THESIS

FUNCTIONAL ANALYSES OF SPLICE VARIANTS OF THE SPLICING REGULATOR SR45 IN ABIOTIC STRESSES IN ARABIDOPSIS

Submitted by

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ABSTRACT

FUNCTIONAL ANALYSES OF SPLICE VARIANTS OF

THE SPLICING REGULATOR SR45 IN ABIOTIC STRESSES IN ARABIDOPSIS

Alternative splicing, a post-transcriptional regulatory mechanism of gene expression, produces multiple mRNAs from a single gene. Alternative splicing increases proteome complexity and regulates gene expression through multiple mechanisms. A number of stresses have been shown to regulate alternative splicing of precursor mRNAs in plants and change transcriptome complexity. Serine/arginine-rich (SR) and SR-like proteins that regulate splicing also undergo extensive alternative splicing in response to various stresses. SR45, an SR-like protein, interacts with several spliceosomal proteins such as U170K, SCL33, U2AF35, and also with an intronic sequence of *SR30* and regulates alternative splicing of pre-mRNAs of several other *SR* genes. It has been previously shown that *SR45* pre-mRNA undergoes alternative splicing and produces two alternatively spliced mRNA isoforms (long and short) and the proteins coded by these two isoforms differ in eight amino acids. The two isoforms have distinct biological functions during development where the long isoform is important for flower development while the short isoform is necessary for normal root growth. In this work, I have studied the roles of SR45 and its splice variants in heat and salt tolerance using *SR45* mutant (*sr45*) and transgenic lines complemented with either the long or short isoform. I have found that at different developmental stages *sr45* shows high sensitivity to heat stress and salt stress as compared to wild type. The sensitivity of *sr45* to heat and salt stresses is rescued by the long isoform but not the short one, suggesting that only the long isoform functions in these stresses. Further molecular analyses have revealed that the relative expression and the splicing pattern of *heat shock factors* (*HSFs*), *heat shock proteins* (*HSPs*), *salt overly sensitive*

(*SOS*) genes, ABA signaling pathway genes, and other stress-responsive genes are affected in the *sr45* mutant and the long isoform is needed for normal splicing and expression of these genes. Furthermore, an *in vitro* binding assay showed that SR45 binds to an alternatively spliced intron of *HsfA2*, suggesting that SR45 directly regulates alternative splicing and expression of *HsfA2* under heat stress. In addition to misregulation of expression and splicing of some salt stress responsive genes in the mutant, new splicing isoforms that are affected in the mutant are identified, suggesting the importance of SR45 in fine-tuning gene expression under salt stress. In conclusion, results presented here demonstrate that SR45 functions as a positive regulator of tolerance to two abiotic stresses by modulating the expression and splicing of several stress responsive genes. Further, I show that only the long isoform confers tolerance to these abiotic stresses.

DEDICATION

This thesis is dedicated to whom I will never forget, my brother Faisal, who past away while I was doing my master research at Colorado State University in 2011.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my committee members who were more than generous with their expertise and precious time. A special thanks to my advisor Professor A.S.N.Reddy for his countless hours of reflecting, motivation, enthusiasm and knowledge. Beside my advisor I would like to thank my co-advisor Associate professor Salah Abdel-Ghany, and Professor Patrick Byrne for their excellent guidance and useful comments in this research. I would like to acknowledge all my colleagues in Dr. Reddy's laboratory especially Dr. Saiprasad, Dr. Kristin Laluk and Dr. Iren Day for their continuous help, fruitful discussion and editing during preparing my thesis. Special thanks goes to Umm Al-Qura University at Kingdom of Saudi Arabia for generous support that allowed my to pursue my study at Colorado State University. Finally, I would like to thank my family members and close friends for their encouraging and supporting to do this work.

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INTRODUCTION

Environmental stresses are the major limiting factors of agricultural productivity (Boyer, 1982). Apart from biotic stresses caused by plant pathogens and insects, abiotic stresses such as drought, salinity and high and low temperatures are the most harmful factors that adversely affect the growth and productivity of crops worldwide (Witcombe et al., 2008), resulting in more than a 50% reduction for most crop plants (Wang et al., 2003).

Because of their sessile nature, plants cannot survive unless they are able to cope with these environmental changes. Coping with these environmental changes involves a wide range of responses at the molecular, cellular and whole-plant levels and, therefore, understanding abiotic stress responses is important for developing stress tolerant crops. At the molecular level, complete sequencing of the *Arabidopsis thaliana* genome and other plants has facilitated access to genome-wide gene expression profiling in response to various abiotic stresses. Using microarrays and RNA-seq technology, genes and splicing variants responding to abiotic stresses have been identified more comprehensively than ever before (Filichkin et al., 2010; Hirayama and Shinozaki, 2010; Iida et al., 2004). Functional studies with some of these stress-regulated genes and splicing variants has been performed using the reverse genetic approach (Hirayama and Shinozaki, 2010).

Abiotic Stresses and Transcriptional Regulation

Most of the stress responsive genes are regulated by a network of transcription factors and several families of transcription factors that are known to translate stress signals into changes in gene expression have been identified (Hirayama and Shinozaki, 2010). One particularly well-studied family is the heat stress transcription factors (HSFs) family. *HsfA2*, which is highly

induced in stressed plants, is considered as one of the key regulators of plant responses to heat and other stresses including oxidative damage (Charng et al., 2007; Ogawa et al., 2007; Schramm et al., 2006; Zhang et al., 2009). *HsfA2* knockout plants are sensitive to heat, high light, oxidative stress and anoxia. In addition, *Arabidopsis* plants overexpressing *HsfA2* showed thermotolerance and increased resistance to other stresses including salt/osmotic stress, oxidative stress and anoxia (Ogawa et al., 2007; Yokotani et al., 2008; Zhang et al., 2009). HsfA2 hetero-oligomerizes with another heat shock transcription factor (HsfA1) to form a super-activator complex, whose activity is much higher than that of the HsfA homodimers (Scharf et al., 1998).

Another family of transcription factors that are induced in response to different abiotic stresses is the APETALA 2/ethylene-responsive element binding factor (AP2/ERF) family. AP2/ERF, plant-specific transcription factors, includes four major subfamilies: the AP2, RAV, ERF and dehydration responsive element-binding protein (DREB) subfamilies (Sakuma et al., 2002). DREBs activate the expression of many abiotic stress-responsive genes via binding to the dehydration-responsive element/C-repeat (DRE/CRT) in their promoters (Yamaguchi-Shinozaki and Shinozaki, 2006). RD29A, one of the DREBs, is induced by exogenously applied ABA and stress stimuli such as cold, dehydration and high salinity (Yamaguchi-Shinozaki and Shinozaki, 1993) and is regulated in both ABA-dependent and ABA-independent ways (Yamaguchi-Shinozaki et al., 1991). Another family of plant-specific transcription factors that has important roles in both abiotic and biotic stress signaling, in addition to development, is NAC [for NAM (no apical meristem), ATAF, CUC (cup-shaped cotyledon)] proteins. Transgenic *Arabidopsis* and rice plants over-expressing stress-responsive *NAC* (*SNAC*) genes have exhibited improved drought tolerance (Nakashima et al., 2012). *RD26*, a NAC gene, is induced in response to drought and ABA treatment and Arabidopsis plants over-expressing *RD26* were drought tolerant

and highly sensitive to ABA indicating that RD26 functions in ABA-mediating gene expression (Fujita et al., 2004).

Alternative Splicing and Abiotic Stress

In addition to changes in transcription in response to abiotic stresses, alternative splicing (AS) of precursor mRNAs (pre-mRNAs) also changes in response to stresses (Ali and Reddy, 2008; Duque, 2011; Mastrangelo et al., 2012; Palusa et al., 2007), Pre-mRNAs of many stress responsive genes, including transcription factors, showed differences in their AS pattern in response to various environmental conditions. For example, *HsfA2*, a heat stress-inducible transcription factor (Schramm et al., 2006), produces two splice isoforms, *HsfA2.I* and *HsfA2.II* (Sugio et al., 2009). Recently, a new splicing variant, *HsfA2-III*, has been reported as a third isoform, which encodes a truncated protein and functions in *HsfA2* self-regulation under severe temperature (Liu et al., 2013).

Recently non-coding RNAs such as microRNAs (miRNAs) and siRNAs are emerging as important candidates in post-transcriptional regulation of gene expression in response to different stresses (reviewed in Khraiwesh et al., 2012). Seventeen stress-inducible miRNAs were detected and *cis*-regulatory elements in their promoters were analyzed (Fujii et al., 2005). A link among miRNAs, alternative splicing and heat stress response was also reported recently in *Arabidopsis* (Yan et al., 2012). The intronic miR400 transcribed with its host gene is down regulated by heat treatment. Under heat stress a specific AS event results in accumulation of miR400 primary transcripts, but a decrease of mature miR400 (Yan et al., 2012). mRNA stability and regulated translation are also other post-transcriptional regulatory mechanisms that have been shown to participate in stress responses in plants (reviewed in Floris et al., 2009).

Regulation of Alternative Splicing

About 80% of protein-coding genes and many non-coding genes in land plants are interrupted with non-coding sequences (introns) (Reddy, 2007; Reddy, 2001; Szarzynska et al., 2009). Pre-messenger RNA splicing is a complex process that generates functional mRNA via the precise removal of introns and ligation of the exons. Many multi-exon genes in plants and animals generate two or more mRNA isoforms through AS of pre-mRNAs (Marquez et al., 2012; Reddy et al., 2012). Recent studies indicate that AS happens in almost every intron-containing gene in humans and about 60% of multi-exon genes in plants undergo AS (Fig 1), which is thought to contribute to transcriptome and proteome diversity (Kalsotra and Cooper, 2011; Marquez et al., 2012). The four common types of alternative splicing events are intron retention (IR), exon skipping (ES), and usage of alternative 5` or 3` splice sites (Fig 2). Among these IR is most common in plants with ~40% of all alternative splicing events (Syed et al., 2012). Splicing is carried out in a large RNA-protein complex known as the spliceosome, which contains five small ribonucleoproteins (U1, U2, U4/U6 and U5 snRNPs) and a large number of auxiliary proteins (Behzadnia et al., 2007; Bessonov et al., 2008; Jurica and Moore, 2003). Assembly of the spliceosome during splicing is driven by sequence features in pre-mRNA: *cis*-regulatory elements including splice sites at the beginning and end of each intron, branch point, polypyrimidine tract, and enhancer and suppressor sequences that may be located both in exons and introns (Fig 3) (Syed et al., 2012). These splicing regulatory sequences in pre-mRNAs are recognized by *trans*-acting splicing regulatory proteins that act as repressors or enhancers of splicing. Heterogeneous nuclear RNPs (hnRNPs) proteins are recognized as one of the major splicing repressors. In addition, the serine/arginine-rich (SR) family of RNA binding proteins

function as splicing regulators, mostly as activators (Long and Caceres, 2009; Reddy, 2007; Reddy and Shad Ali, 2011; Simpson et al., 2010).

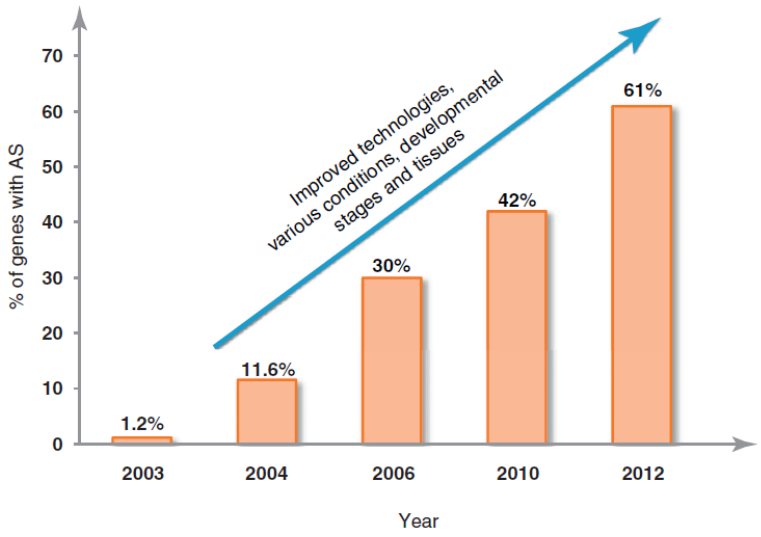


Figure 1: **Increase in discovery of alternative splicing occurrence in *Arabidopsis* during the last decade** (Adopted from (Syed et al., 2012)).

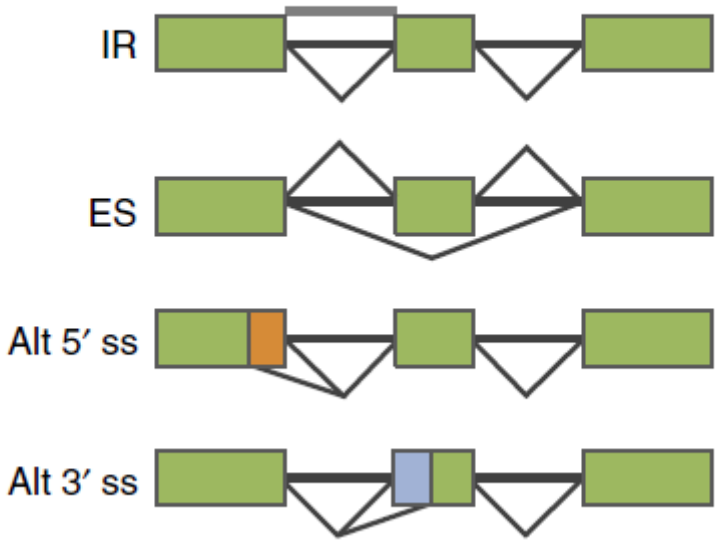


Figure 2: **Types of alternative splicing (AS) events. Schematic diagram shows common AS events in plants.** Abbreviation; Intron retention (IR), exon skipping (ES), and alternative 5` (Alt 5`ss) or 3` (Alt 3`ss) splice sites. Exons (green), introns (solid lines) and spliced segments (triangles). Adopted from Syed et al. (2012).

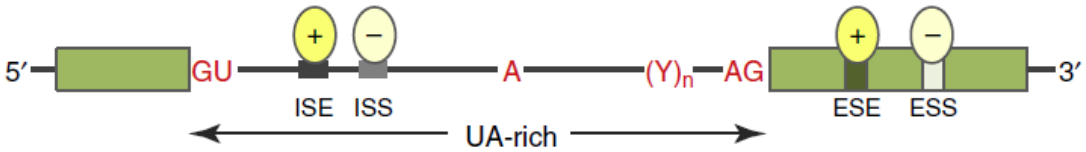


Figure 3: ***Cis*-regulatory elements that contribute in regulation of alternative splicing**. Schematic diagram shows the sequence features in pre-mRNA that drive AS by *trans*-acting regulatory factors. The *cis*-regulatory elements including splice sites at the beginning and end of each intron (GU and AG), branch point (A), polypyrimidine tract (UA-rich), exonic splicing enhancer (ESE), intronic splicing enhancer (ISE), exonic splicing suppressor (ESS), and intronic splicing suppressor (ISS). Adopted from (Syed et al., 2012).

SR Proteins

SR proteins were discovered as regulatory splicing factors in the early 1990s, as a result of *in vitro* investigations of splicing regulation-associated factors (Long and Caceres, 2009). All SR proteins have a unique and conserved structure consisting of one or two N-terminal RNA recognition motifs (RRMs) and a variable length C-terminal arginine/serine-rich (RS) domain (Manley and Krainer, 2010; Shepard and Hertel, 2009) (Fig 4). RRMs recognize and bind *cis*-regulatory elements in mRNAs (Shepard and Hertel, 2009) while RS domains are primarily involved in protein-protein interactions (Golovkin and Reddy, 1999; Haynes and Iakoucheva, 2006; Reddy, 2007). In addition to the importance of the RS domain in promoting spliceosome assembly, they have been shown to participate in protein-protein interaction (Graveley, 2000), modulating interaction with other proteins or mRNA through heavy phosphorylation and de-phosphorylation (Stojdl and Bell, 1999), nuclear localization and nucleocytoplasmic shuttling (Cáceres et al., 1998; Huang et al., 2003). SR proteins play vital roles in splicing regulation *via* recognizing *cis*-sequence elements, namely splicing enhancers or silencers (Fig 3), in a sequence-specific manner and recruiting spliceosome components to the splice sites (Bourgeois et al., 2004; Richardson et al., 2011). Beside their roles in constitutive and alternative splicing, SR proteins in non-plant systems have also been implicated in mRNA export, RNA stability, nonsense mediated decay (NMD) and translation (Barta et al., 2008; Long and Caceres, 2009; Reddy and Shad Ali, 2011). Overexpression of a variety of SR proteins was found to enhance the nonsense-mediated mRNA decay pathway (Zhang and Krainer, 2004). In animal system, SR proteins have been reported to interact with nuclear export factors TAP/NFX1 where they contribute to nuclear exporting of mRNAs (Huang et al., 2003).

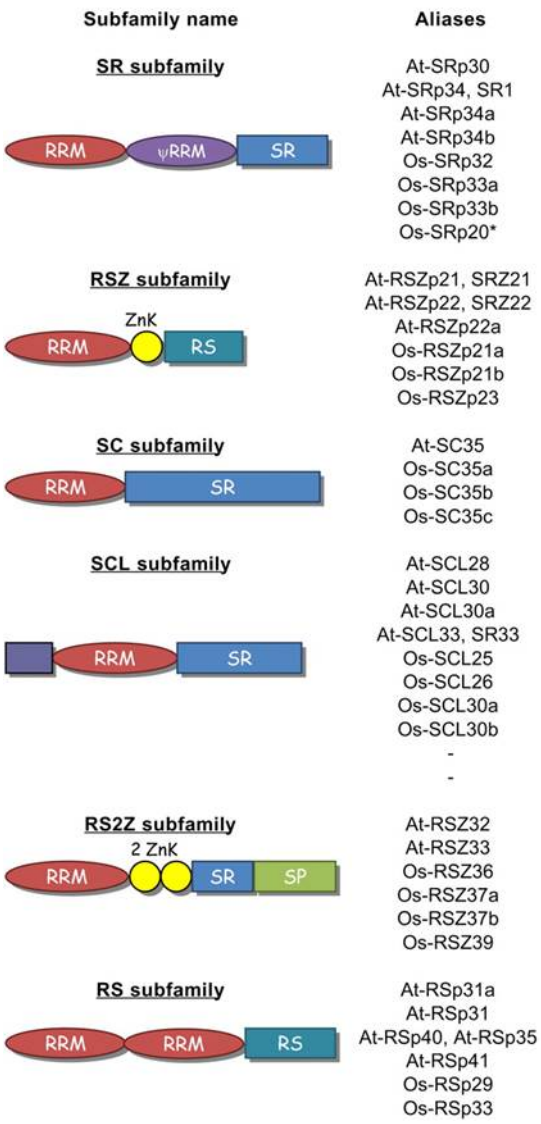


Figure 4: **Schematic diagram showing structural features of Arabidopsis serine/arginine-rich (SR) proteins.** Abbreviations: RRM, RNA recognition motif; RS, arginine/serine-rich domain; Z, zinc knuckle. (Adopted from Barta, 2010)

SR genes are highly conserved throughout eukaryotes (Graveley, 2000; Zahler et al., 1992). An evidence of this conservation is that some plant SR proteins have been reported to complement splicing mechanisms in splicing-deficient HeLa cell extract (Lopato et al., 1996). Analyses of SRs in different lineages suggest that plants possess the highest number of SR proteins of any organism studied with flowering plants (*Arabidopsis*, poplar, rice, soybean, sorghum and maize) containing double or nearly double the number of SR genes as compared to humans (Richardson et al., 2011). In the *Arabidopsis* genome there are 18 SRs, rice has 22 and the highest number was found in soybean (25 SRs) as compared to the human genome, which has 12 SRs (Barta et al., 2008; Richardson et al., 2011). In *Arabidopsis*, some SR pairs are encoded by paralogous genes but it is not known yet if these SRs are functionally redundant or not (Richardson et al., 2011). While there are many SR genes in plants, what remains to be answered is why plants have a higher number of SR genes. It may be an indication of the importance of regulated AS in plants.

