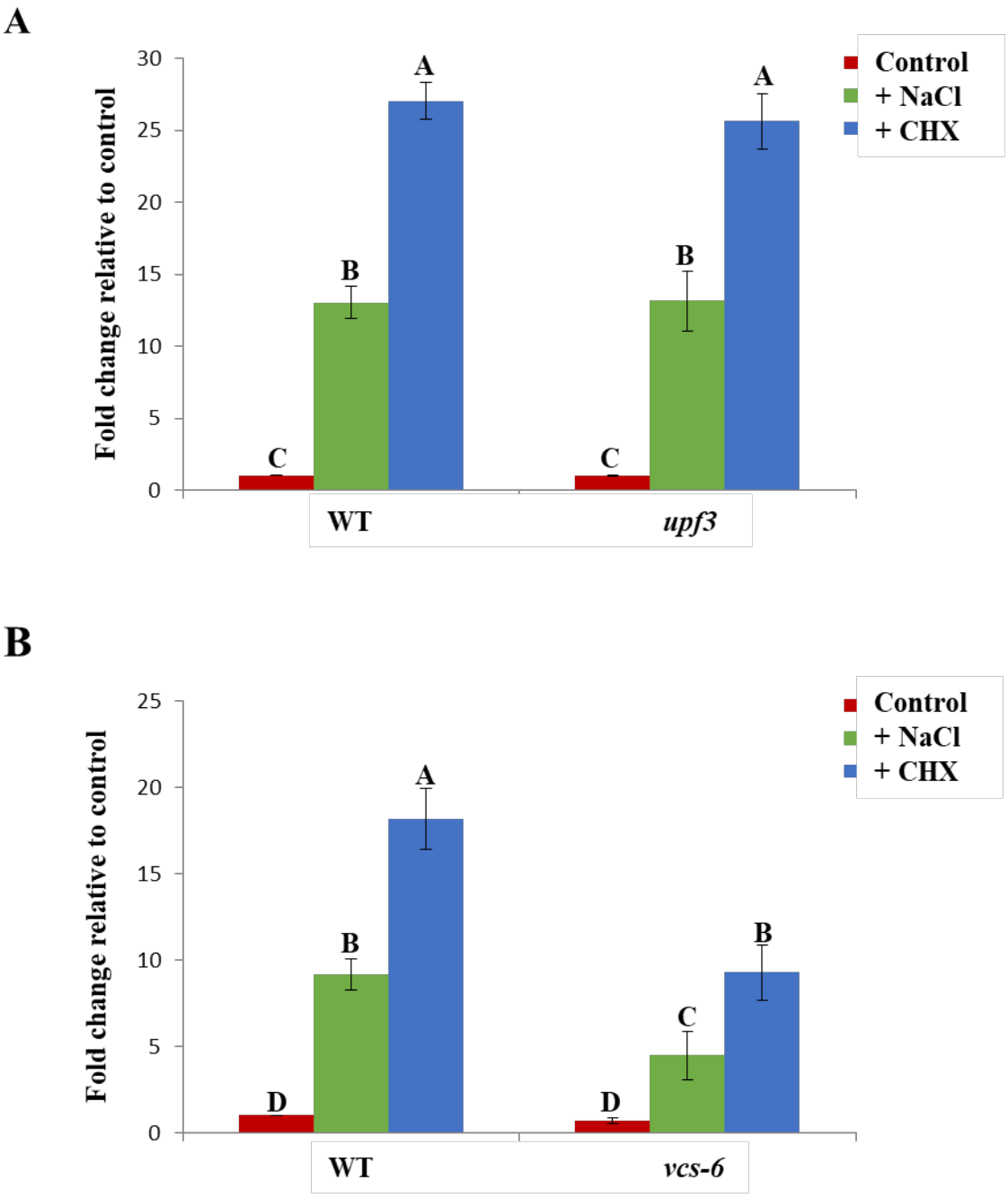


Figure 28. NMD pathway and decapping are not involved in degradation of *SR1* mRNA. Two-week-old WT Arabidopsis as well as *upf3* mutant **(A)** or *vcs-6* mutant **(B)** seedlings were treated with CHX (100 µM) or NaCl (100 mM) for 3h. Presented is the fold change in transcript level relative to untreated WT control based on the RT-qPCR analysis. Untreated WT control transcript levels were considered as 1. Three biological replicates were used for each experiment. Student t-test was performed and significant differences ($P < 0.05$) between treatments and the control are labeled with different letters. The error bars represent SD.

VARICOSE (VCS) is the scaffolding protein enhancing this interaction[478]. Therefore, all three proteins are essential for transcript decapping. Loss of any of these three proteins was found to inhibit mRNA decapping *in vivo* [478]. In this study, we used VCS knockout mutant (*vcs-6*), which was previously demonstrated to accumulate decapped transcripts[478]. Our results showed that *SR1* transcript level in untreated *vcs* mutant was similar to its level in untreated WT seedlings, indicating that decapping is not involved in *SR1* mRNA decay (Fig. 28B). However, a knockout mutation in *VCS* significantly reduced the accumulation of *SR1* transcript in response to salt and CHX (Fig. 28B). These results indicate that mRNA decapping activity is partially required for salt- and CHX-induced accumulation of *SR1* mRNA.

ROS Produced by NADPH Oxidase Mediate CHX-Induced Accumulation of SR1 mRNA

The partial induction of *SR1* mRNA by PUR compared to CHX as well as its induction by the subinhibitory concentration of CHX (Fig. 27 & Fig. 25A) suggest that protein synthesis inhibition contributes to only small part of *SR1* mRNA induction by CHX and another mechanism is involved. Besides being a cytoplasmic protein synthesis inhibitor, CHX also has been demonstrated as an agonist activating signaling pathways that regulate stress-responsive gene expression[539-542]. Hence, we were interested to investigate if ROS mediate the regulation of *SR1* transcript level in response to CHX as it does for salt. To test this, we examined the effect of the antioxidant DMTU as well as the NADPH oxidase inhibitor, DPI on the CHX-induced accumulation of *SR1* transcript. As shown in Fig. 29A, pre-treatment of WT seedlings with the DMTU (20 µM) or DPI (100 µM) significantly decreased the CHX-induced accumulation of *SR1* mRNA, indicating that ROS also mediate CHX-induced accumulation of *SR1* mRNA. Moreover, DMTU and DPI treatments significantly attenuate the superinduction of salt-induced accumulation of *SR1* transcript by CHX (Fig. 29B).