Recent comprehensive comparative analyses of plant SR proteins across all eukaryotes identified five major SR groups that can be subdivided into 11 subfamilies based on sequence similarity and domain organization (Richardson et al., 2011). Five of these subfamilies are plant-specific (RS, RSZ, RS2Z, SCL and SR) with unique domain organization and may have evolved plant-specific functions (Duque, 2011). Five subfamilies are present in non-photosynthetic organisms and one subfamily (SC35/SFSR2) shares members from both metazoans and plants (Richardson et al., 2011). Members of the plant RS subfamily are comparable to the SR subfamily with an imperfect highly conserved SWQDLKD sequence in the second RRM domain and they are highly enriched in RS dipeptides in the RS domain (Barta et al., 2008; Barta et al., 2010). Proteins of plant-specific RS2Z subfamily possess two zinc knuckles, unlike 9G8

orthologs that possess a single zinc knuckle, and also a serine and proline-rich C-terminal extension (Barta et al., 2010; Lopato et al., 2002).

Although SRs are the master regulators of both constitutive and alternative splicing, each SR probably regulating the splicing of several hundreds of pre-mRNAs, it is interesting that pre-mRNAs of SRs are also extensively alternatively spliced (Long and Caceres, 2009; Palusa et al., 2007; Reddy and Shad Ali, 2011; Wang and Brendel, 2006). Analysis of splicing of pre-mRNAs of 18 Arabidopsis *SR* genes revealed that pre-mRNAs from 14 *SRs* undergo alternative splicing to produce over 90 transcripts, resulting in a more than six fold increase in the SR transcriptome complexity (Fig 5) (Palusa et al., 2007). On the other hand, 4 of the 18 SRs (*RSZ21*, *RSZ22*, *RSZ22a*, and *SCL28*) are not alternatively spliced (Reddy and Shad Ali, 2011). Interestingly, *RSZ21, RSZ22*, and *RSZ22a* that are not alternatively spliced in plants are orthologs of the 9G8 family in humans, which undergoes AS, suggesting AS of this family is not conserved in plants (Cavaloc et al., 1994). Most splice variants from *SRs* are generated by AS of introns and a majority of them include a premature stop codon (PTC), suggesting that they may be targeted for degradation by nonsense-mediated decay (NMD) or encode nonfunctional proteins (Palusa and Reddy, 2010). In an *Arabidopsis* mutant that lacks *UPF3*, one of the core components of NMD machinery, Palusa and Reddy (2010) have shown a widespread coupling of alternative splicing with NMD in the SR gene family. In an animal system, NMD has also been reported to modulate the expression of *SRs* (Lareau et al., 2007).

The AS of some of the SR genes is controlled at different developmental stages, in a tissue-specific manner and in response to various abiotic stresses and hormones (Fig 5) (Palusa et al., 2007) suggesting that the AS of SR genes is probably important for development and stress responses in plants (Reddy and Shad Ali, 2011). Loss and gain-of-function mutants have shown

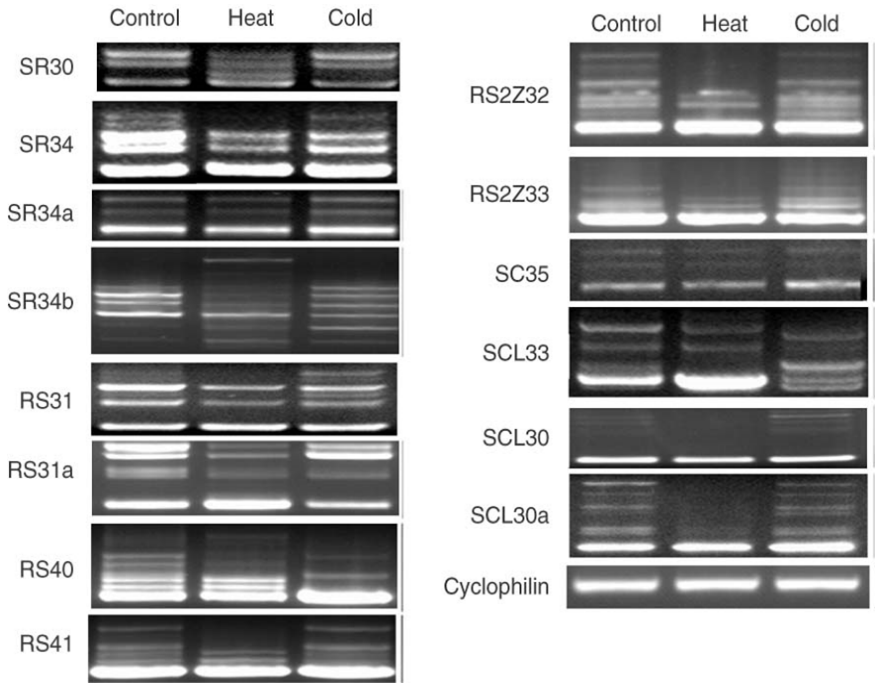


Figure 5: **RT-PCR analysis of splicing of *SR* genes.** Expression and splicing of Arabidopsis *SR* genes in response to abiotic stresses (heat and cold).. (Adopted from Reddy, 2011)

the roles of SRs in root development and vegetative growth as well as flowering, and overexpression lines of some SRs in *Arabidopsis* have shown pleiotropic phenotypes (Reddy and Shad Ali, 2011). For instance, overexpression of *SR30* in Arabidopsis showed defects in transition from vegetative to reproductive phase and changes in AS of other *SR* pre-mRNAs (Lopato et al., 1999). Moreover, ectopic expression of *RS2Z33* was shown to alter the AS of its own pre-mRNA and other *SR* genes with pleiotropic changes in developmental processes such as cell expansion, elongation and division (Kalyna et al., 2003).

The splicing pattern of *SRs* is dramatically changed by either increasing or decreasing specific splice isoforms in response to different stresses. *SR30.1* and *SR30.3* splicing variants are particularly increased under salt and light stress, suggesting alteration in specific mRNA isoforms in response to stresses. Based on the splicing changes in SR pre-mRNAs in response to stresses and also alterations of splicing of many stress-induced genes it is thought that alternative splicing is important in reprogramming the transcriptome and proteome in response to stresses and adaptation of plants to stresses (Ali and Reddy, 2008; Duque, 2011; Iida et al., 2004; Reddy and Shad Ali, 2011). However, the biological functions of most stress-induced mRNA isoforms are not known. To understand the roles of AS in adaptation of plants to stresses it is necessary to investigate the roles of splice variants in abiotic stress response.

SR45, an SR-Like Protein

In addition to SRs, SR-like proteins are also important splicing factors. SR45, one of the plant-specific SR-like proteins, was shown to be an essential splicing factor in a complementation assay (Ali et al., 2007). While SR proteins contain a single RS domain at the C-terminus, SR45 consist of two such RS domains, one at the N-terminus and the other one at

the C-terminus (Fig 6) (Ali et al., 2007; Golovkin and Reddy, 1999). Orthologs of SR45 are found in other flowering plants but absent in algae (Ali et al., 2007). Interactions of SR45 with U1-70K, SC35-like (SR33), AFC2 kinase, U2AF35, and the SR30 intron and its localization to nuclear speckles upon phosphorylation, suggest that SR45 regulates AS, likely by modulating 5' and 3' site selection, by bridging the 5' and 3' components of the spliceosome and likely through other mechanisms (Ali et al., 2003; Ali and Reddy, 2006; Day et al., 2012; Golovkin and Reddy, 1999).

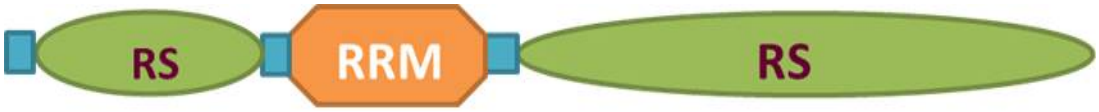


Figure 6: **Schematic diagram showing structural features of Arabidopsis SR-like protein (SR45).** Abbreviations: (RRM) RNA Recognition Motif, (RS) Arginine/Serine rich domain.

By using alternative acceptor 3` splice sites at the beginning of the seventh exon, *SR45* produces two mRNA isoforms that differ by a 21-nucleotide sequence. The long isoform (*SR45.1*) has an additional 21 nts as compared to the short isoform (*SR45.2*) (Palusa et al., 2007; Zhang and Mount, 2009). Because the AS preserves the reading frame, both isoforms are translated and produce functional proteins with a single arginine in the short form substituted by eight amino acids (TSPQRKTG) in the long form (Zhang and Mount, 2009). Loss-of-function of *SR45* affects both alternatively spliced isoforms and the mutant shows pleiotropic developmental phenotypes including narrow leaves and petals, altered number of petals and stamens, delayed root growth and flowering (Ali et al., 2007), indicating that SR45 likely regulates genes that are involved in growth and developmental processes. Isoform-specific complementation of *sr45.1* mutant with green fluorescent protein (GFP) fusion protein provided evidence that the two alternatively spliced isoforms of SR45 have distinct biological functions (Zhang and Mount, 2009). *SR45.1-GFP* complements the flower petal phenotype, but not the root growth phenotype while *SR45.2-GFP* complements the root growth but not floral morphology (Zhang and Mount, 2009). In both root and inflorescence tissues, the AS pattern of other *SRs* (*SRP30, RSP31, RSP31a, SR34* and *SRP34b*) was altered in the *sr45-1* mutant and the isoforms ratio was restored to wild type in transgenic lines that show phenotypic rescue (*SR45.1-GFP*) (Ali et al., 2007; Zhang and Mount, 2009). Recently, *sr45-1* mutants have been linked to establishment and maintenance of DNA methylation (Ausin et al., 2012). In addition, high sensitivity of the *sr45-1* mutant to glucose (Glc) and abscisic acid (ABA) during early seedling development has been reported (Carvalho et al., 2010). In this case, either one of the splice variants can complement the phenotype.

Association of SR45 in glucose (Glc) and abscisic acid (ABA) signaling pathways and increase in SR45 expression in response to different abiotic stresses (Fig 7) suggest that SR45 might have important role(s) in abiotic stress response. Here I investigated the role(s) of SR45 and the biological function of the spliced variants in abiotic stress response using *sr45* and transgenic lines complemented with either long (*sr45::SR45.1)* or short isoform (*sr45::SR45.2*). Compared to WT, *sr45*, at different developmental stages, was highly sensitive to heat stress (38ºC and 45 ºC) and NaCl treatments as well as other ions. Interestingly, this sensitive phenotype was rescued by the long isoform (*sr45::SR45.1*) but not the short one, suggesting that only the long isoform functions in heat and salt stress, likely by regulating the expression and/or the splicing pattern of genes involved in these stresses. Further molecular analyses has revealed that the relative expression and the splicing pattern of heat shock transcription factors (*HSFs*), heat shock proteins (*HSPs*), salt overly sensitive (*SOS*) genes, ABA signaling pathway genes, and other stress-responsive genes were affected in *sr45* and the long isoform is needed for normal splicing and expression of these genes. In addition, *in vitro* analysis showed that the recombinant SR45 binds to the alternatively spliced intron of *HsfA2*. These results suggest an important role for SR45 as a positive regulator of thermotolerance and salt tolerance. Furthermore, our studies indicate that only the long isoform functions in both stresses and the two isoforms might have distinct roles in response to different stresses.

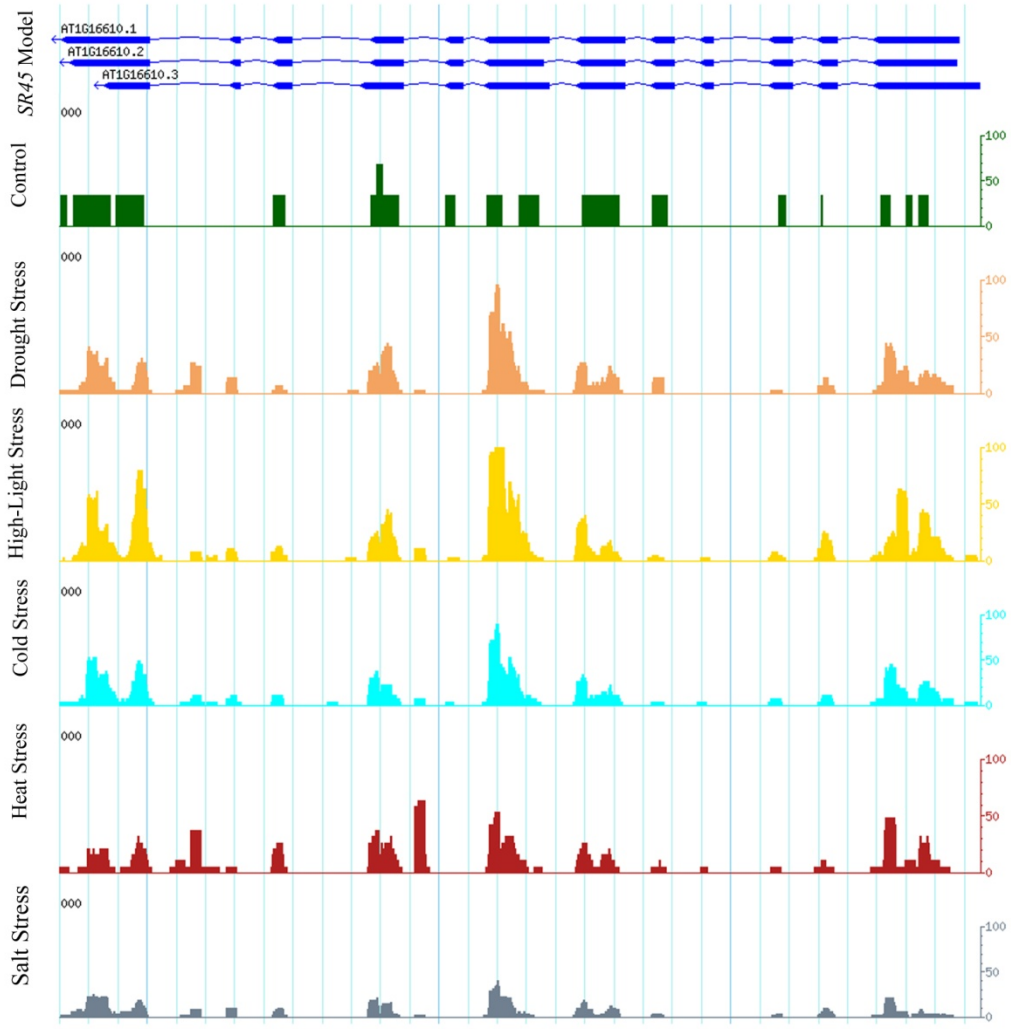


Figure 7: ***SR45* expression in response to different abiotic stresses**. RNA-seq data for *SR45* in response to different abiotic stresses was extracted from the Arabidopsis RNA-seq data at TAIR (http:athal.cgrb.oregonstate.edu/cgi-bin/gbrowse/arabidopsis-gbrowse). Scale to the right represents the read depth and different stresses are shown on the left.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) lines used in this study are in Columbia background. A T-DNA insertion line of *sr45-1* (Salk_004132, http://signal.salk.edu/; Salk_004132) was originally obtained from the Arabidopsis Biological Resource Center (ABRC) and was previously characterized by Ali *et al*. (2007). Seeds of transgenic lines expressing either a long (*SR45.1*) or short (*SR45.2*) isoforms of *SR45* fused to *GFP* (*SR45.1*::GFP and *SR45.2*::GFP) in the *sr45* mutant were provided by Professor Stephen M. Mount at the University of Maryland, College Park (Zhang and Mount, 2009). Mutant and lines complemented with the long or short isoform of *SR45* were genotyped by RT-PCR using gene-specific primers (sequences of the primers are provided in Table 1). Seeds from fully mature siliques were collected and used in all experiments. Seeds of WT and homozygous transgenic lines seeds were surface sterilized with 70% ethanol followed by 15% bleach and stratified for 3 days at 4°C.

Heat Stress Treatment

For thermotolerance assay at seed stage, stratified seeds of all lines (WT, *sr45* and complemented lines) were heat-treated in a water bath either at 38ºC for 24h or at 45ºC for 5h. Control seeds were incubated in a water bath at room temperature for the same period of time. After heat treatment, seeds were sown on MS plates (Catalog No. M524, Phytotechnology) containing 1% sucrose. Plates containing treated and untreated seeds of all lines were grown for 10 days in a growth chamber (100 μmoles m^{-2} s^{-1} light, 16 h/8 h dark/light cycle at 22°C and 70% RH). To analyze heat stress response at seedling stage, sterilized seeds of WT, *sr45*, and

transgenic lines were grown on MS plates for 7 days and then subjected to heat treatment at 38ºC for different time courses (12, 24 and 36 h) and at 45ºC for 4 h. Control plates were kept in the growth chamber all the time. Following treatment the position of root tips was marked and seedlings were allowed to recover under normal growth conditions for 2-3 days. Primary root growth in control and heat-treated seedlings during recovery period was quantified on the 10th day using ruler. To analyze heat stress response in mature leaves, same-stage rosette leaves from 25-day-old WT, mutant, and complemented plants were excised and floated over 3 ml distilled water in a small Petri dish, exposed to 45ºC for 5h and then allowed to recover in the growth chamber for 2 days before data collection. Control plates were kept in a growth chamber at normal growth conditions. For gene expression analysis of thermotolerance-related genes, two-week old seedlings of WT, *sr45* and transgenic lines were heat treated for 3 h at 38 ºC and then left to recover at 22°C for 6 h. Seedlings were harvested, frozen in liquid nitrogen and stored in -80ºC freezer for RNA extraction.

Salt Stress Treatment

To study the effect of salt stress on seed germination and seedling growth, sterilized seeds of WT, *sr45*, and transgenic lines were grown on MS or on MS supplemented with 150 mM NaCl for 10 days in a growth chamber. Images were captured using digital camera on the 10th day and root length was measured manually using ruler. To test *sr45* response to different ions, seeds of WT, mutant and transgenic lines were plated on either MS or MS supplemented with 150 mM KCl or 10 mM LiCl, or 6 mM CsCl. All plates were allowed to grow for 10 days before taking the pictures and measuring root length. To test the effect of salt stress at post-germination stage, seeds of WT and transgenic lines were grown on MS medium for 3 days, transferred under aseptic conditions to either MS or MS plates supplemented with different salts

(150 mM NaCl or 150 mM KCl or 10 mM LiCl or 6 mM CsCl). Seedlings were left to grow for 10 days in a growth chamber under normal growth conditions before taking the images and recording the data.

In order to investigate salt stress tolerance of *sr45* and the complemented lines at late developmental stages, I used a hydroponic setup according to Abdel-Ghany et al. (2008). Surface sterilized seeds of WT, *sr45* and complemented lines were grown vertically on MS plates for 10 days under normal growth conditions. After 10 days seedlings were moved gently to a hydroponic setup containing 1/10 X Hoagland's solution (Hoagland and Arnon, 1983) and allowed to recover for two days. For salinity treatment, 30 mM NaCl was added to 1/10 X Hoagland's solution, while the control set was kept in 1/10 X Hoagland's solution. The growth medium was replaced every week and maintained daily with distilled water to compensate for evaporation. Plants were allowed to grow for 3 weeks at room temperature under 100 µmoles m^{-2} s^{-1} light, 16 h/8 h dark/light cycle. Plant pictures were then taken using digital camera and fresh weight and survival rate were recorded. For expression analyses of genes involved in salt stress, all lines were grown on MS medium for two weeks and then transferred to either MS or MS supplemented with 150 mM NaCl for 6h. Seedlings were harvested, frozen in liquid nitrogen and stored in -80°C freezer for RNA extraction

Pigments Content and Elemental Analysis

Spectrophotometric quantification of chlorophyll *a*, *b* and carotenoids content of control and heat-treated leaves of WT and transgenic lines was conducted in 1 ml 95% ethanol and quantified according to Lichtenthaler (1987). For elemental composition analysis WT, *sr45* and complemented lines were grown on MS medium for 3 days, transferred to either MS or MS

supplemented with 150 mM NaCl and allowed to grow for two weeks. Seedlings were harvested, washed once with 50 mM EDTA, rinsed three times with excess volume of distilled water and dried at 65 ºC for 48 h. Known weights of the dried tissues were digested and analyzed using inductively coupled plasma-atomic emission spectroscopy (ICP) as described by Abdel-Ghany, (2009). Elements were calculated as mg/g dry weight.

RNA Isolation, Reverse Transcription-PCR, and Quantitative PCR (qRT-PCR)

Total RNA was extracted from frozen tissues using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), quantified using a NanoDrop spectrophotometer and used for cDNA synthesis. Total RNA was treated with DNase I (Fermentas, Hanover, MD) to remove residual genomic DNA present in RNA samples. For RT-PCR, 1.5 µg of DNase-treated total RNA was used to synthesize first-strand cDNA with an oligo (dT) primer and SuperScript II (Invitrogen, Carlsbad, CA) in a 20 ul reaction. The PCRs were performed in a final volume of 20 ul using 1 ul from the cDNA as template and gene specific primers. Primers were designed using the PRIMER3 software (http://frodo.wi.mit.edu). The Gene ID number of all genes used in this study and the corresponding primers used in RT-PCR are listed in Table 1. The reaction mixture was heated at 94°C for 3 min, cooled to 55°C, and 2 units of Ex-Taq polymerase (Takara) was added to initiate the amplification reaction. Thirty cycles of amplification were performed in an Eppendorf Mastercycler gradient, each consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C followed by extension at 72°C for a time period based on the expected length of the PCR product. The amplified products were resolved by electrophoresis and gel pictures were captured using ChemiDoc^{TM} XPS^{+} (BioRAD). For semi-quantitative PCR 15 µl of the reactions were collected at different cycles and resolved by 2 % agarose gel electrophoresis. *ACT2* or cyclophilin was used as an internal control to show equal amount of template.

For quantitative PCR, primer pairs were designed using the PRIMER3 software (http://frodo.wi.mit.edu) and the primer sequences are listed in Table 2. The amplification reactions were carried out with LightCycler 480 SYBR Green 1 master mix on a LightCycler 480 (Roche Applied Science). PCR tubes (10 µl) contained the vendor's master mix, 0.5 µmol of each primer, and cDNA corresponding to 20 ng of input RNA in the reverse transcriptase reaction. The PCR conditions were 95 °C for 4 min followed by 40 cycles of 95 °C for 10 s, annealing temp (calculated T_m -5°C) for 10 s, and 72 °C for 30 s. The fluorescence was measured at each cycle at 72 °C. Melting curves were performed after the PCR reaction to assess the presence of a unique final product. The cycle number at which fluorescence signals crossed the threshold (C_T) value was determined. To compare data from different PCR runs or cDNA samples, C_T values for all tested genes were normalized to the C_T value of the *ACT2* gene, a constitutively expressed "housekeeping" gene. Gene expression was normalized to *ACT2* expression by subtracting the C_T value of A*CT2* from the C_T value of the tested gene (ΔC_T).

***In vitro* Transcription**

In vitro transcription of RNA from a PCR template was performed according to Beckert and Masquida, (2011). Oligonucleotides were designed to amplify the intronic rejion of *HsfA2* where the SP6 promoter sequence (underlined) was added upstream of the forward primer. Sense (5'ATTTAGGTGACACTATAGGTAAGTACCTCTTTGCTTC3') and antisense (5'AAACAA GTTTCATCAGCTAC3') primers were used for amplification of alternatively spliced first intron and the PCR product was purified and verified by sequencing (Proteomics and Metabolomics Facility, Colorado State University).

Radiolabeled capped RNA from the PCR-amplified intronic DNA template was transcribed *in vitro* with 45 μCi [α-^{32}P] UTP (800 Ci mmol^{-1}, Perkin-Elmer, www.perkinelmer.com) using SP6 RNA polymerase (Fermentas, www.fermentas.com) in the presence of 500 μM ATP, 500 μM CTP, 50 μM GTP, 50 μM UTP and 7mGpppG (7 methyl di-guanosine triphosphate). The amplified product was digested with DNase and gel-purified as previously described (Wilusz and Shenk, 1988). Unlabeled competitor RNA was generated in the same manner, but without 7mGpppG or radiolabelled nucleotide, and the concentrations of UTP and GTP were increased to 500 μM.

Expression and Purification of Recombinant SR45 Protein

SR45 cloned in p-ET28a was constructed previously in our laboratory (Golovkin and Reddy, 1999). Overnight bacterial cultures were grown at 37 °C until OD_{600} reached to 0.5-0.6, after which 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the cultures were incubated at 30 °C for 4h to induce protein expression. Cultures were centrifuged (5000 xg) at 4^{o}C for 5 minutes. Pellet was rinsed with 50 mM Tris-HCl (pH 8.0), re-suspended in one-tenth of the culture volume of lysis buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 100 μg ml lysozyme and 0.1% Triton X-100) in presence of the protease inhibitors and incubated at 4°C for 15 min. The extract was sonicated, centrifuged and used for purification. S-protein agarose beads were added to the supernatant and incubated for 1 h at 4°C. The beads were then washed three times with binding buffer, and the bound protein was eluted using 0.2 M citrate buffer (pH 2.0) and neutralized by adding a 1/20th volume of 2 M Tris base (pH 10.4). The eluted protein was dialyzed using phosphate buffer (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl pH 7.4), quantified with Bradford reagent (Bradford, 1976) and used in electrophoretic mobility shift assay.

Electrophoretic Mobility Shift Assay (EMSA)

Radio labeled RNA of the *HsfA2*-intron was incubated with increasing amounts of purified recombinant SR45 in the presence of RNase inhibitor (Invitrogen), 15 mM spermidine and gel shift buffer (15 mM HEPES pH 7.9, 8% glycerol, 100 mM KCl and 2 mM $MgCl_2$) for 5 min at 30°C in a 14 µl reaction volume. Subsequently 4 µg μl^{-1} of heparin sulfate (Sigma, www.sigmaaldrich.com) was added to each sample, and then the sample was cooled on ice for 5 min. 10X loading dye (1.5 µl) (30% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol) was added and samples were resolved on a 5% native polyacrylamide gel at room temperature in 1X TBE (Tris-Borate-EDTA) buffer (200 V for 2–6 h). Gels were dried and visualized using Storm PhosphoImager (Molecular Dynamics, www.amershambiosciences.com).

RNA-seq Analysis

The gene models and RNA-seq coverage of all analyzed genes in this study were visually inspected using Arabidopsis RNA-seq GBrowse (http://athal.cgrb.oregonstate.edu/cgi-bin/gbrowse/arabidopsis-gbrowse/). RNA-seq of *sr45* and its control (WT) were extracted from RNA seq data (manuscript in preparation) and were visually inspected using Integrated Genome Browser (IGB) (http://bioviz.org/igb/). The nucleotide sequences of studied genes were obtained from Arabidopsis TAIR10 annotation (http://www.arabidopsis.org).

Statistical Analysis

Statistical analyses were conducted using JMP 10 software (http://www.jmp.com). All experiments were repeated at least three times and statistical significance was determined using analysis of variance followed by Tukey's test for mean separation ($P<0.05$).

Accession Numbers

Arabidopsis gene ID numbers were extracted from Arabidopsis TAIR10 for the genes discussed in this thesis are as follows: *SR45 (*AT1G16610), *HSFA2* (AT2G26150), *HSP101* (AT1G74310), *HSP70B* (AT1G16030), *HSP18* (AT5G59720), *HSP17.7* (AT5G12030), *HSP25* (AT4G27670), *APX2* (AT3G09640), *GOLS1* (AT2G47180), *HSFA1a* (AT4G17750), *HSFA1b* (AT5G16820), *HSFA1e* (AT3G02990), *HSFA4a* (AT4G18880), *HSFA4c* (AT5G45710), *HSFA5* (AT4G13980), *HSFA6a* (AT5G43840), *HSFA7a*(AT3G51910), *HSFB2a* (AT5G62020), *HSFB2b* (AT4G11660), *HSFB4* (AT1G46264), *HSFC1* (AT3G24520), *SOS1*(AT2G01980), *SOS2* (AT5G35410), *SOS3* (AT5G24270), *SOS4* (AT5G37850), *RD29A (*AT5G52310), *RD29B* (AT5G52300), *RD20* (AT2G33380), *ADH1* (AT1G77120), *DREB2A* (AT5G05410), *ABF1* (AT1G49720), *ABF2* (AT1G45249), *ABF3* (AT4G34000), *ABI5* (AT2G36270), and *ACTIN2* (AT3G18780).

Table 1: Primers used for RT-PCR.

ATG	name	Primer sequence (5'-3')	Annealing Temperature
AT1G16610	SR45	AAGTCCTGCTGGACCTGCTA	58.2 ºC
		CCTTCTTCGAACAGGACTGC	55.7 ºC
AT2G26150	HSFA2	GCTTTGTGGTGTGGGATTCT	55.6 ºC
		CATCCCAGATCCTTGCTGAT	54.5 ºC
At1g74310	HSP101	GGCTCATGTTGCTGTCTTCA	55.5 ºC
		GACTGCCAAAGCAACTCCTC	56.3 ºC
At5g59720	HSP18	ACGTCTTTGATCCGTTCTCG	54.8 ºC
		CGTAAGCACACCATTCTCCA	54.9 ºC
AT5G12030	HSP17.7	CCCTGAAGAACAAACCGAGA	54.6 ºC
		GGCTCAGGAGGAGGAAGTTT	56.6 ºC
AT4G27670	HSP25.3	CCATGGACGTCTCTCCTTTC	55.0 ºC
		ACGCTTCTTCCAGACCAAGA	56.2 ºC
AT1G16030	HSP70B	TGGACTTGAGACAGCAGGTG	57.0 ºC
		ACCTTCCTTTGTCGTTGGTG	55.4 ºC
AT2G47180	GOLS1	CGTGAAAGGAGTCGTTGGTT	55.3 ºC
		ATCACCGCGTACAAATAGCC	55.3 ºC
AT3G09640	APX2	TAAGGCATCCCCAAGAGCTA	55.4 ºC
		GTGTGTCCACCAGACAATGC	56.5 ºC
AT4G17750	HSFA1a	GACGGGTTCTCATCTCCAAA	54.4 ºC
		GAACCACCATCAAGGAATGG	55.6 ºC
AT5G16820	HSFA1b	AACATTGGCCGAGTTTTCAC	55.9 ºC
		CATTCATCTTGCCCTCACCT	54.9 ºC
AT3G02990	HSFA1e	GTGGGGCAGAAAGTTCATGT	55.6 ºC
		GTTTGTTGCATCGTTCATCG	54.7 ºC
AT4G18880	HSFA4a	ACGCAAACCAGTTCATAGCC	55.8 ºC
		GCGATAGGTTCAAAGCAAGC	54.6 ºC
AT5G45710	HSFA4C	CCAAGAGCAAGAACGGAAAG	55.9 ºC
		TCTCGCATGATTCCGATACA	55.4 ºC
AT4G13980	HSFA5	TGGAGCGCTAACAACAACAG	55.6 ºC
		TGCTCTTGCAACACTGCTCT	57.2 ºC
AT5G43840	HSFA6A	TGAGCTGCGAAGAGACAGAA	56.1 ºC
		ACCATGGCTTGAGATCACCT	56.2 ºC
AT3G51910	HSFA7a	AGGTGGAAGCCAAACTCTCA	56.3 ºC
		AGCAATGCAGAGTCCTTCGT	56.8 ºC
AT1G67970	HSFA8	ATGATGGAGCATGGGAAAAG	54.9 ºC
		CCAGCAATTCCATCTGCTCT	55.2 ºC
AT5G62020	HSFB2A	TGGAACGAAGATGGTTCCTC	54.4 ºC
		CCGGAATTTGACGGTGATAC	53.5 ºC
AT4G11660	HSFB2B	ACCAGAGCTGGTTGAGGAGA	57.9 ºC
		CTCGCTTGACCCCAATAGAA	55.5 ºC

Table 1, continued: Sequences of primers used for RT-PCR.

ATG	Name	Primer sequence (5'-3')	Annealing Temperature
AT1G46264	HSFB4	CTCCGTTCATGTCACACCAC	55.9 ºC
		CAGGCTTGACATGGTTTTGA	55.5 ºC
AT3G24520	HSFC1	GCAACGAATCTTACCTGCTT	55.9 ºC
		CTAGCTCCCTCTGCTCTTCTT	55.9 ºC
AT5G24270	SOS3	CGGTCCTTAGGTGTCTTCCA	56.2 ºC
		CTTGCACGAAAGCCTTATCC	54.1 ºC
AT5G37850	SOS4	TTCCACAAGGACCACAATCA	54.1 ºC
		CCCCAACAATTGAAGAGGAA	55.7 ºC
AT2G33380	RD20	CGGAACGATTTGGAGGAAACA	58.0 ºC
		AGTATCCATTCAACTTTGTTTG	55.1 ºC
AT1G45249	ABF2	CGAGAATCAGCTGCAAGGTC	56.0 ºC
		AAGGTCCCGACTCTGTCCTC	58.3 ºC
AT5G24270	SOS3	CGGTCCTTAGGTGTCTTCCA	56.2 ºC
		CTTGCACGAAAGCCTTATCC	54.1 ºC

Table 2: Primer sequences used for qPCR.

ATG	Name	Primer sequence (5'-3')	Annealing Temperature
AT2G26150	HSFA2	AGCTCAATACTTATGGATTCAGAAAGA	59.4 ºC
		AAA CCG TAT TGC CCA ACC TC	58.6 ºC
At1g74310	HSP101	GAGGAGTTGCTTTGGCAGTC	58.3 ºC
		CAGCGCCTGCATCTATGTAA	59.1 ºC
At5g59720	HSP18	GGTTACCGGAGAATGCAAAGATG	58.1 ºC
		CGATGGACTTGACTTGAGGCTTC	58.7 ºC
AT5G12030	HSP17.7	TCAGGTCCAGATAGAGAACGAGAAC	57.4 ºC
		CCTCTCCATCCTCACAAACTTCAC	57.3 ºC
AT4G27670	HSP25.3	ACGGAGTCCTCTTTATCACTATCCC	57.4 ºC
		GATCGAGTCCTACTGAATCTGG	58.2 ºC
AT1G16030	HSP70B	GCAGAAGATTGAGAAGGCGATTG	58.3 ºC
		CGCTCTAATCCACCTCTTCGATC	60.01 ºC
AT2G47180	GOLS1	AGCCGTTCATCACCGCTCTTAC	59.4 ºC
		ACTCCTGGCAACATTCAAGCAG	57.9 ºC
AT3G09640	APX2	AATATGCTGCAGATGAGGATGC	58.4 ºC
		CAAGAATCAAGGAGGTAGGAGATG	58.7 ºC
AT2G01980	SOS1	AAAGTCGCACTTGGAGCTGT	60.06 ºC
		CATGACCGTCAAAACACCAG	60.0 ºC
AT5G35410	SOS2	TTGGTCGCACAATAGGTGAA	60.11ºC
		GTTCGGGTGACGAACAATCT	59.97 ºC
AT5G24270	SOS3	CGGTCCTTAGGTGTCTTCCA	60.1 ºC,
		CTTGCACGAAAGCCTTATCC	59.85 ºC
AT5G52310	RD29A	TCTTGCTCATGCTCATTGCT	59.76 ºC ,
		CAACACACACCAGCAGCACCC	60.32 ºC
AT5G52300	RD29B	GGTCATGAGCAAGCAGAAGAACC	59.76 ºC
		ACTTCTGGGTCTTGCTCGTC	60.32 ºC
AT1G77120	ADH1	ACCACCGGACAGATTATTCG	59.76 ºC
		AGCTTCATGGCCGAAGATAC	60.32 ºC
AT5G05410	DREB2A	GGAAAACTCGAAAAAGCTACACA	59.76 ºC
		AGCCACAGTAGTACCGTCACC	60.32 ºC
AT1G49720	ABF1	AGATCAAGGGCTCGAAAACA	59.76 ºC
		ATTGTCTTTTGGCCAGCAAT	60.32 ºC
AT4G34000	ABF3	CAGGTGCAGTTCTGGAGAAAG	59.76 ºC
		CAGGGACCCGTCAATGTC	60.32 ºC
AT2G36270	ABI5	GGTCCAGTGGAGAAAGTAGTGG	59.76 ºC
		TCT CCC GTT CGA TTT CGG CAA	60.32 ºC
AT3G18780	ACTIN2	GGCAAGTCATCACGATTGG	58.0 ºC
		CAGCTTCCATTCCCACAAAC	58.3 ºC

RESULTS

PART 1

sr45 is Thermosensitive at Different Developmental Stages and This Phenotype is Rescued Only by the Long Isoform

SR45, an SR-like protein, is an essential splicing factor involved in regulating alternative splicing of pre-mRNAs (Ali et al., 2007; Golovkin and Reddy, 1999; Reddy and Shad Ali, 2011). Alternative splicing of *SR45* pre-mRNA generates two spliced isoforms that differ in 8 amino acids and each isoform has distinct biological functions in plant development (Zhang and Mount, 2009). Several studies suggest that stresses significantly modulate alternative splicing and this stress-regulated alternative splicing is thought to be important for stress adaptation of plants (Ali and Reddy, 2008; Duque, 2011; Iida et al., 2004; Palusa et al., 2007). Earlier studies have shown that glucose and the phytohormone ABA modulate various biological processes including response to changes in environmental conditions (Koch et al., 1996; Lopez-Molina et al., 2001). Carvalho et al., (2010) have found that loss-of-function mutant, *sr45-1*, confers hypersensitivity to glucose and ABA. As reported in the introduction, RNA-seq data from plants exposed to different abiotic stresses (drought, high light, cold, heat and salt) has revealed that the *SR45* expression is induced in response to diverse stresses with the highest expression in response to drought and high light. Based on the suggested roles of alternative splicing in plant stress responses and induction of SR45 expression in response to diverse stresses I hypothesized that SR45 may have a regulatory role in plant stress responses. To test this hypothesis, I investigated the role of SR45 in two abiotic (heat and salt) stress responses and the biological roles of the *SR45* mRNA isoforms in these stress response.