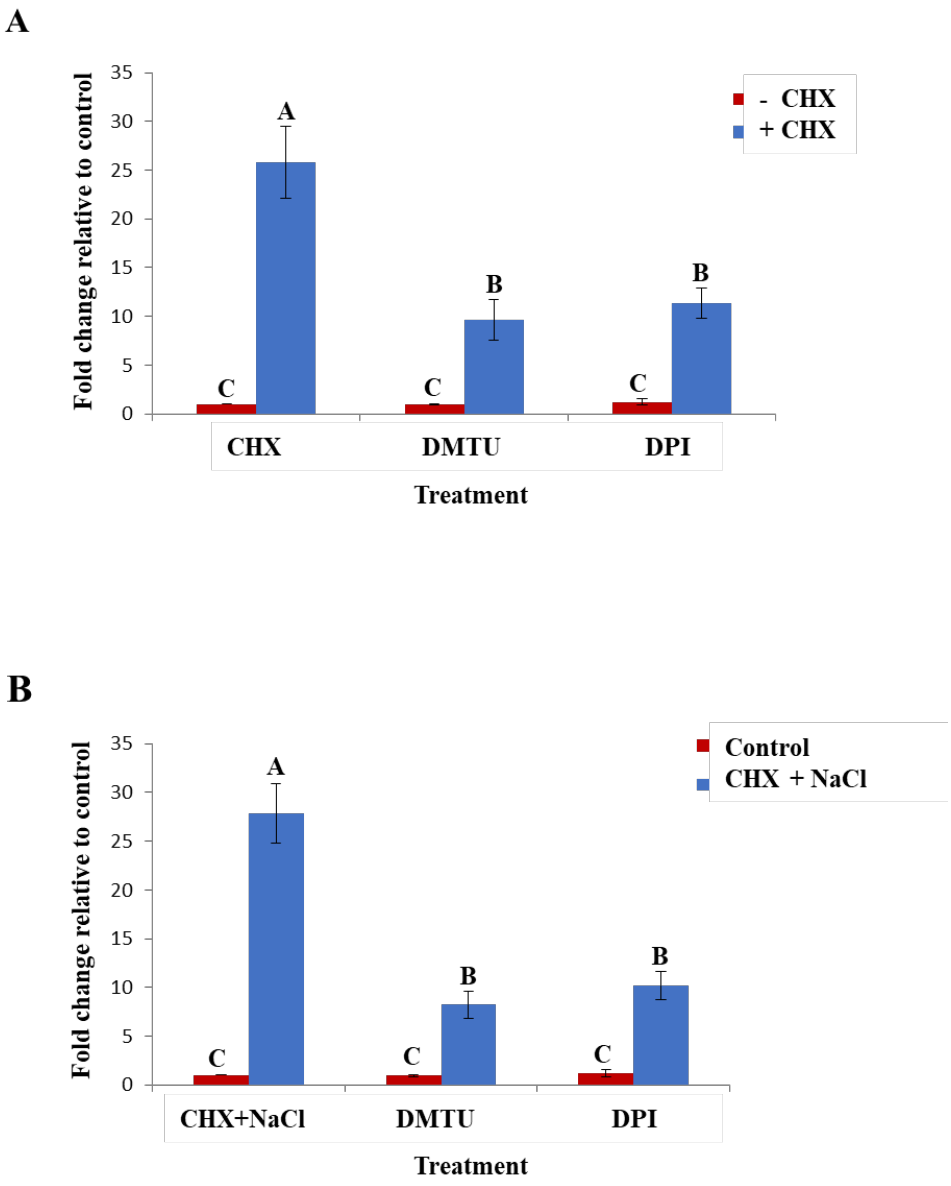


Figure 29. Reactive oxygen species produced by NADPH oxidase mediate NaCl-induced accumulation of *SR1* mRNA. Two-week-old WT Arabidopsis seedlings were pretreated with DMTU (20 µM) or DPI (100 µM) for 2h followed by incubation for additional 2h with **A)** DMTU (20 µM) plus NaCl (150 mM) or DPI (100 µM) plus NaCl (150 mM), respectively. OR **B)** DMTU (20 µM) plus NaCl (150 mM) plus CHX (100 µM) or DPI (100 µM) plus NaCl (150 mM) plus CHX (100 µM). Sole treatments with DPI (100 µM) or DMTU (20 µM) for 4h as well as NaCl (150 mM) or NaCl plus CHX for 2h were also performed. The transcript level of *SR1* was measured by RT-qPCR. Presented is the fold change in *SR1* transcript level relative to its level in untreated control seedlings. *SR1* transcript level in untreated control seedlings was set to 1. The presented values represent the average of three biological replicates, and the error bars represent the SD. Student t-test was performed and significant differences ($P < 0.05$) between treatments and the untreated control are labeled with different letters.

A 500-nts Region at the 3' End of SR1 Open Reading Frame is Required for its Stability

The stability of mRNA is determined by structural elements found at the 5′- and 3′- ends (the 7- methyl-G cap and the polyadenylate [poly(A)] tail, respectively) [584,579] as well as specific sequence elements (*cis*-acting elements) within the transcript and trans-acting factors. Contrary to the 5′-cap and poly(A) structures that are found on all mRNAs, *cis*-acting elements are specific sequence motifs found only in a subset of transcripts. These *cis*-acting elements can be located within the 5′-untranslated region (UTR), ORF, and 3′-UTR regions of the transcript and modulate the stability of mRNA by binding to RNA-binding proteins (*trans*-acting factors), which have a stabilizing or destabilizing effect[473]. In this study, the accumulation levels of SR1-YFP transcript in transgenic line in response to salt and CHX were equivalent to the levels induced in WT seedlings. These results suggest that salt- and CHX induced stabilization of *SR1* mRNA is likely attributed to *cis*-elements in the coding region of *SR1* rather than the UTRs as the expression cassette of *35S:SR1-YFP* contained only the coding region of *SR1* cDNA. To identify the *cis*-element(s) responsible for salt- and CHX-induced *SR1* mRNA stability, we generated Arabidopsis transgenic lines expressing the coding region for the N-terminus (amino acids 1–517; nucleotides 1-1551) or C-terminus (amino acids 518–1034; nucleotides 1549-3099) of SR1 protein in the *sr1* mutant background. As shown in Fig. 30A, only the C-terminus transcript but not N-terminus transcript accumulated in response to NaCl or CHX treatments. These results suggest that the *cis*-element responsible for salt- and CHX-induced stability of *SR1* mRNA are present in the region encoding the C-terminus of SR1 protein. We further generated 3 truncated versions (500 nts each) of each of the N- and C-terminus versions and expressed each in the *sr1* mutant. Then, we tested the accumulation of each of these truncated transcripts in response to salt or CHX. Among all of these truncated versions, only one fragment (nucleotide 2584-3099) at the 3' end of *SR1* coding