To investigate the role of *SR45* in abiotic stress tolerance and the biological role of two mRNA isoforms, I used WT, *sr45* loss-of-function mutant (*sr45-1*) and two transgenic lines that are expressing either the long (*sr45::SR45.1*) or short (*sr45::SR45.2*) isoform in the mutant background. Genotypes of all lines were tested for *SR45* expression by RT-PCR using gene-specific primers (Fig 8). No amplification was observed in *sr45* (second lane) suggesting that this is a loss-of-function mutation. A single product of predicted size was detected in both complemented lines (lane 3 and 4), indicating that each line is expressing a specific isoform. The size of the product is slightly higher in the complemented line expressing the long isoform (Fig 8). The short isoform was not detected in the WT as this isoform is normally expressed at a low level.

To perform heat stress studies, all four lines were exposed to either 38 ºC or 45 ºC at different developmental stages (seeds, seedlings or adult plants) for different time periods. To test the heat stress response at germination stage, sterilized seeds were heat treated at 38ºC for 24h, grown on MS plates for 10 days and then root length was measured to quantify heat stress response. The root length of *sr454* and the line complemented with the short isoform is significantly reduced compared to WT (~50% and ~35%, respectively compared to 20 % reduction for WT) (Fig 9). On the other hand, no significant difference from WT was observed in the mutant complemented with the long isoform suggesting that SR45 is important for heat stress tolerance and the long isoform, but not the short one, rescues the heat sensitive phenotype when seeds were exposed to heat stress. To test the heat stress response at post-germination stage, seeds were grown at 22ºC for 7 days, transferred to 38ºC for different time periods (12, 24 or 36 h) and allowed to recover for 2 days (Fig 10). The longer the treatment time the more inhibition

was observed in all lines (Fig 10C). At 36 h the leaves of both *sr45* and the mutant complemented with the short isoform were bleached with

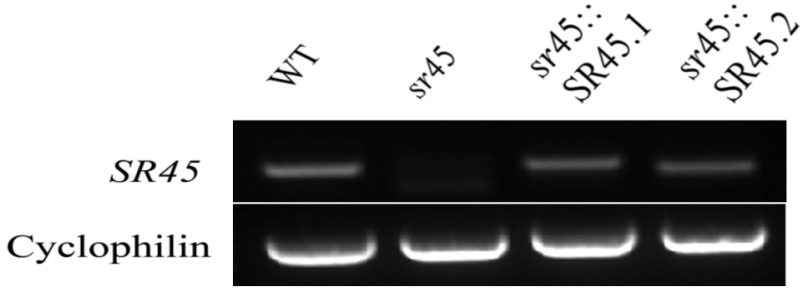


Figure 8: **Genotype analysis of *sr45* mutant and the complemented lines using RT-PCR.** Total RNA was isolated from wild type (WT), *sr45* mutant, *sr45* complemented with either the long isoform (*sr45::SR45.1*) or the short one *(sr45::SR45.2*) and used for cDNA synthesis. An equal amount of cDNA template was used as a template for PCR using gene specific primers. An equal amount of cDNA in different samples is verified by amplified cyclophilin.

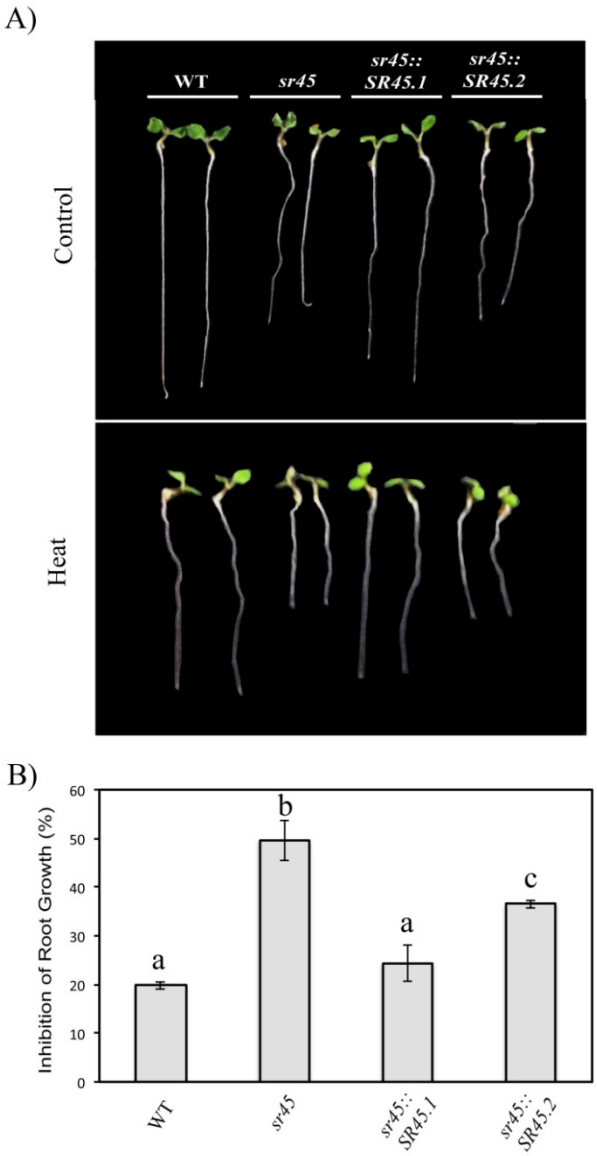


Figure 9**: Role of *SR45* isoforms in thermotolerance of Arabidopsis seeds.** Surface-sterilized seeds of WT, *sr45*, and complemented lines were heat-treated at 38ºC for 24h, and grown on MS plates for 10 days. Plant images (A) were taken and percent of inhibition of root growth (B) in heat-treated samples as compared to controls was calculated on the 10th day. Untreated seeds were shown as a control. Long isoform fully rescued the root length phenotype. Experiment was repeated three times and data represent means ±SE from a minimum of 30 seedlings. Statistical significance was determined using analysis of variance and Tukey's test and indicated by letters associated with bars.

no root growth during recovery (Fig 10B). Although a significant reduction in primary root length was observed in the mutant exposed to 12 h and 24 h of heat (~40% and ~90% inhibition, respectively), no significant difference was observed for long and short complemented lines (Fig 10A, C). However, the heat-treated long isoform complemented line showed an increased number of lateral roots and root hairs, suggesting that the long isoform induces lateral root formation in response to heat stress (Fig 10A, arrowheads). To test the response of *sr45* and complemented lines to a higher temperature (45ºC) during early germination, seeds were exposed to heat treatment for 4 h and allowed to recover for 10 days (Fig 11). Similar to 38ºC treatment, *sr45* and the mutant complemented with short isoform displayed enhanced sensitivity at 45ºC. Significant reduction in root growth following heat treatment was observed in the mutant and short form expressing line, whereas no inhibition of root growth was detected in the mutant expressing the long isoform (Fig 11A and B). Similarly, 7 day old seedlings of *sr45* mutant and short isoform-complemented line showed reduced root growth and chlorotic leaves when exposed to 45ºC for 4h (Fig 12A and B) and also this reduced root growth was fully rescued by the long isoform. These results suggested that SR45 is required for thermotolerance at early growth stages (seeds and seedlings), and the long isoform is the one that complements the defect of thermotolerance in *sr45*, not the short one.

To test the involvement of *SR45* in heat stress responses at later stages of development (i.e., in adult plants) rosette leaves from 25-day-old seedlings were exposed to 45ºC for 5h and allowed to recover for 2 days as described in Methods. Compared to untreated leaves the treated ones showed chlorosis with a severe effect observed in leaves from mutant and mutant complemented with the short isoform (Fig 13A). However, leaves from complemented lines with the long isoform were similar to WT, suggesting that only the long isoform complements this

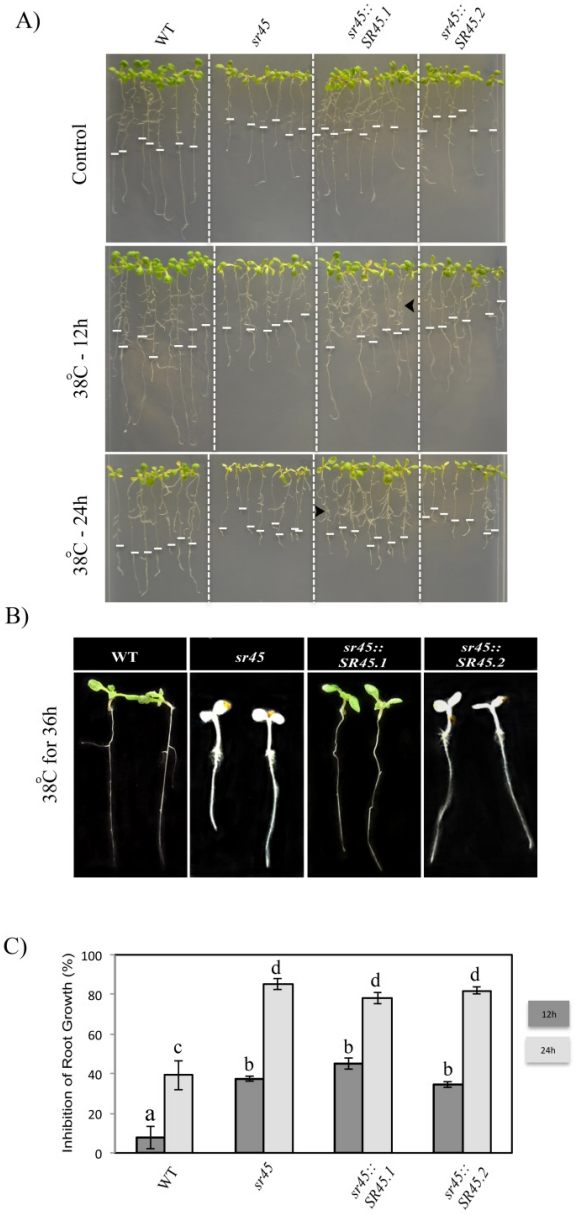


Figure 10: **Role of *SR45* isoforms in thermotolerance of *Arabidopsis* seedlings.** WT, *sr45*, and complemented lines were grown at 22 ^{0}C for 7 days and then transferred to 38^{0}C for different time periods (12, 24, or 36 h). Tips of the main root were marked then plants were allowed to recover at 22ºC for 2 more days. Plant images (A & B) were taken and percent of inhibition in root growth was calculated (C). Enhancing lateral root growth in long isoform is indicated by arrowhead. Experiment was repeated three times and data represent means ±SE from a minimum of 30 seedlings. Statistical significance was determined using analysis of variance and Tukey's test and indicated by letters associated with bars. No root growth was recorded for seedlings that were treated for 36 h during the recovery period.

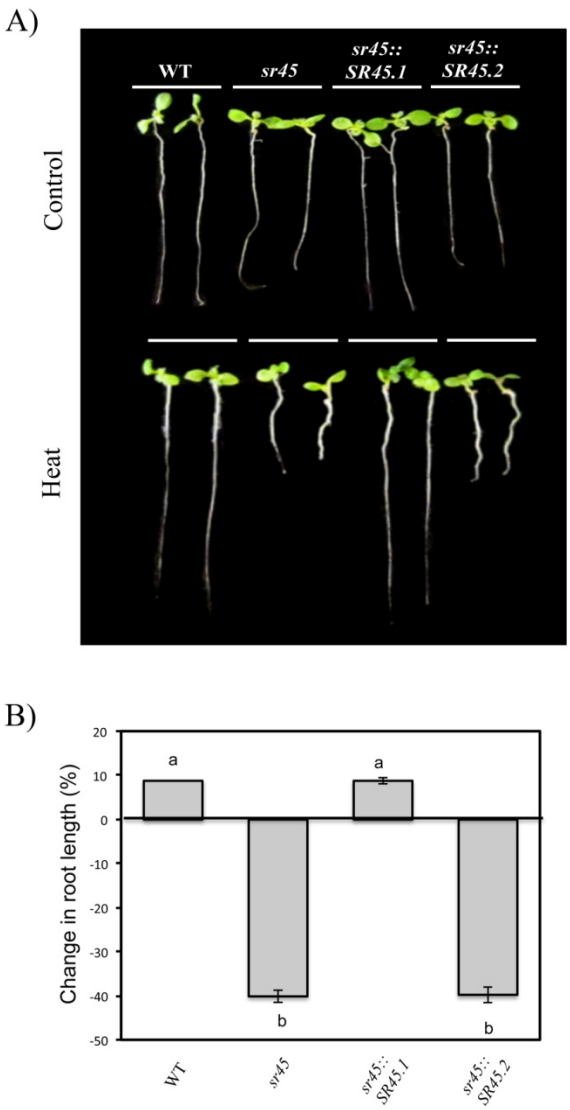


Figure 11: **The effect of high temperature (45ºC) on seeds of WT, *sr45* and complemented lines.** Surface-sterilized seeds of WT, *sr45*, and complemented lines were heat-treated at 45^0C for 5h, and grown on MS plates for 10 days. Plant images (A) were taken and change in root growth (B) was recorded. Experiment was repeated three times and data represent means ±SE from a minimum of 30 seedlings. Statistical significance was determined using analysis of variance and Tukey's test and indicated by letters associated with bars. Untreated seeds were used as a control.

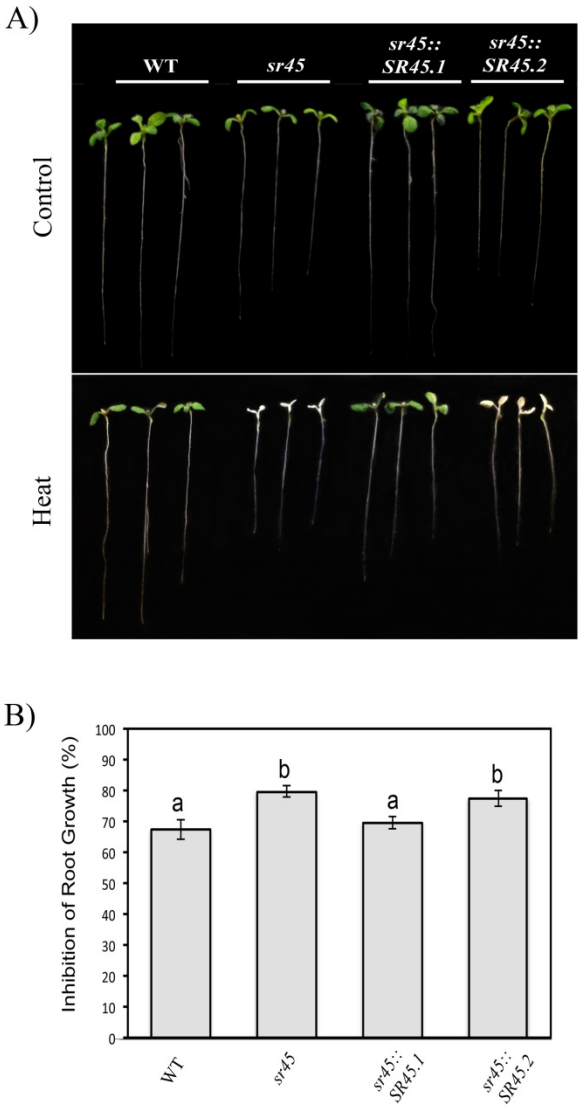


Figure 12: **The effect of high temperature (45ºC) on seedlings of WT, mutant and complemented lines.** Seven day-old seedlings of WT, *sr45*, and complemented lines were heat-treated for 4h at 45ºC and then allowed to recover for two days at 22 ^{0}C. Un-treated seedlings were used as a control. Images were taken after 2 days of recovery (A) and inhibition of root growth was quantified (B). Experiment was repeated three times and data represent means ±SE from a minimum of 30 seedlings. Statistical significance was determined using analysis of variance and Tukey's test and indicated by letters associated with bars.

phenotype. Since chlorosis is dependent on the degradation of photosynthetic pigments I quantified pigment levels. To quantify the amount of degradation in pigments I measured chlorophyll a, b and carotenoid content in the same leaves under both heat stress and control conditions (Fig 13B, C and D). Under heat stress conditions reduction in pigment content was observed in all lines but the reduction was more dramatic in *sr45* and the mutant complemented with the short isoform compared to WT and the mutant complemented with the long isoform. Taken together these results suggest that SR45 is essential for heat stress tolerance and functions as a positive regulator in thermotolerance. Furthermore, the long isoform appears to have a major role in thermotolerance at different developmental stages.

Splicing and Expression Patterns of *HsfA2* are Misregulated in *sr45* in Response to Heat Stress

Previous studies have established that *Arabidopsis* heat–inducible transcription factor *HsfA2* is a key regulator of heat stress response and other abiotic stresses (Nishizawa et al., 2006; Nover et al., 2001; Ogawa et al., 2007). Interestingly, recent studies have shown that *HsfA2* pre-mRNA undergoes alternative splicing in response to heat treatment and produces three splice variants (*HsfA2-I*, *II*, and *III*) (Liu et al., 2013; Sugio et al., 2009). *HsfA2-I* isoform encodes the functional protein, and *HsfA2-II* has a premature termination codon (PTC) and is likely targeted for degradation by NMD. *HsfA2-III* encodes a small protein that is important for autoregulation of its expression under high temperature (Fig 14A). As SR45 is a splicing factor, *sr45* is thermosensitive and *HsfA2* is alternatively spliced in response to heat, I have investigated the expression and alternative splicing patterns of *HsfA2* in WT, *sr45* and the two complemented lines under control and heat stress conditions to test if SR45 is important for *HsfA2* splicing and regulation.

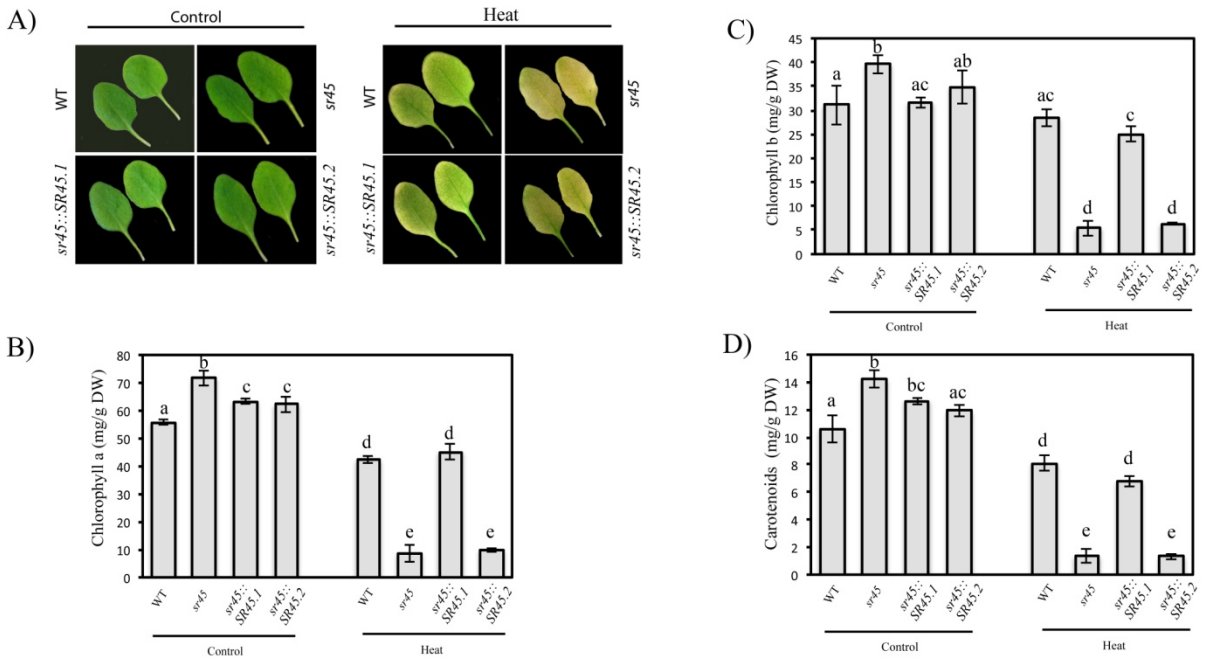


Figure 13: **Heat stress response of 25 day-old leaves of WT, *sr45* and complemented lines.** Same age leaves of WT, *sr45*, and complemented lines were excised, floated in Petri dishes containing water and incubated at either 22^0C or 45^0C for 5 h. After 2 days of recovery at 22^0C the images were taken (A) and the chlorophyll "a", "b" and carotenoids (B, C, D, respectively) were quantified. Experiment was repeated at least three times with similar results and the statistical analysis was determined using analysis of variance and Tukey's test, significance is indicated by letters associated with the error bars.