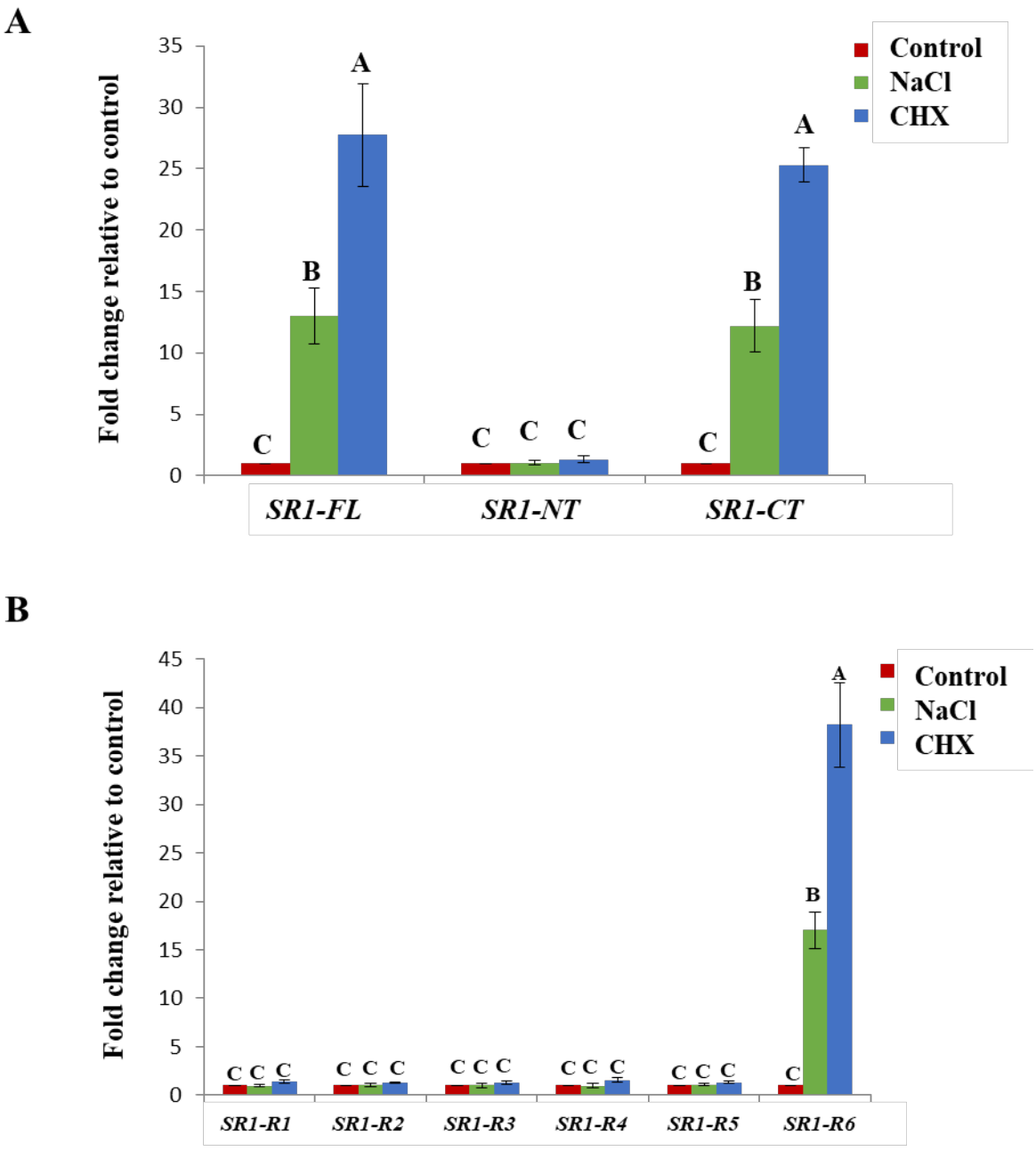


Figure 30. A 500-nts region at the 3' end of *SR1* open reading frame is required for its stability. Two-week-old seedlings expressing **(A)** *SR1-FL*, *SR1-NT* or *SR1-CT* and **(B)** truncated versions of *SR1-NT* (*SR1-R1*, *SR1-R2*, *SR1-R3*) or *SR1-CT* (*SR1-R4*, *SR1-R5*, *SR1-R6*) were treated with CHX (100 µM) or NaCl (100 mM) for 3h. Fold change in transcript level relative to untreated control based on the RT-qPCR analysis is presented. Untreated control transcript levels were considered as 1. Three biological replicates were used for each experiment. Student t-test was performed and significant differences ($P < 0.05$) between treatments and the control are labeled with different letters. The error bars represent SD.

region confers salt and CHX-inducibility (Fig. 30B). It should be noted that all other truncated versions, which lack this salt and CHX-inducible region did not accumulate the truncated transcript neither in response to CHX nor under normal growth conditions. As the absence of this salt and CHX-inducible region did not confer stability of other truncated mRNA versions under normal growth conditions, therefore this region responsible for increased stability in response to NaCl and CHX, but is not responsible for *SR1* mRNA instability under normal growth conditions.

CHX-Induced SR1 Transcript Accumulation is Reflected at the Protein Level

To determine if the salt- or CHX- induced accumulation of *SR1* transcript is reflected at the protein level, we treated two-week-old WT seedlings with NaCl (150 mM) and CHX (100 µM), alone or in combination, and the level of SR1 protein was monitored by Western blotting using anti-SR1 antibody. Our results showed that CHX as well as NaCl plus CHX treatments increased SR1 protein level considerably as compared to the untreated seedlings (Fig. 31). However, the accumulation level of SR1 protein in seedlings treated with NaCl alone was similar to its level in the control untreated seedlings (Fig. 31).

Discussion

NaCl Treatment Increases SR1 mRNA Level

Plants use a wide array of mechanisms to regulate gene expression in response to stresses, including transcriptional, post-transcriptional and translational mechanisms[468,469]. Post-transcriptional mechanisms such as pre-mRNA processing and editing, nuclear export, mRNA localization and stability are critical for fine-tuning gene expression in eukaryotes[468-472,585]. Among these post-transcriptional control mechanisms, the control of mRNA stability is highly regulated and can be modulated by extracellular and intracellular stimuli leading to changes in expression pattern of many genes[473-476]. Regulation of mRNA stability was found to be critical for the control

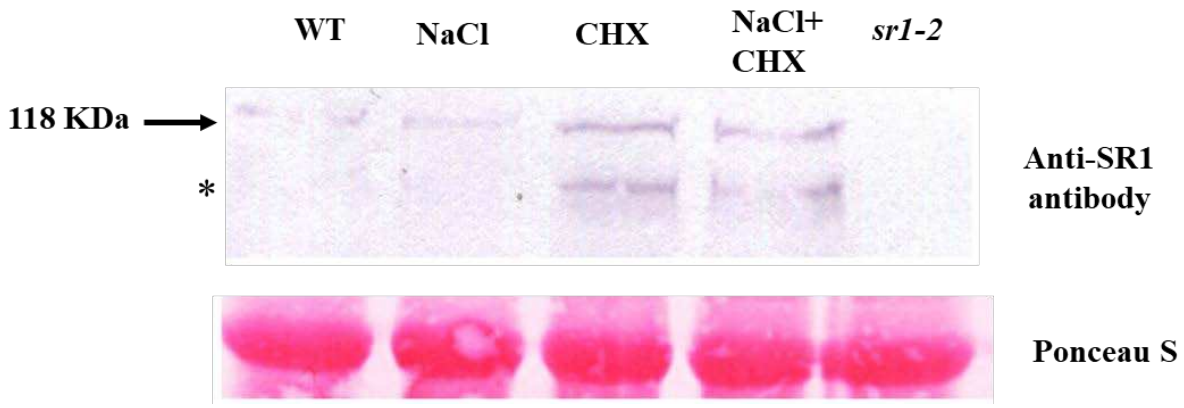


Figure 31. CHX-induced *SR1* transcript accumulation is reflected at the protein level
Two-week-old WT Arabidopsis seedlings were treated with NaCl (150 mM) and/or CHX (100 µM) for 3h. The level of SR1 protein in the treated and untreated WT seedlings as well as untreated *sr1-2* mutant was detected by Western blotting using anti-SR1 antibody. Ponceau S staining of the RuBisCO large subunit was used as a loading control. The arrow points to full length SR1 band and the star refers to a degraded product of SR1.

of gene expression during development and in response to chemical and environmental stimuli[472,477-482]. However, the mechanisms and signaling pathways regulating mRNA turnover during stress conditions are still not well understood in many cases.

In this study, we show that salt stress enhances the *SR1* transcript levels by stabilizing *SR1* mRNA (Fig. 20 & Fig. 23). Post-transcriptional regulation of gene expression in response to salt stress has been demonstrated in the last few decades[504]. Similar to our results, Chun *et al*., (2007) showed that salt stress enhances the stability of *salt overly sensitive 1* (*SOS1*) mRNA[508]. In addition, osmotic stress alters the activity of the mRNA decapping machinery, which subsequently modulates transcript levels of different genes involved in salt stress response[505]. Furthermore, SnRK2 and MAP kinases, which are activated by salt, are involved in regulation of mRNA stability pathways[488,501-503]. The post-transcriptional regulation of gene expression by salt stress is essential for plant salt stress tolerance. Mutations in the mRNA decay machinery alter the levels of specific transcripts and enhance plant sensitivity to salinity stress[480,586].

New Protein Synthesis is not Required for NaCl-Induced SR1 mRNA Level

Our results show that treatment with a protein synthesis inhibitor, CHX, like NaCl, induced *SR1* mRNA level, but the level of *SR1* transcript is much higher in CHX treated seedlings (Fig. 24). Treating seedlings with both CHX and salt did not result in an additive effect on *SR1* transcript level, suggesting that both may share some common mechanism in regulating *SR1* mRNA (Fig. 24). Cycloheximide blocks translation elongation by inhibiting the translocation step[587]. However, there is increasing evidence that CHX and other protein synthesis inhibitors (PSIs) such as anisomycin and puromycin can interact with signaling pathways and induce accumulation of specific gene transcripts[588-590]. Similar to our results, various auxin-responsive genes including two in pea[591], one in tobacco[592] and small auxin-responsive (SAUR) mRNAs in soybean[593]

accumulate if protein synthesis is inhibited. In addition to these, transcripts of several other genes in Arabidopsis, cucumber and maize are also accumulated by CHX treatment[537,539,594,595] In mammals, CHX induces transcripts of immediate early genes including interleukin 8, *c-fos*, *c-jun* and *junB*[538,596-600], several stress-responsive protein kinases[534,535] and other genes [599,601-603].

Accumulation of some transcripts by PSI can occur in the presence or absence of a co-stimulant such as growth factors or other stimulating factors. For example, in case of *par* and *SAUR* genes induction, the transcripts levels can be induced by CHX, both in the presence or absence of auxin[593,604]. Similar to these cases, the *SR1* transcript was induced by CHX both in the presence or absence of salt. The expression of the ABA-induced *HVA22* gene in the barley aleurone tissue can be induced by treatment with ABA or CHX and a combination of both factors has a synergistic effect on the induction[605]. In contrast to this, the induction of transcripts in some cases requires the presence of a co-stimulant with CHX. For example, accumulation of *catalase* transcript in *Schizosaccharomyces pombe* occurred only in presence of H_2O_2 and CHX, whereas CHX alone did not affect the level this transcript[606]. Similarly, in rice seedlings, the level of the heat shock transcripts, *Oshsp17.4-CI*, *Oshsp17.9A-CI* and *OsClpB-cyt/hsp100*, was superinduced by co-treatment with heat stress and CHX. However, CHX alone did not affect the expression of these transcripts[607].

The accumulation of transcripts in response to PSI was attributed to increased mRNA stability[608,609], enhanced transcription[588], decreased synthesis of labile gene repressors[536] or activation of signaling cascades. Results reported here (Fig. 26) indicate that CHX-induced *SR1* transcript accumulation is due to enhanced mRNA stability rather than enhanced transcription. Similar to our results, treatment of cultured rice suspension cells with CHX enhanced the accumulation of the α-amylase gene (*αAmy3*) transcript in the presence or absence of sucrose[486].

Cycloheximide did not affect transcriptional rates of αAmy3. Additionally, in mammalian systems, CHX superinduces immediate early genes (IEGs) such as *c-jun* and *junB* by increasing their mRNA stability[596-598]. In other cases, CHX -induced accumulation of certain mRNA levels reflected enhanced transcriptional activity. For example, in *Schizosaccharomyces pombe*, CHX has been shown to superinduce *catalase* gene expression in presence of H_2O_2 by enhancing transcription[606,610]. Also, in Arabidopsis, CHX treatment enhanced the accumulation of *CBF* transcripts by stimulating transcription[611]. Hence, the mechanism of induction varies according to the gene being induced as well as the cell type[531,541,612-614].

CHX-Induced SR1 mRNA Level is Partially Mediated by Protein Synthesis Inhibition

Although CHX is a widely used protein synthesis inhibitor[587], in some cases CHX-induced increase in transcripts may not be related to protein synthesis inhibition[541,542]. CHX can also function as an agonist to initiate a signaling pathway that regulates the expression of genes responsive to extracellular stimuli[540]. Our results showed that puromycin, another protein synthesis inhibitor, induced *SR1* transcript levels to only 27-40% of CHX-induced levels (Fig. 27). This result suggests that protein synthesis inhibition contributes to part of *SR1* transcript increase and that another mechanism is involved in the CHX-induced transcript increase. Furthermore, the increase in the *SR1* transcript at a low CHX concentration that does not affect protein synthesis supports this conclusion. Consistent with our results, treatment of maize seedlings with a subinhibitory concentration of CHX induces the accumulation of three cold-inducible transcripts[539]. Similarly, low concentrations of CHX superinduce the transcript level of the alpha-epithelial Na^+ channel (*α-ENaC*) in dexamethasone-treated canine kidney cells, whereas treatment with the protein synthesis inhibitor puromycin did not influence *α-ENaC* transcript level[542]. Thus, in some cases protein synthesis inhibition does not seem to be necessary to enhance transcript

accumulation. The partial increase in *SR1* mRNA level observed in this study by protein synthesis inhibition suggests that one or more labile proteins that are affected by protein synthesis inhibition may be involved in *SR1* mRNA decay. An additional possibility is that inhibition of translation by CHX enhanced *SR1* mRNA protection by loading it up with ribosomes[605,615,616]. CHX is known to bind to the larger ribosome subunit and prevent the movement of ribosome relative to mRNA, thus blocking translational elongation and protect the mRNA from ribonucleases[587,605,617].

Several studies have provided evidence that mRNA translation and decay are tightly linked and that mRNA decay can be modulated by alterations in translation[580,581]. Initially, it was proposed that being engaged in translation will protect the mRNAs from decay and translational repression is a prerequisite for mRNA decay[581]. This notion has been supported by the fact that the poly(A) tail and the methyl-7-guanosine cap at the mRNA 3'- and 5'- ends, respectively, have functional roles in both translation initiation and mRNA stability[578,579]. The 5'-cap binds to the translation initiation factor eIF4F, which in turn binds to the poly(A) tail through the poly(A) binding protein (Pab1p) that directly associates with eIF4G[578,579,618]. Therefore, with the aid of specific binding proteins the 5'-cap and the 3'-poly(A) acts as assembly points for translation initiation factors and promotes translation. Furthermore, this leads to formation of the closed mRNP loop, which inhibits mRNA decay by protecting the ends from deadenylation and mRNA decapping enzymes[580-582]. Hence, inhibition of translation and release of these factors is a prerequisite for deadenylation and decapping to occur[619,620]. In support of this hypothesis, the decapping regulators Dhh1p and Pat1p repress translation and this activity is required for its function in promoting mRNA decapping[621,622]. In addition, inhibition of translation initiation by inserting a stem-loop in the mRNA 5'-UTR[581] or by mutations in the genes encoding the eIF4E, eIF4G, or eIF3 complex, promote deadenylation, decapping and subsequently mRNA

decay[581,623,624]. Also, many of the *trans*-acting factors involved in mRNA degradation either have the ability to repress translation or recruit factors that affect translation[625]. Therefore, it was suggested that repression of translation drives mRNA towards degradation by enhancing the accessibility of its ends to decay factors that replace the translation initiation machinery[626].