Semi-quantitative RT-PCR analysis showed that the expression and splicing pattern of *HsfA2* is altered in response to heat treatment where the expression of the functional isoform (isoform I) is increased while the expression of other isoforms (II and III) is reduced (Fig 14 B) in WT and the complemented line expressing the long isoform. In contrast, the level of induction was much less in the mutant and in the mutant complemented with the short isoform. qPCR quantification of isoform I using isoform-specific primers also indicated that this isoform is induced in response to heat treatment and the level of induction is significantly less in the mutant and mutant complemented with the short isoform (~25%) (Fig 14 C) suggesting that the proper expression and splicing of *HsfA2* might be mediated through the splicing factor SR45. These findings further support the heat sensitivity phenotype of *sr45*, which is rescued by the long isoform at most stages.

The Expression of HsfA2 Target Genes is Down-Regulated in *sr45* and is Rescued in the Complemented Line Expressing the Long Isoform

Several of HsfA2 target genes have been implicated in environmental stress response; including many chaperone proteins (HSPs) (Nishizawa et al., 2006), ascorbate peroxidase (*APX2*), which encodes a scavenging enzyme of stress-induced reactive oxygen species (ROS) (Shigeoka et al., 2002), and *GolS1*, which is a heat-inducible gene and encodes an enzyme that catalyzes the synthesis of protective osmolytes such as raffinose family oligosaccharides (RFO) (Taji et al., 2002). The defect in thermotolerance that is observed in *sr45* and short-isoform complemented lines is likely to be caused by misregulation of *HsfA2* expression and splicing (Fig 14).

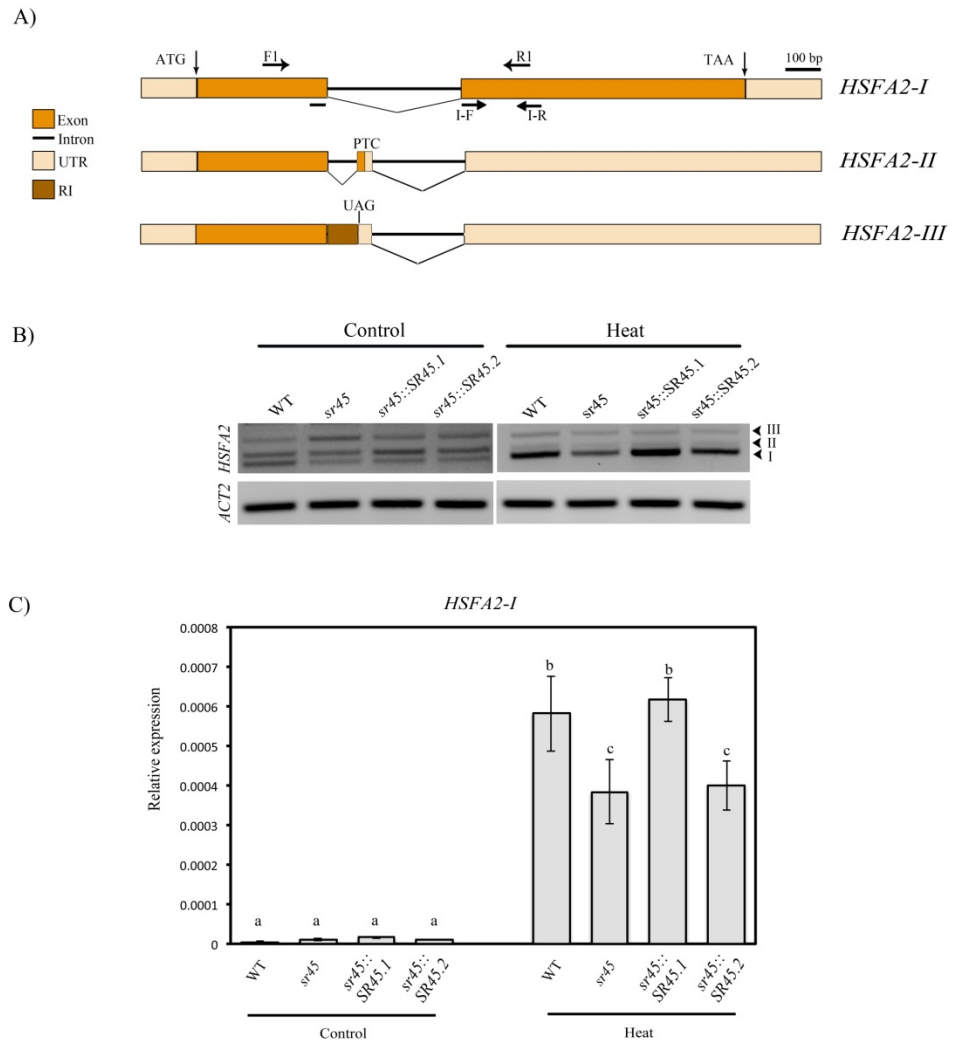


Figure 14: **Expression and alternative splicing of *HsfA2* in WT, *sr45*, and complemented lines in response to heat stress**. (A) *HsfA2* gene structure showing three different splice variants, exons (orange boxes), introns (thick solid lines), UTR, untranslated regions (light orange); RI retained intron (dark orange); and the premature stop codons (PTC). B) RT-PCR analysis of *HsfA2* isoforms in control and heat-treated seedlings showing that the functional isoform (isoform I) is significantly reduced in both *sr45* and mutant complemented with the short isoform (*sr45::SR45.2*). (C) Expression analysis of *HsfA2*-functional isoform (isoform I) by qPCR. *ACT2* was used as an internal control and arrowheads in "A" represent the primers used in RT-PCR and quantitative real time PCR. Experiment was repeated three times and statistical analysis was performed using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

To further investigate the role of SR45 and its spliced isoforms in heat stress tolerance, I analyzed the expression levels of Hsfa2 target genes in all lines under control and heat stress conditions. I performed semi-quantitative (Fig 15) and quantitative RT-PCR (Fig 16) to monitor the expression of *HsfA2* downstream genes. In general, expression levels of the tested genes, including some HSPs (*HSP101, HSP70B, HSP18, and HSP17.7, HSP25,*), *APX2*, and *GOLS1*, are induced in response to heat treatment and the induction is less in *sr45* and the complemented line expressing the short isoform (Fig 15 and Fig 16) as compared to WT and the line complemented with the long isoform. Notably, constitutive expression of the long isoform restored the WT expression level of all tested genes, and induction of some of these genes was significantly as compared to WT such as *HSP101*, *HSP17.7*, *APX2*, and *GOLS1* (Fig 16), suggesting that the long isoform is important for expression levels of these genes under heat stress. Disruption of *HsfA2* splicing and expression, reduction of its target genes' expression in *sr45* and the short isoform expressing line, and rescue of splicing and expression of *HsfA2* and its targets in the long isoform-complemented line suggest an important positive role for *SR45* long isoform in *HsfA2* regulation and thermotolerance.

SR45 Binds the Intronic Region of *HsfA2*

Since the splicing of *HsfA2* pre-mRNA is changed in the *sr45* mutant it is possible that SR45, a known splicing regulator (Ali et al., 2007; Day et al., 2012), might bind to the alternatively spliced intron and regulate alternative splicing. To test this possibility, I prepared ^{32}P-labeled RNA of the *HsfA2*-intron (Fig 17) and performed electrophoretic mobility shift assays (EMSA) using purified recombinant SR45 protein. Protein/RNA complexes with reduced movement were detected when purified SR45 was incubated with the RNA probe (Fig 18A).

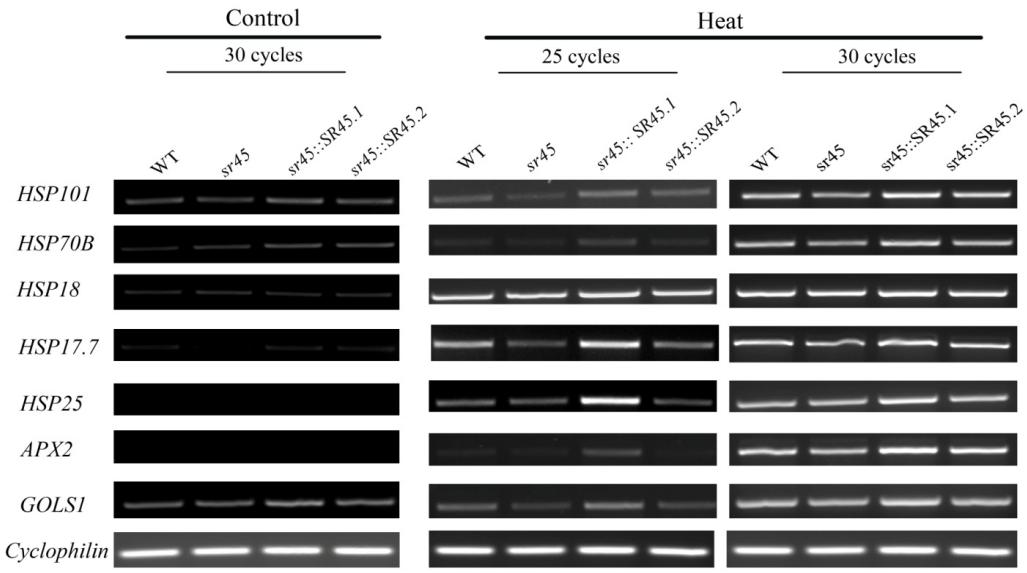


Figure 15: **Expression analysis of *HsfA2* target genes in WT, *sr45* and complemented lines in response to heat stress by semi-quantitative PCR**. Total RNA prepared from treated and untreated samples was reverse transcribed and used as a template for PCR. Number of cycles used for amplification is shown on the top and gene names are shown on the left. Cyclophilin was used to verify equal amount of template in different samples.

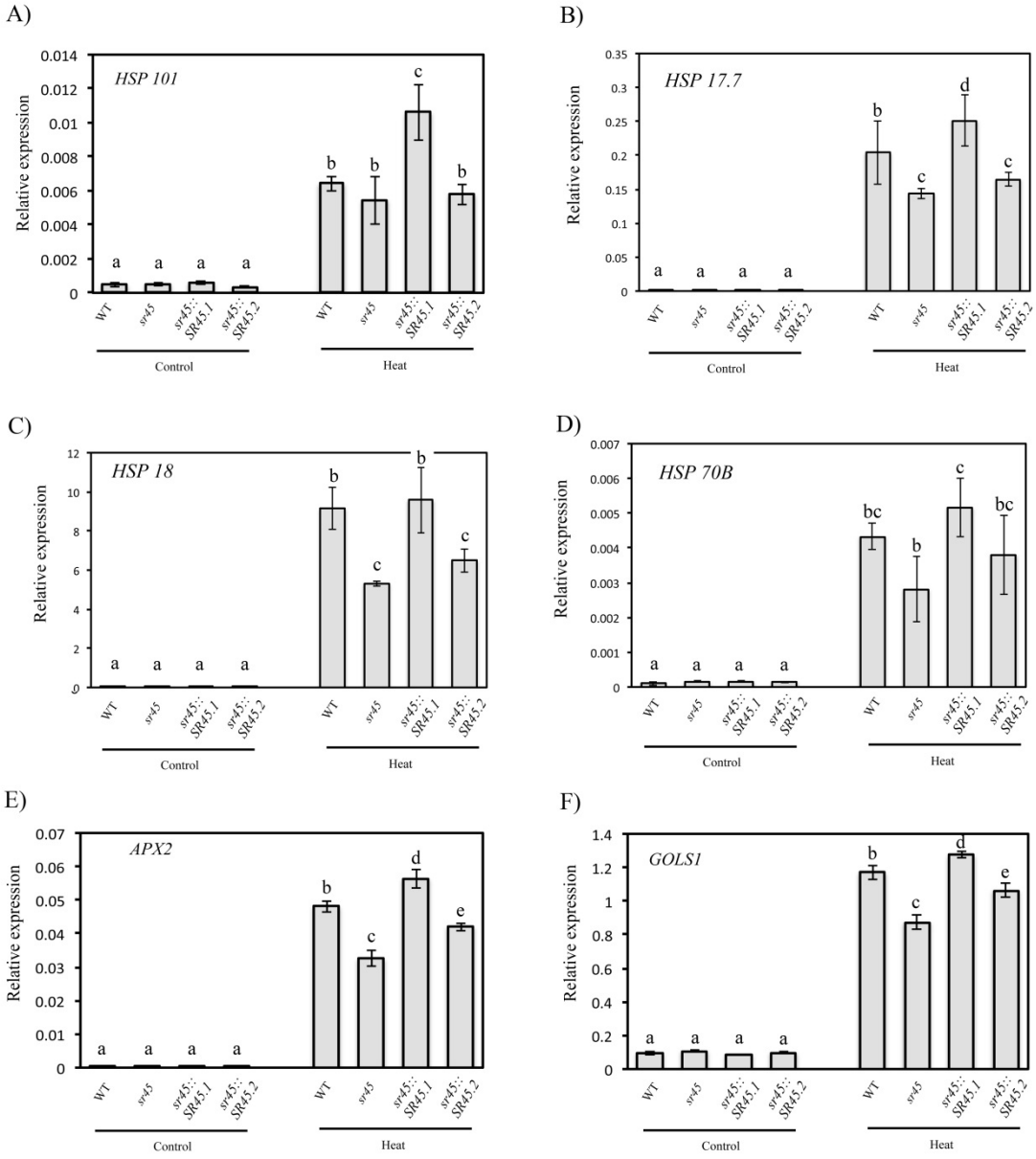


Figure 16: **qPCR expression analysis of *HsfA2* target genes in WT, *sr45* and complemented lines in response to heat stress.** cDNAs used in the semi-quantitative analysis (Fig 15) were used as templates for qPCR using primers designed for qPCR (Table 3). Samples were normalized to actin expression and relative expression was calculated. Experiment was repeated three times and data represents the mean ±SE from the average value over all experiments. Statistical analysis was performed using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

However, when SR45 recombinant protein was incubated with cold competitor RNA, it completely abolished the binding (Fig 18B). These results suggest that the SR45 interacts with *HsfA2* RNA and the interaction is specific.

Splicing Factor SR45 Widely Regulates Heat Shock Transcription Factors (HSFs)

Heat stress transcription factors (HSFs) are essential regulatory factors that function in response to heat stress and other environmental stresses (Li et al., 2013). The heat stress response phenotype of *sr45* as well as *in vitro* interaction between recombinant SR45 and *HsfA2* suggested that splicing regulator SR45 might have global impacts on other HSFs. To investigate the global role of SR45 I have compared the expression level of several other HSFs in *sr45* with WT plants grown under normal growth condition. Analysis of available RNA-seq data of both WT and *sr45* grown under normal growth conditions has shown that expression of ten HSF genes (*HSFA1a, HSFA1b, HSFA1e, HSFA4a, HSFA4c, HSFA5, HSFA6a, HSFA7a, HSFB2b,* and *HSFB4)* was reduced in the mutant (Fig 19, left panels) as compared to WT. These findings were further validated by RT-PCR analysis, and shown in (Fig 19, right panels).

To test the alternative splicing of these transcription factors I designed primer pairs to flank the exon- exon junction of *HSFB2a* and *HSFC1,* both of which undergo alternative splicing in this region. Interestingly, the splicing pattern of these transcription factors is altered. One isoform of *HSFB2a* was detected only in the *sr45* mutant while another isoform was present in both complemented lines and was increased in the mutant. In the case of *HSFC1* also both its expression and splicing is altered in the mutant as compared to WT.

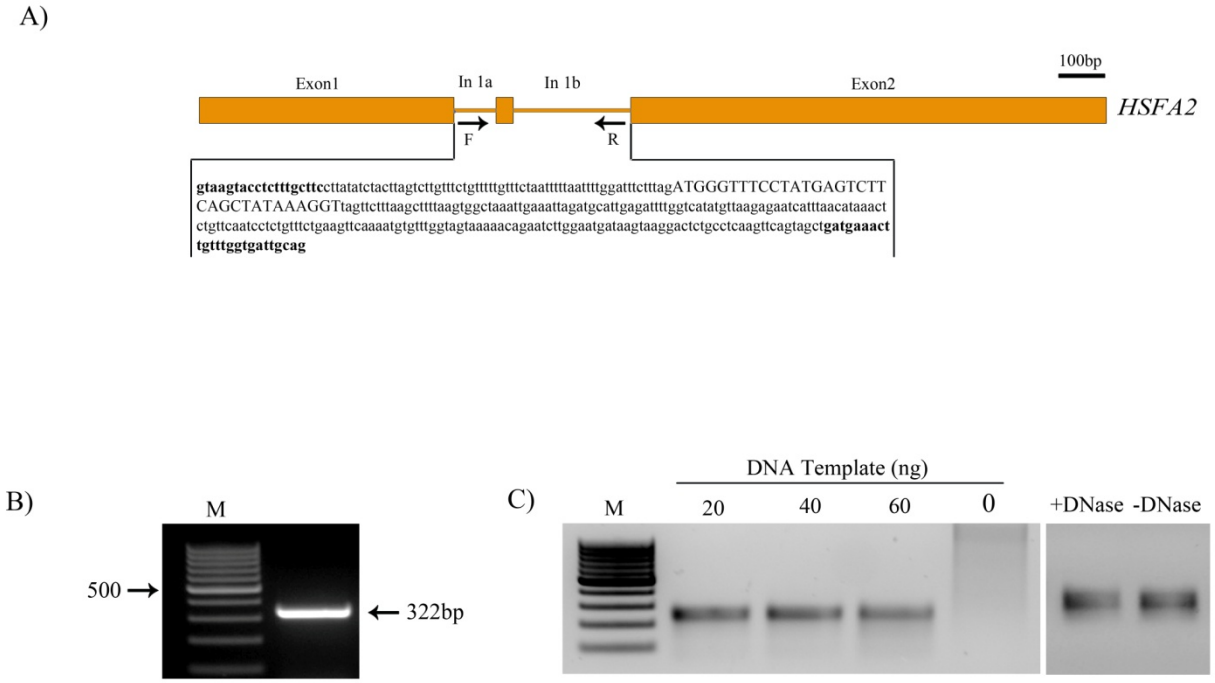


Figure 17: ***in vitro* transcription of *HsfA2 intron* for electrophoretic mobility gel shift assay (EMSA).** (A) Schematic diagram of *HsfA2* gene (upper) showing exons, introns, forward (F) and reverse (R) primers and DNA sequence of the amplified region (lower). (B) Gel image of the amplified PCR product showing the predicted size in base pairs (bp). (C) *in vitro* transcribed RNA with increasing concentrations (20, 40, 60 ng) of DNA template (left) and DNase treatment of the *in vitro* transcribed product (right) confirming that the observed band is RNA and not DNA template.