However, later on, the relationship between translation and mRNA decay was found to be much more complex than that. Recent studies established that mRNA decay is not limited to non-translating mRNA and can occur also on translating mRNA[626]. Furthermore, some mRNAs are highly stable although they remain untranslated[626]. For example, inhibiting translation with the antibiotic cycloheximide[581,627] or a mutation in the gene encoding eIF5A[628,629] promotes stabilization of some mRNAs. Additionally, in response to environmental stresses, yeast and other eukaryotes are known to repress translation while at the same time stabilizing some mRNAs[585,630-635]. In this regard, glucose deprivation[491,636], osmotic stress[491,492,637] as well as heat shock[491,638] have been demonstrated to enhance mRNA stability in yeast cells. However, the mRNA stabilization under these stress condition was suggested to be directly induced by the stress and is not attributed to translation repression by the stress[491].

ROS Mediate Salt- and CHX- Induced Increase in SR1 Transcript

Furthermore, our results showed that ROS mediate salt- and CHX induced accumulation of *SR1* transcript (Fig. 22 & Fig. 29). It is well established that ROS including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) are generated in excess in response to many biotic and abiotic stress[447,639-651]. Particularly, salinity stress is known to induce ROS production in plants, which subsequently mediate plant tolerance to salt tolerance[509-513]. Similar to our results, Chun et al., (2007) showed that salt-induced stabilization of *SOS1* mRNA is mediated by ROS produced by NADPH oxidase activity[508]. Also, ROS has been reported to

enhance salt stress tolerance by regulating the expression of salt-responsive genes[652-654]. In addition, CHX was demonstrated to strongly induce oxidative burst in plants[655].

There are two major sources for ROS production in response to stress. ROS can be produced as a result of disruptions in the metabolic activity[2] or by the activity of the plasma membrane-bound NADPH oxidases as a result of stress perception[656]. Our results reveal that NADPH oxidase activity is required for the salt- and CHX-induced accumulation of *SR1* mRNA (Fig. 22 & Fig. 29). Abiotic stresses including salt stress can activate NADPH oxidase by inducing a Ca^{2+} spike that can be specifically recognized by NADPH oxidases that contain two EF-hand calcium-binding motifs[656-659]. Activation of NADPH oxidase induces the production of superoxide anion (O_2^-), which is then converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Subsequently, the extremely reactive hydroxyl radical (OH) can be generated from H_2O_2 in the presence of transition metals such as Fe^{2+}.

ROS generated by stresses could directly alter gene expression by altering the function of key regulatory proteins via ROS-derived redox modifications[660,661]. Also, ROS may act as a messenger in regulating various intracellular signal transduction pathways. Multiple signaling pathways involved in regulation of plant responses to various environmental stresses are activated by ROS[662-664]. In this regard, the MAPK signaling pathway is one of the well-characterized ROS activated signaling pathways[665-668]. In plants, MAPK pathway is a key signal transduction pathway involved in cell proliferation, developmental processes, hormone responses, as well as plant responses to a variety of stresses[669-673]. Salt stress is known to activate different MAPKs which in turn regulate plant stress response[674-678]. Interestingly, Chun *et al.*, (2007) showed that MAPK signaling pathway is involved in the salt-induced stabilization of *SOS1* mRNA[508]. Also, CHX induces accumulation of several transcripts through activation of different MAPKs[599,679]. Together,

these data suggest that salt- and CHX-induced *SR1* transcript levels may be mediated through activation of MAPK signaling pathway. Further experiments will be required to test this possibility.

Salt and CHX Increase SR1 Transcript Level Likely by Inhibiting Deadenylation

It has been proposed that stresses promote mRNA stability by inhibiting deadenylation, which is a prerequisite for mRNA decay[491,680]. In support of that, the enhanced mRNA degradation in an eIF3 translation initiation mutant is reversed by stress application or by inhibiting deadenylation[491,623]. Also, hyperosmolarity, heat shock, and glucose deprivation stabilize multiple mRNAs in yeast by inhibiting the function of the deadenylases Ccr4p/Pop2p/Notp and Pan2p/Pan3p[142]. Furthermore, inhibition of deadenylation in response to stress seems to be conserved among eukaryotes as similar observations were made in mammalian cells and *Drosophila* under stress conditions[490,681,682]. However, it worth mentioning that only specific mRNAs are stabilized under stress conditions and some mRNAs can resist stabilization under these conditions by recruiting specific decay factors or by using alternative decay pathways[492,636,637,683].

After deadenylation, the transcripts can be degraded in the 3′ to 5′ direction by the exosome, a multienzyme complex. The second pathway, which is more predominant, involves deadenylation-dependent removal of the 5' cap leading to the transcript degradation in 5' to 3' direction by the exonuclease XRN4[583]. Our results show that *SR1* transcript level in untreated decapping mutant is similar to its level in untreated WT seedlings, indicating that decapping is not involved in *SR1* mRNA decay (Fig 28B). Therefore, *SR1* mRNA is likely degraded in 3' to 5' direction by the exosome complex. Based on this result and the aforementioned reports that showed inhibition of mRNA deadenylation by multiple stresses, we suggest that salt stress and CHX treatment lead to *SR1* mRNA stability by inhibiting its deadenylation. CHX has been reported to stabilize several mRNAs by preventing decapping[478,627]. However, CHX has been also

demonstrated to stabilize the mammalian *c-myc* and *c-fos* mRNAs by inhibiting deadenylation[684,685]. Therefore, individual mRNAs respond differently to CHX treatment.

Decapping Activity is Partially Required for NaCl- and CHX-Induced SR1 mRNA Levels

Knockout mutation in *VCS* significantly reduced the accumulation of *SR1* transcript in response to CHX and NaCl as compared to CHX and NaCl treated WT seedlings (Fig. 28B). These results indicate that mRNA decapping activity is required for salt- and CHX-induced accumulation of *SR1* mRNA. Consistent with our results, decapping has been demonstrated to be involved in many stress responses[493,494,505,686]. For example, Xu & Chua, (2012) reported that osmotic and dehydration stresses enhance phosphorylation of DCP1 by MPK6[687]. Phosphorylation of DCP1 promotes its binding to DCP5, DCP2 and enhances their decapping activity *in vivo*[687]. They also demonstrated that this decapping activity is required for stress-induced changes in the transcriptome as well as stress adaptation. For example, similar to our results, they found that the dehydration and osmotic stress-induced increase in dehydration responsive element binding 1b (*DREB1b*;) and *DREB2a* transcripts was largely attenuated in the decapping mutant (*dcp5-1*) as compared to WT[687]. They proposed that DCP5 may specifically target a subset of mRNAs for decapping and subsequent degradation, thereby decreasing the competition for polysome occupancy[687]. Therefore, the osmotic stress-responsive mRNAs can enter the polysome for translation, which inhibits their degradation. A similar mechanism may be involved in the salt-induced stabilization of *SR1* transcript.