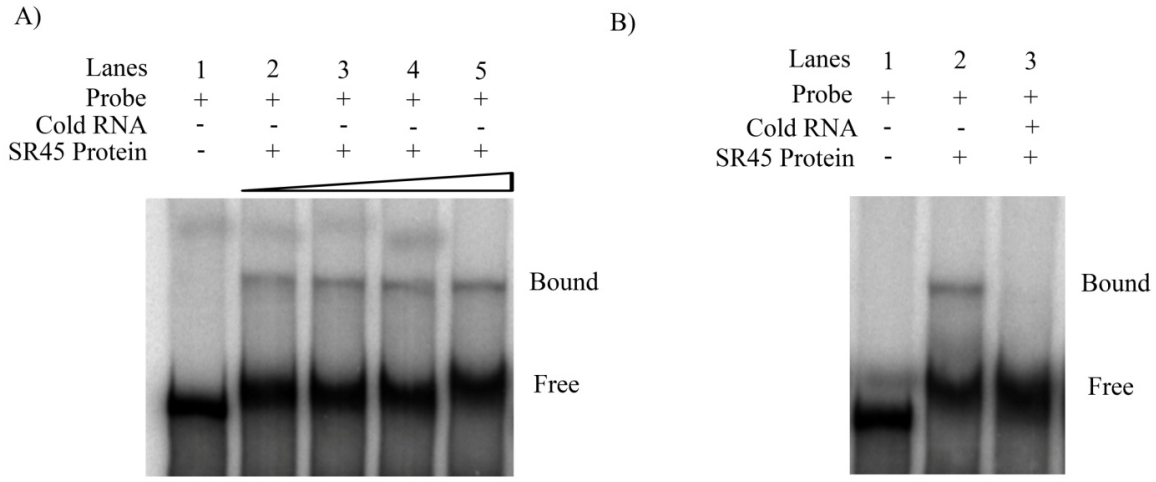
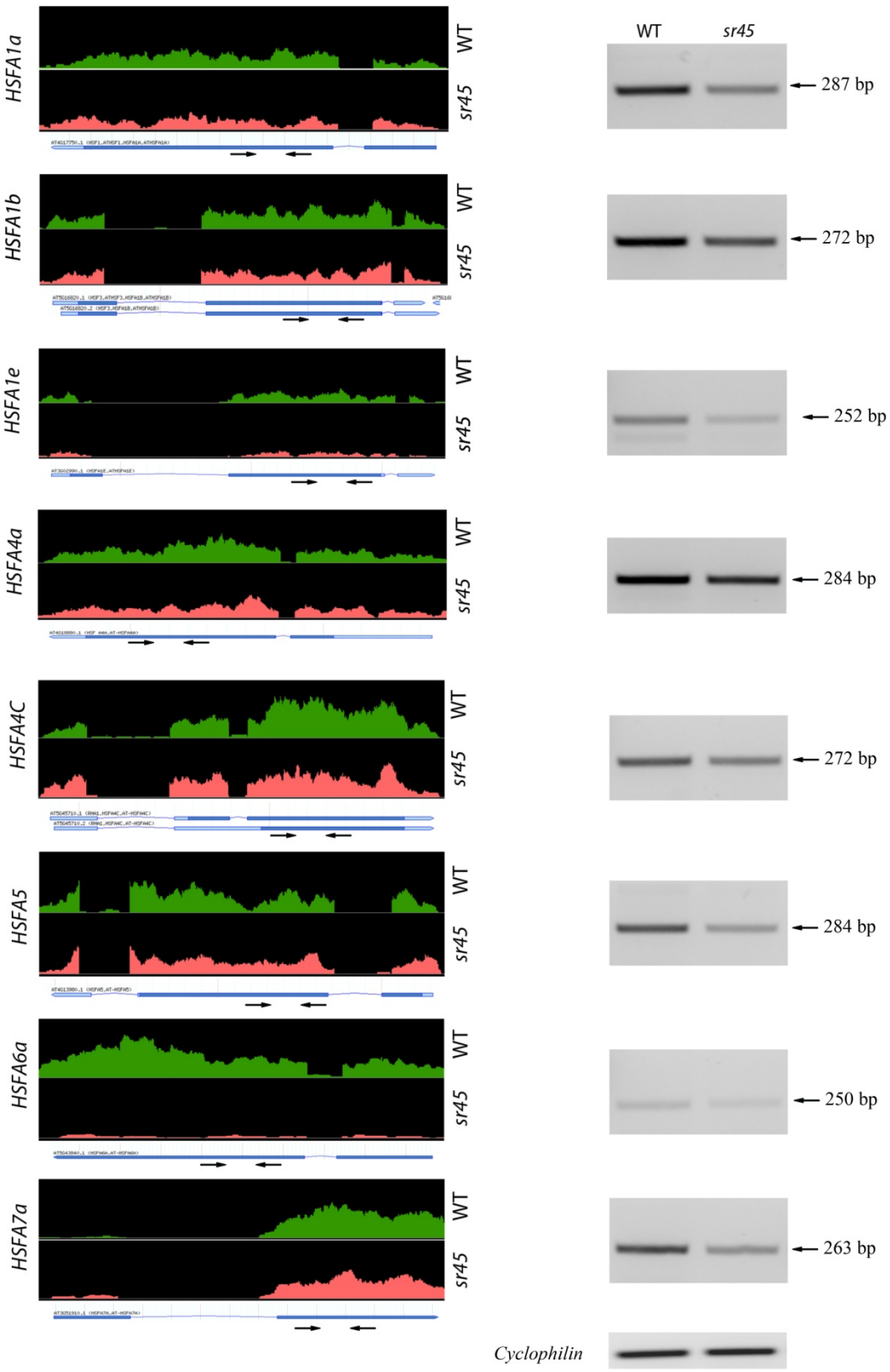


Figure 18: **SR45 binds *HsfA2* intron.** (A) EMSA with labeled *HsfA2* intronic RNA and purified recombinant SR45 protein. *HsfA2* probe was incubated with purified recombinant SR45 protein, resolved on a 5% native polyacrylamide gel and visualized using a Storm PhosphoImager. Lane 1, free probe; lanes 2-5 have an increasing concentration of SR45 (400, 800, 1200 and 1600 ng). Free probe and the RNA-protein complex (bound) are shown on the right. (C) Binding of SR45 to *HsfA2* probe is competed by cold *HsfA2* probe; lane 1, free probe; lane 2, probe plus SR45 protein (1600 ng); lane 3, as in lane 2 with 25x of cold *HsfA2* probe.

From all these analyses it is clear that the expression levels and alternative splicing of several HSFs are misregulated in the *sr45* mutant, suggesting a global role for SR45 in regulating expression and alternative splicing of many HSFs.


HSFA1a
WT
sr45
HSFA1b
WT
sr45
HSFA1e
WT
sr45
HSFA4a
WT
sr45
HSFA4C
WT
sr45
HSFA5
WT
sr45
HSFA6a
WT
sr45
HSFA7a
WT
sr45
WT
sr45
287 bp
272 bp
252 bp
284 bp
272 bp
284 bp
250 bp
263 bp
Cyclophilin

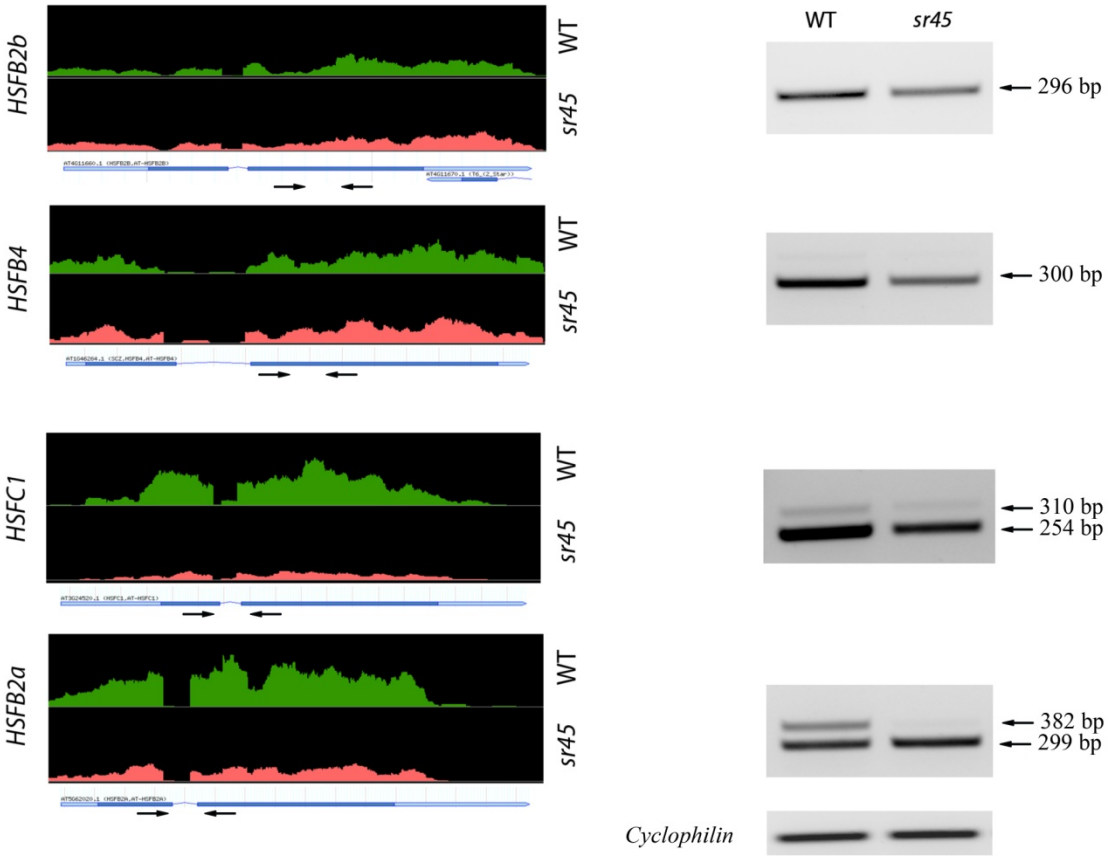


Figure 19: **RNA-seq read depth of heat shock transcription factors (HSFs) in WT and *sr45* and validation by RT-PCR.** Gene structure was extracted for *Arabidopsis thaliana* from TAIR10 and HSTFs read depth was extracted from RNA-seq data obtained in our laboratory (left panel). RT-PCR expression and the splicing pattern of HSTFs were conducted to validate the RNA-seq (right panel). Primer location for each gene was indicated by arrowheads. The size of PCR products is indicated. Cyclophilin was used as an internal control.

PART 2

SR45 Long Isoform is Required for Salt Stress Tolerance in *Arabidopsis*

As SR45 is important for heat stress tolerance and bioinformatics data suggests that its expression is induced in response to salt stress (Fig 7), I aimed to investigate the role of SR45 and its splicing variants in salt stress response. To test the effect of salt on germination, seeds of WT, mutant and complemented lines were germinated on MS or MS supplemented with 150 mM NaCl for 10 days (Fig 20). Both *sr45* and the mutant complemented with the short isoform displayed enhanced sensitivity (Fig 20 A), which is reflected in significant inhibition of root growth and reduction in emergence of green leaves (Fig 20 B and C) as compared to WT and long isoform-complemented plants. Interestingly, the same trend was also observed for germination on MS medium supplemented with KCl, LiCl, or CsCl (Fig 21 A-D), suggesting sensitivity of *sr45* to all tested salts.

Next, I evaluated the role of SR45 and its splice variants in salt stress during post-germination and vegetative stages. Following the transfer of the germinated seedlings to MS media supplemented with different salts, both *sr45* and short isoform showed decreased tolerance to growth on CsCl, LiCl, and KCl (Fig 22 B-E). However, no significant differences were observed when grown on medium supplemented with the same concentration of NaCl (Fig 22A) suggesting that this concentration of NaCl is inhibitory in germination stage but less during post-germination.

To test the role of SR45 and the spliced isoforms at later stages of plant development I used a hydroponic system where the germinated seedlings were transferred to a hydroponic set up containing 1/10X Hoagland solution supplemented with 30 mM NaCl for 3 weeks (Fig 23).

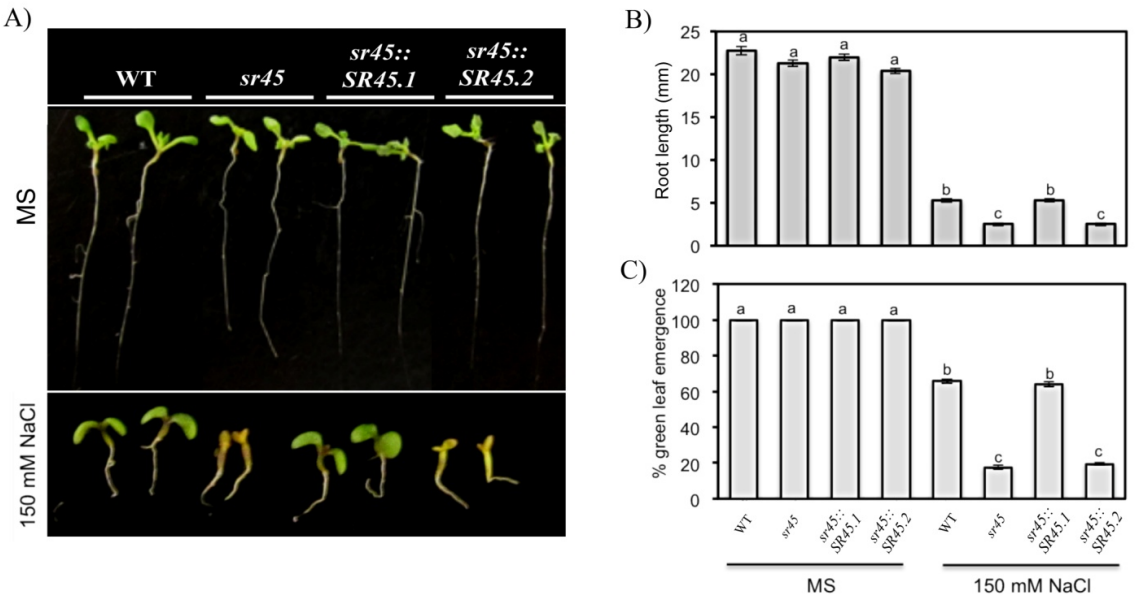


Figure 20: **Response of *sr45* and complemented lines to salt stress at germination stage.** Seeds of WT, *sr45* and the complemented lines were germinated on either MS or MS supplemented with 150 mM NaCl for 10 days. Plant images (A) were taken and root length (B) and the number of the green leaves (C) were calculated. Experiment was repeated three times with similar results. Data represents the mean ±SE from the average value over all experiments. Statistical analysis was performed using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

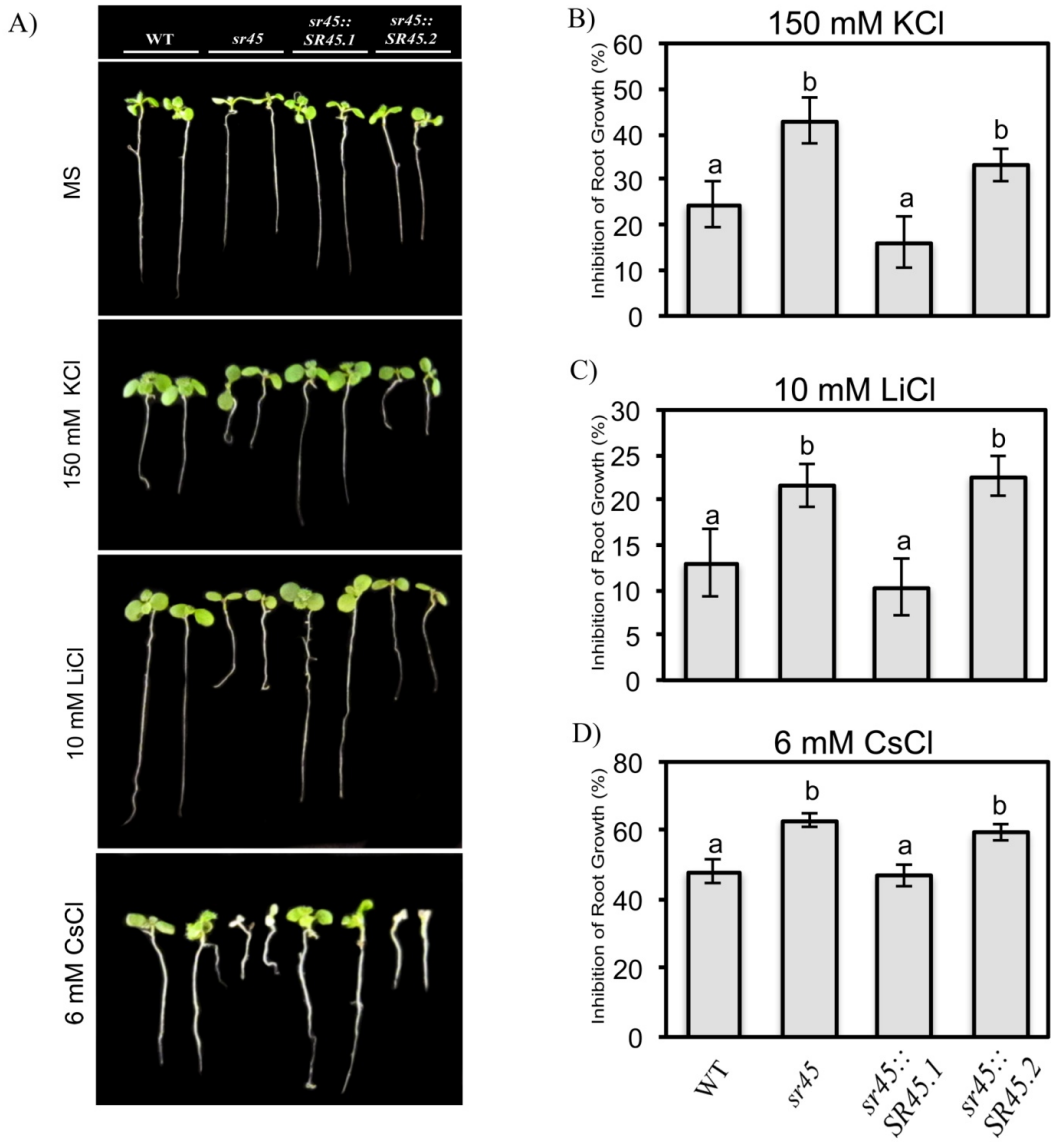


Figure 21: **Role of SR45 isoforms in ion stress tolerance during germination phase.** Seeds of WT, *sr45* and the complemented lines were grown on either MS or MS supplemented with different salts (150 mM KCl, 10 mM LiCl or 6 mM CsCl) for 10 days. Plant images (A) were taken and inhibition of root growth in the presence of different salts was calculated. Experiment was repeated three times and data represents the mean ±SE from the average value over all experiments. Statistical analysis was performed using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

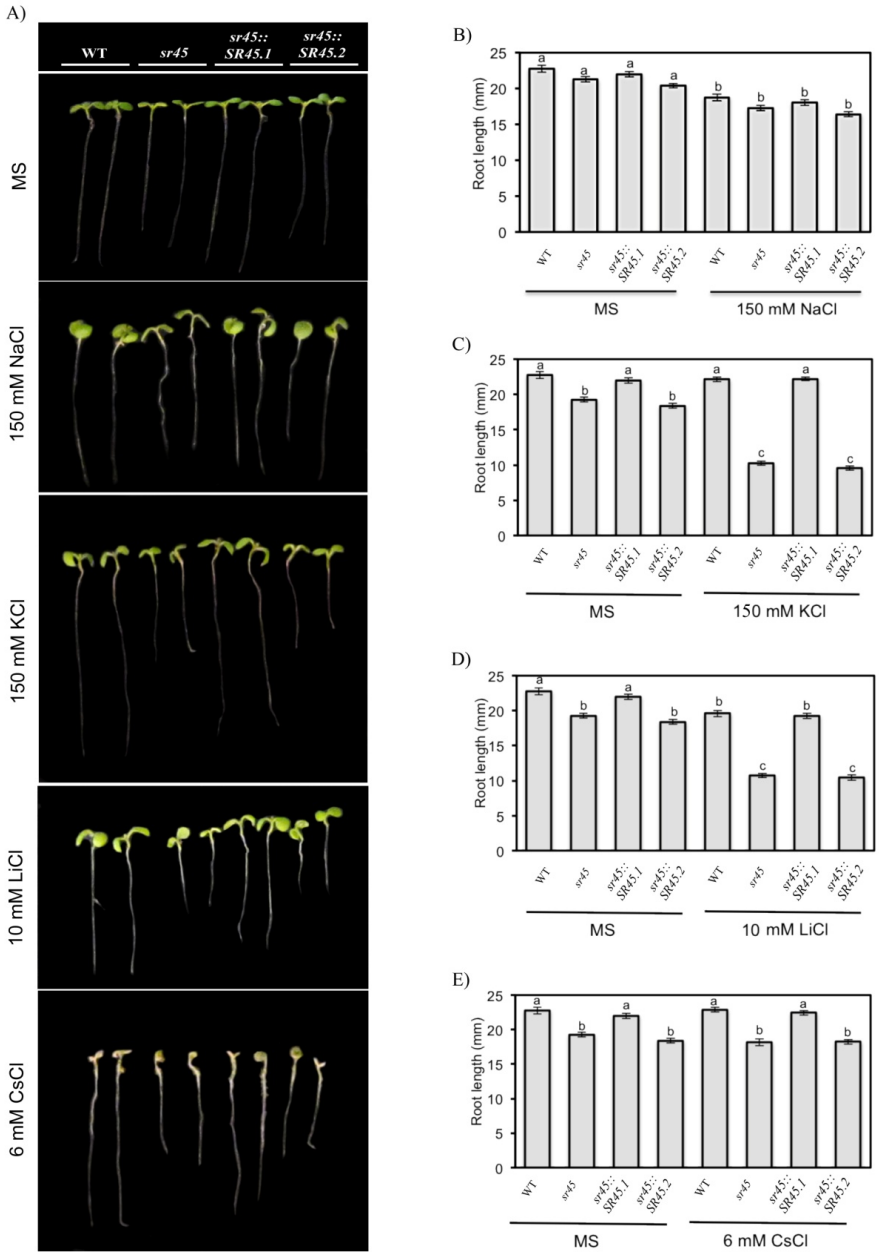


Figure 22: **Post germination response of WT, *sr45* and the complemented lines to different ions.** Three-day old MS-grown seedling of WT, *sr45* and the complemented lines were transferred to MS medium supplemented with different salts (150 mM NaCl, 150 mM KCl, 10 mM LiCl or 6 mM CsCl) for 10 days. Seedlings transferred to MS were used as controls. Plant pictures (A) were taken and the root length was measured (B, C, D, and E). Experiment was repeated three times with similar results. Data represent the mean ±SE from the average value over all experiments. Statistical analysis was performed using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

Shoot growth, and the survival rate of *sr45* and the mutant complemented with the short isoform were significantly reduced compared to both WT and the complemented line with the long isoform (Fig 23A, B, C and E). The leaf size of the mutant and the short isoform expressing line is reduced (Fig 23 B). On the other hand, no significant reduction was observed in the root fresh weight (Fig 23D and F). Thus, similar to its function in heat stress responses, SR45 is important for salt stress tolerance and may be involved in the maintenance of ionic homeostasis during salinity. Only the long isoform is able to fully rescue the growth sensitivity phenotype.

Mutation of *SR45* Affects Ions Homeostasis under Salt Stress

Maintaining cellular ions homeostasis is a fundamental process in salt tolerance and one of the splice forms (long form) of SR45 is necessary for an adaptation mechanism in response to salt stress (Fig 20-23). In an effort to investigate the mechanism of salt tolerance I assayed whether the splicing regulator SR45 long isoform participates in the regulation of intercellular ion homeostasis under salt stress. The WT, *sr45* and mutant lines complemented with the short or long isoform were grown on either MS or MS supplanted with 150 mM NaCl for 14 days and used for metal analysis using ICP. As predicted high Na^+ and less K^+ accumulation was observed in all tested lines when plants were grown in medium supplemented with NaCl and an opposite pattern was observed when plants were grown on MS (Fig 24 A and B). Surprisingly the mutant and mutant complemented with the short isoform accumulated significantly less Na^+ when grown in the presence of NaCl compared to WT and the mutant complemented with the long isoform (Fig 24 A). In addition, K^+ accumulation also was insignificantly different between all lines. This is likely due to using whole seedling to measure ion contents. It would be of interest to investigate the ion accumulation in root verses shoot to understand if SR45 has roles in ion translocation and distribution of sodium within the plant.

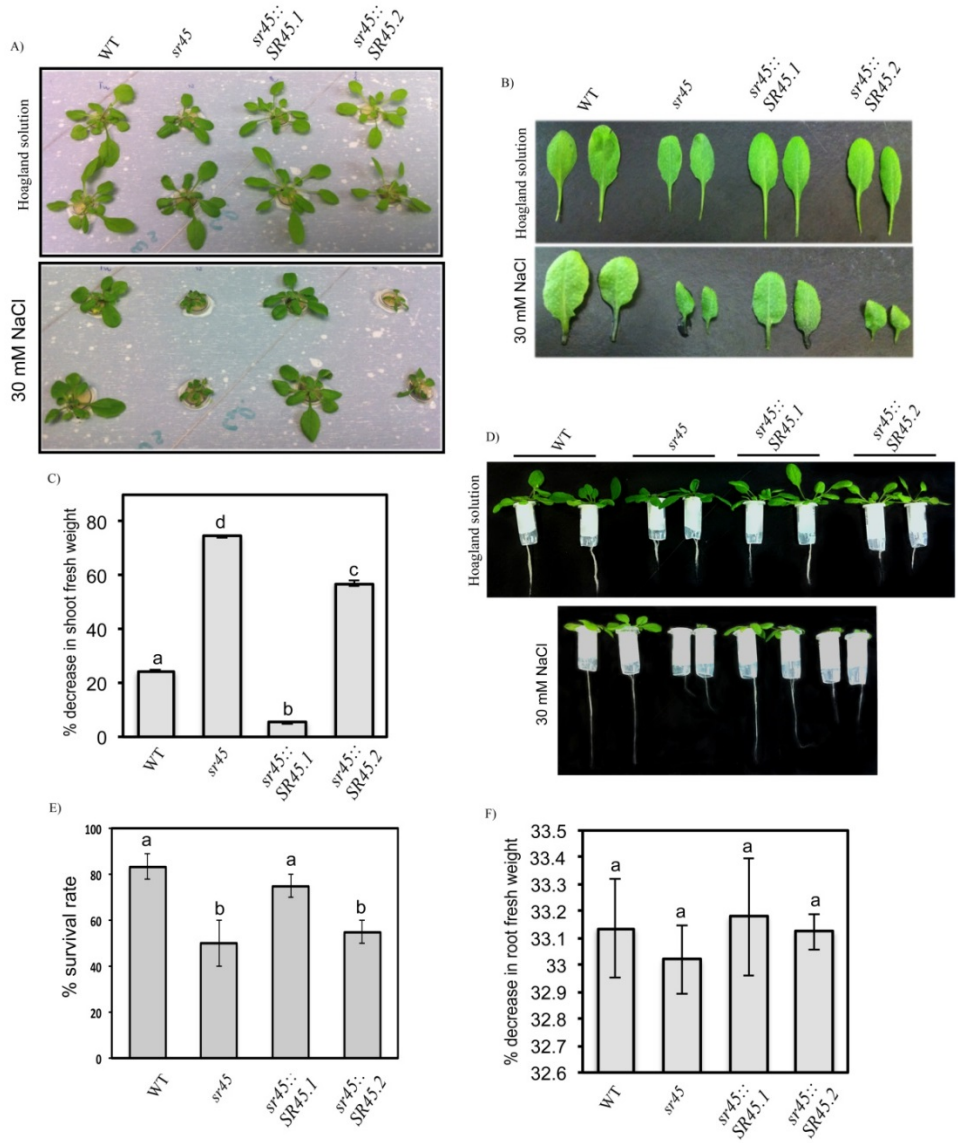


Figure 23: **Response of *sr45* and complemented lines to salt stress tolerance during vegetative phase.** (A) Three-week old seedlings of WT, *sr45* and complemented lines grown in a hydroponic set up containing 1/10X Hoagland solution (control) or 1/10X Hoagland solution plus 30 mM NaCl. (B) Close up images of rosette leaves excised from plants in panel A. (C) Reduction in shoot fresh weight at the end of the experiment (three weeks); (D) Plant images showing the root length under control (upper) and treated (lower) conditions. (E) Percent survival rate, (F) Percent reduction in root fresh weight. All quantifications represent the mean ±SE from the average value over three independent experiments. Statistical analysis was performed using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

Divalent cations, especially calcium, have been shown to be important in plant adaptation to salinity (Shabala et al., 2005); therefore, I measured Ca^{2+} and Mg^{2+} content. *sr45* and the short isoform accumulated significantly less Ca^{2+} and Mg^{2+} (Fig 24 C, D) compared to the mutant complemented with the long isoform but not the WT when grown on medium supplemented with NaCl and no significant changes was observed when plants were grown on MS medium. These findings suggest that over-expression of SR45 long isoform may affect ion accumulation under salt stress.

SR45 Long Isoform Restores Normal Function of the SOS Signaling Pathway

Earlier studies have shown that the Salt Overly Sensitive (SOS) genes have an essential role in controlling ion homeostasis and conferring salt tolerance (Qiu et al., 2004). To further investigate the role of SR45 in ion homeostasis and salt tolerance, I examined the expression levels of *SOS1, SOS2,* and *SOS3* in NaCl-treated seedlings of WT and *sr45*, as well as the two complemented lines. As shown in Figure 25 A and C the expression of *SOS1* and *SOS3* genes is significantly reduced in both *sr45* and the mutant complemented with the short isoform compared with those in WT and the long isoform-complemented mutant in response to salt stress. On the other hand, no significant reduction was observed for *SOS2* in mutant and short isoform-complemented lines although it was significantly induced in long isoform (Fig 25 B) as compared to WT in response to salt stress. In addition, based on gene annotation, *SOS3* is alternatively spliced producing two isoforms. Using junction primers that flank the spliced introns, I performed RT-PCR using RNA isolated from salt-treated and untreated seedlings. No splicing changes were observed among all tested lines (Fig 25 D).

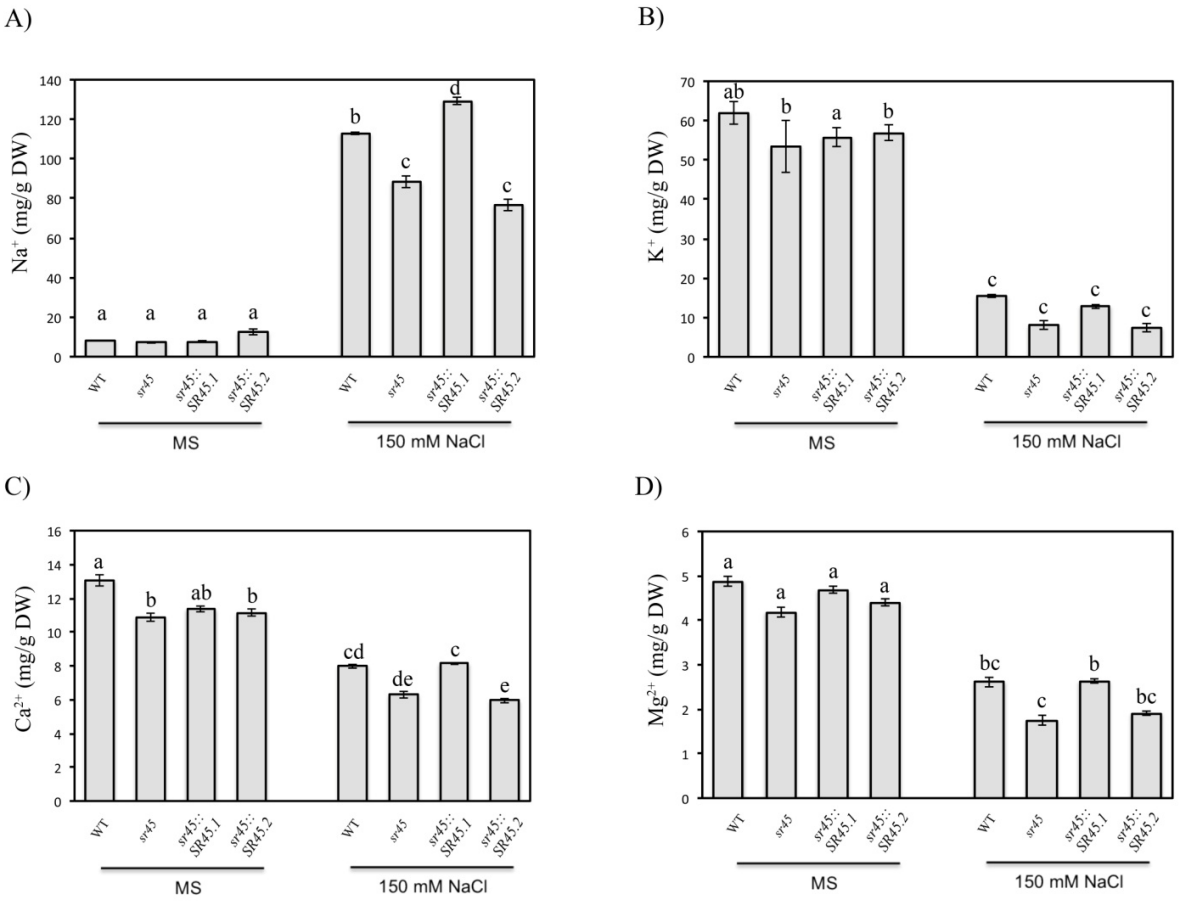


Figure 24: **Ion content (mg/g dry weight) of WT, *sr45* and the complemented lines in plants grown in the presence and absence of NaCl.** Three day old-MS grown seedlings were transferred to MS or MS supplemented with 150 mM NaCl and allowed to grow for two weeks before harvesting. Harvested seedlings were rinsed once with excess volume of 50 mM EDTA followed by 2 rinses with bi-distilled water. Known weight of the oven-dried tissues were digested and used for ICP analysis. Data represent the mean ±SE from the average of three independent experiments. Significance was calculated using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

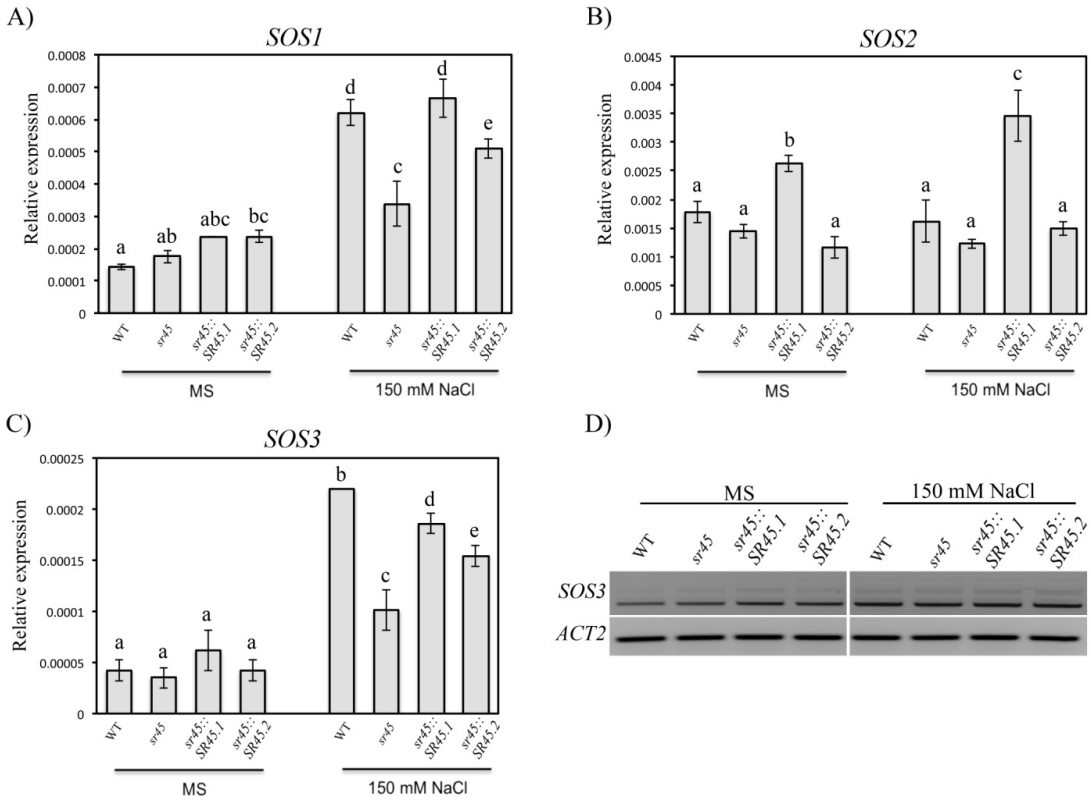


Figure 25: **Relative expression and alternative splicing of SOS signaling pathway genes in seedlings grown under normal and salt treatment conditions**. Two-week-old seedlings of WT, *sr45* and the complemented lines were treated with 150 mM NaCl for 6 h and then total RNA was prepared and used for cDNA synthesis. Relative expression of *SOS1* (A), *SOS2* (B), and *SOS3* (C) as well as the alternative splicing of *SOS3* (D) are shown. Data on relative expression represent the mean ±SE from the average of three experiments. Statistical analysis was performed using analysis of variance and Tukey's test, significance is indicated by letters associated with bars.

SOS4 is another gene that has been shown to be involved in salt stress response and *sos4* mutants are hypersensitive to several ions including Na^+, K^+, and Li^+ and show a defect in ion homeostasis (Shi et al., 2002b). *SOS4* encodes a pyridoxal (PL) kinase that functions in the biosynthesis of pyridoxal-5-phosphate (PLP), which is a vitamin B6 salvage enzyme (González et al., 2007). Interestingly, pre-mRNA of *SOS4* was shown to be alternatively spliced within the first intron resulting in two transcripts encoding two proteins with a 34-amino acid difference in their N-termini (Fig 26A) (Shi et al., 2002b). *SOS4.1* is localized in the chloroplast where it maintains the phosphorylated vitamin levels (Rueschhoff et al., 2012). Based on the role of *SOS4* in ion sensitivity and AS of *SOS4*, I tested the expression level and AS events of *SOS4* in WT, *sr45* and two complemented lines in response to salt treatment. Interestingly, the expression and splicing of *SOS4* is misregulated in *sr45* and the mutant complemented with the short isoform (Fig 26 B) where isoform IV is accumulated and the expression of other isoforms is reduced (Fig 26 B). On the other hand, this misregulation of *SOS4* expression and splicing was fully rescued by the *SR45* long isoform. Together these results indicate that the loss-of-function mutation of *SR45* affects the expression and splicing of *SOS* genes in NaCl-treated plants, and the *SR45* long isoform rescues these phenotypes to the WT levels, suggesting the importance of SR45 in salt tolerance *via* regulating *SOS* genes.