Also, decapping might be involved in the degradation of an mRNA of a protein that inhibits a *trans*-factor required for stabilization of *SR1* transcript. In this case, the observed difference between *SR1* transcript levels in the decapping mutant in response to salt and CHX may be related to stabilizing *SR1* mRNA by CHX through two different mechanisms while only one of them is

shared with salt and requires decapping activity. However, we also cannot exclude the possibility that the reduction in salt and CHX induced accumulation of *SR1* mRNA in the decapping mutant might be related to growth arrest caused due to the abolition of decapping activity in the mutant. Loss of decapping activity leads to severe developmental phenotypes and growth arrest[478,688].

A 500-nts Region at the 3' End of the SR1 Open Reading Frame is Required for its Stability

The effect of salt and CHX on *SR1* transcript could be attributed to the presence of specific *cis*-acting elements in the mRNA or the *trans*-factors binding to those elements. In this study, we demonstrated that a 500 nts region at the 3' end of *SR1* mRNA is required for salt- and CHX-induced stability of *SR1* transcript (Fig. 30). Interestingly, deletion of this region from *SR1* gene did not confer stability in all other truncated versions. This result suggests that the 3' end region is responsible for enhanced stability in response to NaCl and CHX but is not responsible for *SR1* mRNA instability under normal growth conditions. Based on these results, we propose that salt and CHX treatments might enhance the stability of *SR1* mRNA by enhancing binding of one or more *trans*-factors to *cis*-elements in this 500 nts region. Binding of this *trans*-factor to *SR1* mRNA might enhance *SR1* transcript stability by making it less available to the deadenylase complex. Salt and CHX might enhance modification in this *SR1* mRNA binding protein, which in turn activates its binding activity. Also, salt and CHX might modify *cis*-elements (e.g, methylation of adenine) in the *SR1* mRNA that facilitates its binding to the *trans*-factor or inhibit binding of the deadenylases. Future work will be required to test these possibilities and gain a detailed mechanism through which salt and CHX increase *SR1* transcript stabilization.

CHX-Induced Accumulation of SR1 mRNA is Reflected at the Protein Level

Our results showed that treatment with CHX or NaCl plus CHX increased *SR1* mRNA as well as its protein levels in the treated seedlings. However, treatment with NaCl alone enhanced

SR1 mRNA accumulation but not its protein level (Fig 31). Similar to our results, Hershko *et al*., (2004) reported that treatment of Caco-2 cells with CHX increased the accumulation of the cytokine interleukin-6 (IL-6) at mRNA and protein levels[541]. Previously, we reported that the Ca^{2+}/CAM activated SR1 acts as a negative regulator of salt stress tolerance by directly repressing the expression of salt-responsive genes. On the other hand, Ca^{2+}-mediated signaling is involved in plant salt stress response and elevation of Ca^{2+} concentration in response to salt is a key signaling event for salt stress tolerance[418]. Also, Galon *et al*., (2010)[327] and Pandey *et al*., (2013) [325] identified SR2/CAMTA1, another member of SR family TF, to be a positive regulator of salt stress. Together, these data indicate that Ca^{2+} signaling acts both as a positive and negative regulator of plant salt stress response. Therefore, there should be a coordination for Ca^{2+}-mediated signaling during salt stress, so the plant can overcome this negative regulation by SR1 to achieve efficient stress tolerance.

Zhang *et al*., (2014) have reported that the negative regulation of plant immunity by SR1 is released at the time of pathogen infection by temporary removal of SR1 via the pathogen-induced SR1IP1 that binds to SR1 and facilitates its ubiquitination and degradation by the 26S proteasome to help establish an effective plant defense against the attacking pathogens[387]. Our results here suggest that a similar mechanism might be involved in relieving SR1 negative effect during salt stress. The accumulation of *SR1* mRNA but not SR1 protein in NaCl treated seedling suggests that the SR1 protein is likely subjected to degradation under salt stress conditions. In support of this notion, several RING-type E3 ubiquitin ligases are known to be salt inducible and positively regulate plant response to salt stress[689-691]. Moreover, the accumulation of SR1 protein in CHX and NaCl plus CHX treated seedlings further supports this notion as CHX is known to block ubiquitin synthesis and decrease the total cellular content of ubiquitinated proteins, thereby

reducing proteasomal degradation[692,693]. The function of SR1 as a negative regulator for salt stress is likely to balance and fine-tune plant stress response and prevent unnecessary overactivation of stress-responsive genes, which can negatively affect the plant and its growth. The stabilization of SR1 mRNA may be a mechanism to set SR1 in a ready to go state so it can be rapidly translated once the salt stress is removed without requiring the mRNAs to be transcribed again[694]. Therefore, SR1 can control the expression of salt-responsive genes to return back to basal levels during the recovery from stress.

It is well established that many cellular stresses repress translation initiation by phosphorylation of the initiation factor eIF2α[695,696]. The untranslated mRNAs are subsequently sequestered into cytoplasmic foci like the processing bodies (PBs) or stress granules (SGs), which are mainly formed under stress conditions[697,698]. PBs are enriched in mRNA degradation factors like DCP1, DCP2 and XRN4; and is mainly involved in mRNA degradation[699-701]. SGs are sites for mRNA storage and are enriched in poly(A) mRNAs, 40S ribosomal subunits, translation initiation factors, as well as RNA-binding proteins[699,702,703]. PBs and SGs have a key role in enhancing stress tolerance as well as recovery from stress[704,705]. Sequestering mRNA in PBs and SGs allows selective degradation and translation of specific mRNAs, which in turn fine-tunes gene expression patterns to stress conditions[699,706,707]. Recently, it has been demonstrated that SGs formation is required for salt stress adaptation in Arabidopsis by modulating mRNA levels of specific transcripts[705]. SGs act as storage sites for untranslated mRNA under stress condition, thereby protecting untranslated mRNAs until the cell recovers. Once stress is relieved the stored mRNA can be recruited for translation or targeted for degradation by transferring to PBs[694,708,709]. Together, these data and results in this study support our hypothesis that salt stress stabilizes *SR1* mRNA by inhibiting deadenylation so this mRNA can be stored in the SGs and re-enter the

translation pool after stress relief. Based on our results as well as previous puplished data, we proposed a model (Fig. 32) to explain how salt stress and CHX regulate *SR1* at the posttranscriptional level.

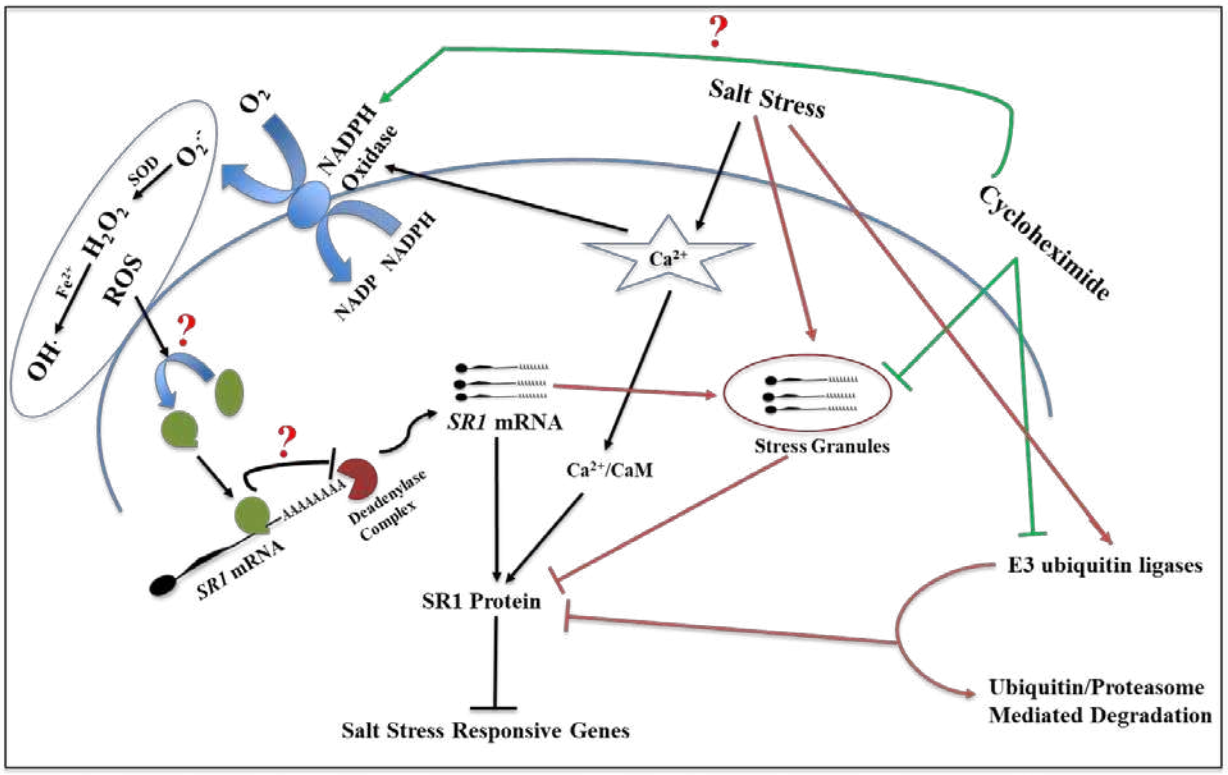


Figure 32. Proposed model for *SR1* transcript and its encoded protein regulation at the posttranscriptional level by salt stress and CHX. Salt stress elevates cytosolic Ca^{2+} level[418], which in turn activates NADPH oxidase, a calcium-binding protein[656], to generate ROS. Also, CHX activates NADPH oxidase to generate ROS. The generated ROS induce modification(s) in *SR1* mRNA binding protein, which enhances its binding to the *SR1* transcript. The binding of this protein to the *SR1* mRNA prevents deadenylation of the *SR1* transcript and stabilizes it. Then, the stabilized *SR1* transcripts can be translated to form SR1 protein that can be activated by Ca^{2+}/CAM and suppresses the expression of salt-responsive genes conferring salt sensitivity. To release this negative effect of SR1 under salt stress condition, the cell can prevent accumulation of SR1 protein by two ways. First, instead of being translated, the stabilized *SR1* mRNA can be stored in stress granules (SGs), which are formed in response to salt stress[705]. Alternatively, SR1 protein can be targeted to proteasome degradation through ubiquitination by E3 ligases, which are activated by salt stress[689-691]. On the other hand, CHX inhibits the formation of SGs[710] and prevents ubiquitination and proteasome degradation[692,693] leading to accumulation of SR1 protein. Arrows indicate positive effect, whereas the lines terminated with a bar indicate inhibitory effect.

Materials and Methods

Plant Material and Growth Conditions

Three Arabidopsis genotypes - WT (Columbia-0), *sr1-2* mutant in Col-0 background, and a complemented line (SR1-YFP) - used here were developed earlier in our lab[316,547,572]. Also, *upf3* mutant (SALK_025175) was used in a previous study in our lab[565,566]. *Arabidopsis thaliana* knockout T-DNA insertion line *vcs6-1* (SAIL_831) was obtained from Arabidopsis Biological Resource Center (ABRC) at Ohio State University. *upf3* and *vcs6-1* mutants were genotyped using primers listed in Table 4.

Surface sterilized seeds were plated on ~70ml of Murashige and Skoog (MS) medium (GibcoBRL, Grand Island, NY, USA) supplemented with 1% sucrose and 0.8% (w/v) Phytoblend in square sterile Petri dishes. All the plates were incubated at 22±2°C with 60% humidity, 200 μmoles/m^2 /sec white light under 16h-light/8h-dark cycles. Two-week-old seedlings grown under these conditions were used for all treatments. However, for the experiment involving *vcs6-1* line, we used one-week-old seedlings because the seedlings of homozygous knockout lines of this mutant start to die after one week of germination.

Treatments

For NaCl and CHX treatments, the seedlings were transferred to a 12 well plate, which contained in each well 5 ml of liquid MS medium with or without NaCl (150 mM) or CHX (100 μM) for 3h. For H_2O_2 and PQ treatments, the seedlings were transferred to liquid MS medium with or without H_2O_2 (2-100 mM) or PQ (2.5-50 μM) for 1h. For Act D and CP treatments, the seedlings were preincubated for 2h with NaCl (150 mM) or CHX (100 μM) to induce high levels of *SR1* mRNA. Then the seedlings were extensively washed three times and incubated for an

additional 2h in the presence of NaCl (150 mM), CHX (100 µM), Act D (100 µg/ml) or CP (200 µg/ml) or in combinations (NaCl plus Act D, NaCl plus CP, CHX plus Act D and CHX plus CP). For DMTU and DPI treatments, two-week-old seedlings were preincubated for 2h with DMTU (20 µM) or DPI (100 µm) for 2h, then treated for additional 2 h with NaCl (150 mM), CHX (100 µM), DMTU (20 µM) and DPI (100 µm), alone or in combinations (NaCl plus DMTU, NaCl plus DPI, CHX plus DMTU, CHX plus DPI, NaCl plus CHX plus DMTU and NaCl plus CHX plus DPI) for 2h. After all treatments, seedlings were collected and flash frozen in liquid nitrogen for RNA extraction.

RNA Extraction and Expression Analyses

Total RNA was isolated from seedlings using TRIzol reagent (Invitrogen, US)[711]. Total RNA samples were treated with an RNase-free DNase (Promega) to remove any genomic DNA contamination. Two µg of the DNAse-treated RNA was used for cDNA synthesis using oligo dT primers and Superscript II reverse transcriptase (Invitrogen) as per manufacturer instructions. The cDNA was diluted with 80 µl sterile nuclease-free water and 2.5 µl/reaction was used as a template. For every qPCR reaction, 10 µl of 2X LightCycler 480 SYBR Green I Master mix (Roche) was used along with 1µl of 5 µM of each primer in a final reaction volume of 20 µl. Expression analysis was performed using RT-qPCR in a Roche LC480 machine (Roche) using the preprogrammed "SYBR green-I 96 well program". *ACTIN2* was used as a reference gene as this gene does not exhibit any difference in its expression levels among the various genotypes. Fold change in expression was calculated and plotted with respect to control treatments. A minimum of three biological replicates were used for each experiment. Primers (Table 4) for Real-time qPCR (RT-qPCR) were designed using Primer Quest web tool (http://www.idtdna.com/Primerquest/Home/Index) from IDT (USA).

Plasmids Construction

Eight truncated versions of *SR1* coding sequence were PCR amplified from Col-0 Arabidopsis cDNA using primers indicated in Table 4. First, we started with two truncated versions, N-terminus (nucleotides 1-1551) or C-terminus (nucleotides 1549-3099), then we generated 3 truncated versions (500 nt each) from each of the N- and C-terminus versions. The fragments were cloned into pFGC 5941 vector between the *Asc1* and *BamH1* sites using the restriction sites for *Asc1* and *BamH1* that were added to the forward and reverse primers, respectively.

Generation of Transgenic Lines

Each of these constructs was transformed into *Agrobacterium* strain GV3101. Subsequently, the transformed *Agrobacterium* was used to stably transform *sr1-2* mutant plants by flower dipping method[712]. Transgenic plants were selected on MS plates containing Basta (10 µg/ml) and genotyped by RT-PCR using the primers listed in Table 4. The selected plants were then selfed to get homozygous lines.

Protein Extraction and Western Blot Analysis

Seedlings were flash frozen and ground to fine powder in tissue lyser and dissolved in 100 µl protein extraction buffer (40 mM K_2HPO_4, 10 mM KH_2PO_4, 1mg/ml ascorbate, 0.05% β-mercaptoethanol (v/v) 0.2% Triton, 1mM PMSF) containing 1% protease inhibitor cocktail (P9599; Sigma-Aldrich). The extract was clarified by centrifugation for 10 min at 16,000g at 4ºC. Protein concentration was determined using the Bradford reagent (Bio-Rad). Thirty µg of total protein from each sample was resolved in 12% SDS gels and blotted onto a PVDF membrane. The blot was blocked with 5% non-fat milk in TBST buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl,

0.05% Tween-20). Then, the membrane was probed with anti-SR1 antibody and detected with secondary antibody conjugated with alkaline phosphatase detection system. The anti-SR1 antibody was raised in rabbit against a specific peptide of SR1 (NH_2-VLEKVILRWRRKGAGLRGFK-OH) that resides in the SR1 CaM-binding domain and affinity purified using the same peptide (Biosynthesis Incorporation, Texas, USA).

Table 4. List of primers used for chapter 3

Name	Sequence	Restriction Enzyme
Genotyping		
VCS FW	5'-CTACCTGTTCTCACTTTCAAGTCATCATCG-3'	
VCS RV	5'-CGGCTGAATCTCACCTTGTAATCTCACATC-3'	
LB3 FW	5'-AGCATCTGAATTTCATAACCAATCTCGATACA-3'	
RB3 RV	5'-GTCATAGCTGTTTCCTGTGTGAAATTGTTA-3'	
RT-PCR		
UPF3 FW	5'-GGGAGGTTGATCAAGGGAATAC-3'	
UPF3 RV	5'-CTTCTGAGGGTTCCACTCTTTG-3'	
Cloning of *SR1* truncated versions		
FL-SR1 FW	5'-TTGGCGCGCCATGGCGGAAGCAAGACGA-3'	Asc1
FL-SR1 RV	5'-CGGGATCCTTAACTGGTCCACAAAGATGAGGA-3'	BamH1
NT-SR1 FW	5'-TTGGCGCGCCATGGCGGAAGCAAGACGA-3'	Asc1
NT-SR1 RV	5'-GGGATCCTCAAATACCATTAGATATAACATCTG-3'	BamH1
CT-SR1 FW	5'-TTGGCGCGCC ATGATTCTCCAATGTGTTGCTCC-3'	Asc1
CT-SR1 RV	5'-CGGGATCCTTAACTGGTCCACAAAGATGAGGA-3'	BamH1
R1-SR1 FW	5'-TTGGCGCGCCATGGCGGAAGCAAGACGA-3'	Asc1
R1-SR1 RV	5'-CGGGATCCTCAAGAAGCATAACCATCATGTTC-3'	BamH1
R2-SR1 FW	5'-TTGGCGCGCCATGTGCAGCTTCAATCAAAATGA-3'	Asc1
R2-SR1 RV	5'-CGGGATCCTCATGAATTTAACTCCTGATTCTG-3'	BamH1
R3-SR1 FW	5'-TTGGCGCGCCATGGGACTTACATCTGATCGTACC-3'	Asc1
R3-SR1 RV	5'-CGGGATCCTCAAATACCATTAGATATAACATCT-3'	BamH1
R4-SR1 FW	5'-TTGGCGCGCCATGATTCTCCAATGTGTTGCTCCT-3'	Asc1
R4-SR1 RV	5'-CGGGATCCTCAATCAACGCTTACACCAGCGAT -3'	BamH1
R5-SR1 FW	5'-TTGGCGCGCC ATGTTTCGCGATGTAAATGGTTG-3'	Asc1
R5-SR1 RV	5'-CGGGATCCTCAGTTCTGAATCCGAATAGCAGC-3'	BamH1
R6-SR1 FW	5'-TTGGCGCGCCATGAAGTTCCGAGGTTACAAGGG-3'	Asc1
R6-SR1 RV	5'-CGGGATCCTTAACTGGTCCACAAAGATGAGGA-3'	BamH1
RT-qPCR		
FL-SR1 qFW	5'-CAGGCTCATGTGAGAGGTTATC-3'	
FL-SR1 qRV	5'-CCTTTCCGTCTCCAACGTAAT-3'	
NT-SR1 qFW	5'-GAACCTACTGGGAAGAAGTTGAG-3'	
NT-SR1 qRV	5'-CTTTGAGAGGGAAGGACTCATAAC-3'	
CT-SR1 qFW	5'-CAGGCTCATGTGAGAGGTTATC-3'	
CT-SR1 qRV	5'-CCTTTCCGTCTCCAACGTAAT-3'	
R1-SR1 qFW	5'-CACTACCTCGAAGTTAAGGGTAGTAGAGTT-3'	
R1-SR1 qRV	5'-CGGGATCCTCAAGAAGCATAACCATCATGTTC-3'	
R2-SR1 qFW	5'-TGGGAAATTGTGGTTCTGGAGTTGAAGCCT-3'	
R2-SR1 qRV	5'-CGGGATCCTCATGAATTTAACTCCTGATTCTG-3'	
R3-SR1 qFW	5'-GAACCTACTGGGAAGAAGTTGAG-3'	

R3-SR1 qRV	5’-CTTTGAGAGGGAAGGACTCATAAC-3’	
R4-SR1 qFW	5’-GATAGCAGAAGGTGGAAAAGGTCCAAGTGT-3’	
R4-SR1qRV	5’-CGGGATCCTCAATCAACGCTTACACCAGCGAT -3’	
R5-SR1 qFW	5’-GGAGATAAAAAGCTTGGAATGTCGGAGGAG-3’	
R5-SR1 qRV	5’-CGGGATCCTCAGTTCTGAATCCGAATAGCAGC -3’	
R6-SR1 qFW	5’-CAGGCTCATGTGAGAGGTTATC-3’	
R6-SR1 qRV	5’-CCTTTCCGTCTCCAACGTAAT-3’	
ACTIN FW	5’-GGCAAGTCATCACGATTGG-3’	
ACTIN RV	5’-CAGCTTCCATTCCCACAAAC-3’	

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