Using available RNA-seq data to analyze *SOS4* expression in response to other abiotic stresses I found that its expression is relatively induced by heat stress (Fig 26A). Thus, I have also tested *SOS4* expression in response to heat stress in *WT, sr45* and complemented lines (Fig 26 C). Clearly *SR45* mutation affects the splicing pattern and the ratios of the spliced isoforms in both treated and untreated seedlings where isoform IV is clearly accumulated and isoform II is reduced. A similar trend is also observed for mutants complemented with the short isoform (Fig

26C). This change in expression was rescued to the WT level in plants complemented with the long isoform. Since *SOS4* is known to produce three isoforms based on the gene annotation and previous studies. From RT-PCR I observed four spliced variants (Fig 26). I purified and sequenced the fourth product. Interestingly, the fourth isoform is indeed a spliced variant that has the first intron retained and it is the one accumulated in *sr45* and in the mutant complemented with the short isoform (Fig 26 A, Fig 27). This retained intron is predicted to produce nonfunctional protein. These results showed that the *SR45* long isoform is important for splice site selection of *SOS4* splicing and this is required for stress tolerance.

Expression and AS of Stress-Inducible Genes is Misregulated in *sr45* and Rescued by the Long Isoform

The salt stress phenotypes of *sr45* suggested that stress responsive genes might be affected in the mutant in response to salinity treatment. In order to test this hypothesis, I analyzed the expression level of several stress-inducible genes in WT, *sr45* and the two complemented lines grown in MS or MS supplemented with NaCl. *RD29A, RD29B, ADH1* and *DREB2A* have been identified as marker genes with protective functions in abiotic stress response. As shown in Fig 28 (A-D), the expression of these stress-responsive genes was induced by salt treatment, but the level of induction is lower in *sr45* and in the mutant complemented with the short isoform, compared to WT and plants complemented with the long isoform. These results suggest that the *SR45* long isoform regulates the expression of these stress responsive genes, hence is likely contributing to the salt sensitivity phenotype of *sr45* and the short isoform-complemented plants.

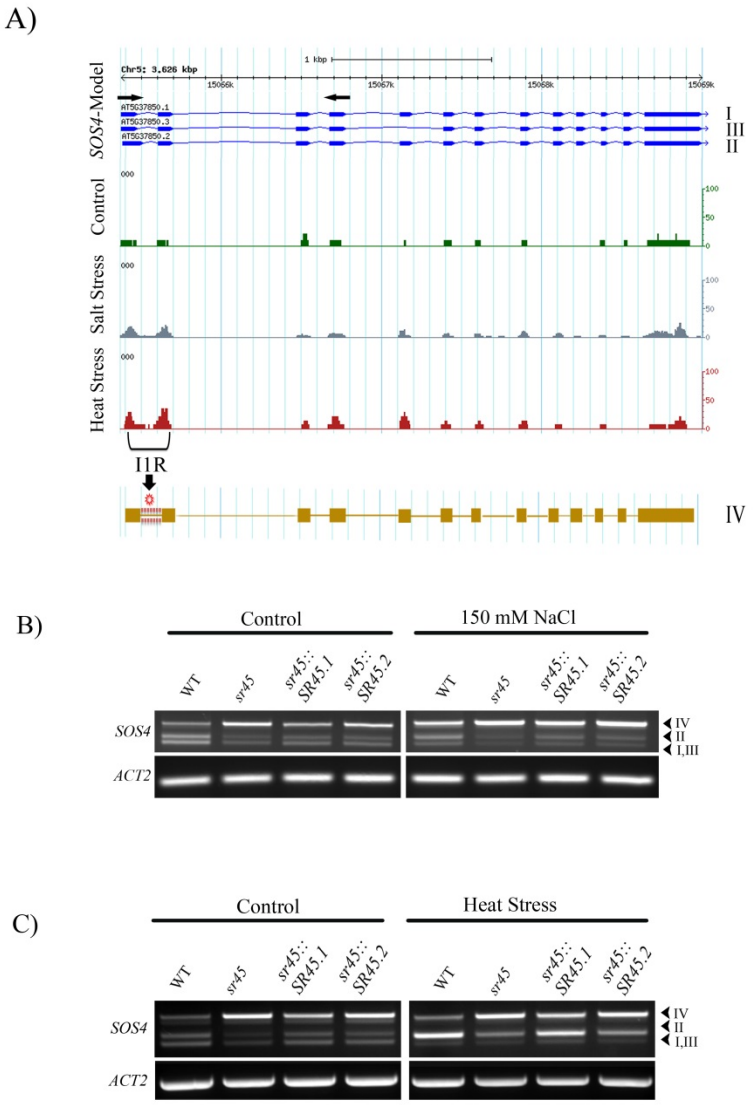


Figure 26: **Expression and alternative splicing analyses of *SOS4* in response to different abiotic stresses.** (A) *SOS4* gene structure and the RNA-seq reads in response to heat and salt stresses retrieved from *Arabidopsis* GBrowse. Novel isoform (IV) with intron 1 retained (IR) is indicated. (B & C) RT-PCR of *SOS4* in response to NaCl and heat treatment, respectively. Primers are indicated by arrowhead and actin was used as an internal control. Different isoforms are indicated by Roman numerals.

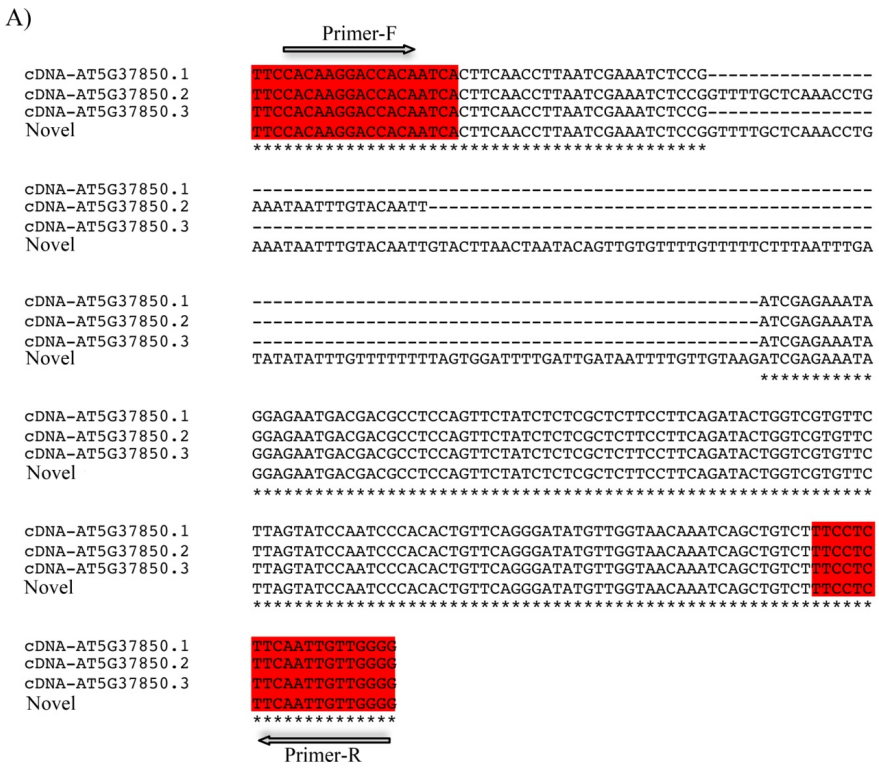


Figure 27: **Sequence alignment of SOS4 isoforms**. PCR was conducted using primer pair (indicated by arrowhead). The amplified products were resolved in a gel and different bands were purified and sequenced. Sequences were aligned and a new isoform (IV) that include the first intron was confirmed. Primers used for PCR were highlighted.

Since *SR45* is a splicing factor and important for splicing of *SOS4*, I analyzed AS of Responsive to Dehydration 20 (*RD20*), a stress-responsive gene. Based on the *Arabidopsis* genome annotation, *RD20* is alternatively spliced producing two mRNAs, and RNA-seq analysis showed that *RD20* is spliced under different abiotic stress (Fig 29 A). RT-PCR analysis has shown that AS of *RD20* is altered in *sr45* with enhanced expression of splice variant II under salt stress as compared with that of WT (Fig 29 B). In complementation lines, the *SR45* long isoform partially complemented the expression and AS change of *RD20*. In addition, RT-PCR analysis has revealed that exon five of *RD20* undergoes AS leading to generation of a novel isoform with 146 amino acids, whereas isoforms I and II produce proteins with 236 and 194 amino acids, respectively (Fig 30 A, B). These results indicate that the AS of *RD20* is misregulated in the loss-of-function mutant of splicing regulator SR45, and partially complemented by the long isoform.

SR45 Loss-of-Function Mutant Affects ABA-Related Genes in Response to Salt Stress

Abscisic acid (ABA) is a plant hormone synthesized in all parts of plants and it has been shown to mediate the adaptation of plants to different stresses (Finkelstein et al., 2002). To understand the role of the splicing factor SR45 in the ABA signaling pathway, I analyzed the expression levels and AS of some ABA-responsive transcription factors, including *ABF1, ABF2, ABF3* and *ABI5* in salt-treated seedlings of WT, *sr45* and the two transgenic lines. The expression levels of *ABF1* (Fig 31 B), *ABF3* (Fig 31 C), and *ABI5* (Fig 31 D) were significantly induced in the mutant complemented with the long isoform as compared to *sr45* and the line complemented with the short isoform under high salinity conditions. In an opposite way, *ABF1*-

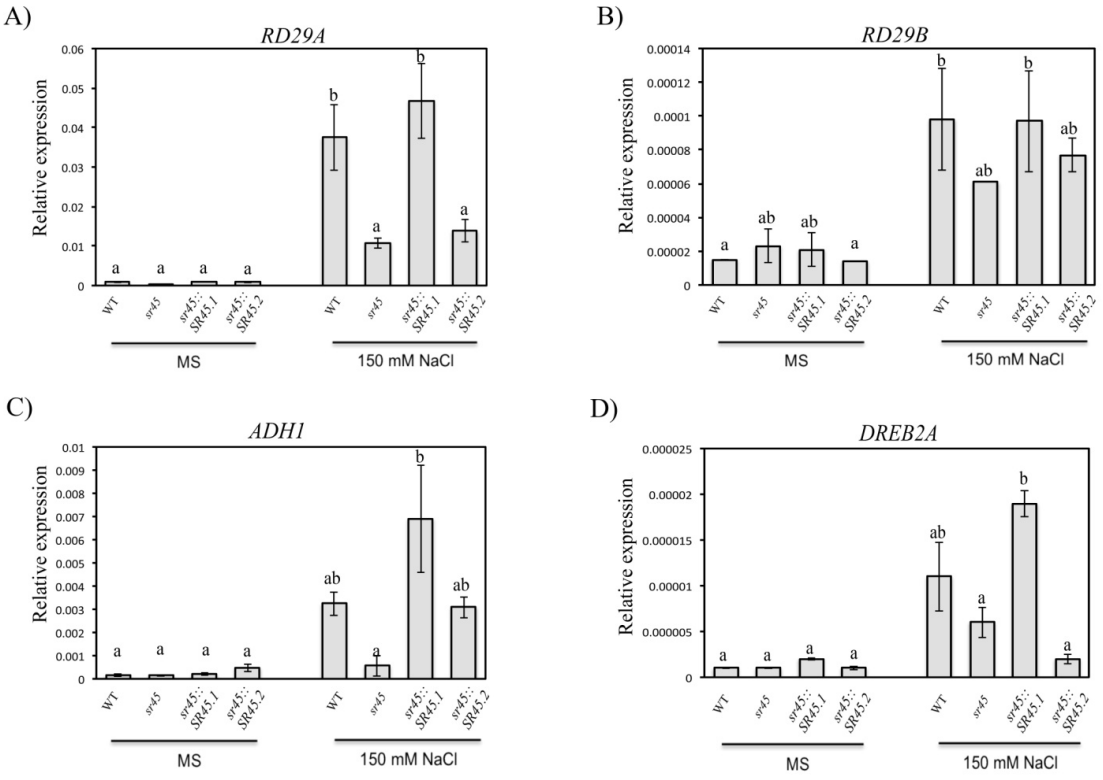


Figure 28: **Relative expression of stress responsive genes in response to salt stress.** cDNAs used in Figure 25 were used for relative expression analysis of stress-responsive genes. (A, B, C, and D) represent the relative expression of *RD29A*, *RD29B*, *ADH1* and *DREB2A* in WT, *sr45* and complemented lines, respectively. *ACT2* was used as an internal control. Statistical analysis was conducted as before and experiment was repeated three times with similar results.

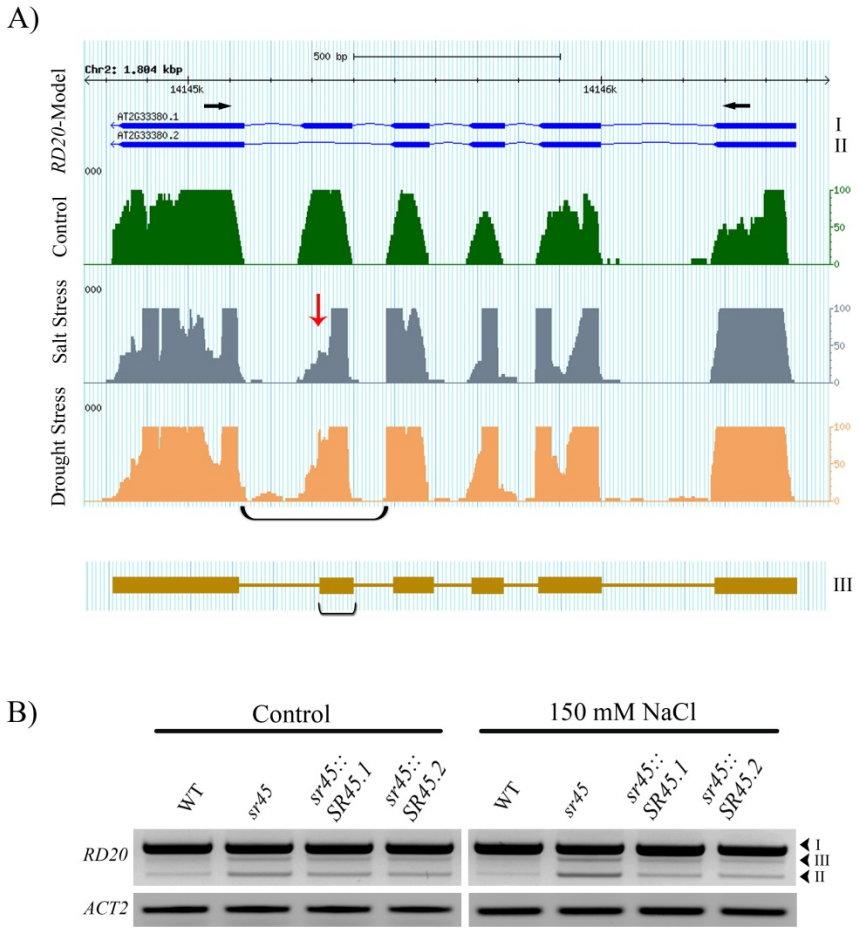


Figure 29: **Alternative splicing of *Responsive Dehydration* (*RD20*) gene under abiotic stresses.** (A) *RD20* gene structure and the RNA-seq reads retrieved from *Arabidopsis* GBrowse in control and stressed tissues. Different isoforms are indicated by Roman numerals and a novel isoform (III) is indicated. (B) Alternative splicing of *RD20* in response to NaCl treatment. Actin was used as an internal control.

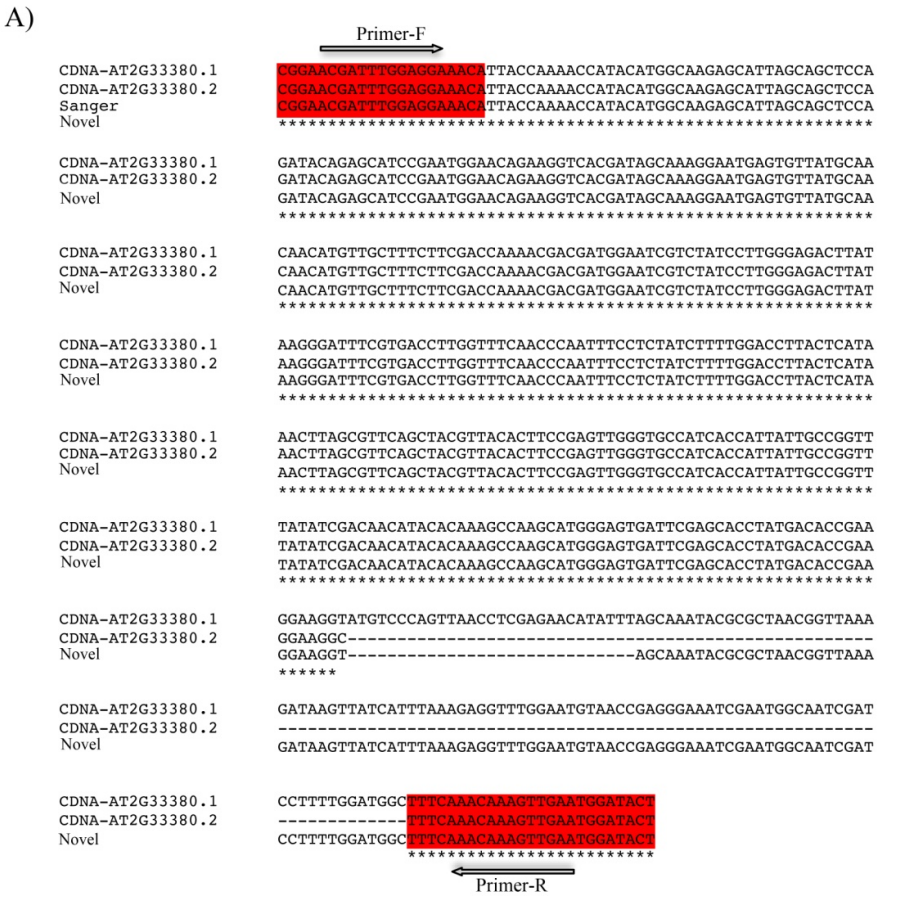


Figure 30: **Sequence alignment of *RD20* isoforms.** (A) cDNAs of known isoforms are extracted from TAIR-10 and aligned to the novel isoform. Primers used for PCR are highlighted. (B) Protein alignment of the two known spliced isoforms of *RD20* with the predicted amino acid sequence of the novel isoform.

(Fig 31 B) and *ABF3* (Fig 31 C) were significantly reduced in the line complemented with the short isoform as compared to other lines. These results suggest that over-expression of long isoform has positive effect while overexpression of short isoform alone has negative effect on ABA-responsive transcription factors. The splicing of *ABF2*, known to be alternatively spliced with two isoforms, is changed in *sr45* as compared to WT (Fig 31 A). In addition, I observed a new isoform (IV) (Fig 31 A), which is more abundant in *sr45* and the two complemented lines as compared to WT. However, the expression level and the alternatively spliced isoforms of *ABF2* have changed in the *sr45* and the two complemented lines (Fig 31 A) as compared to wild types. These findings also indicate that *ABF2* function in stress response may require both isoforms of SR45. Together, these results indicate a role for SR45 in the ABA signaling pathway under salt stress.

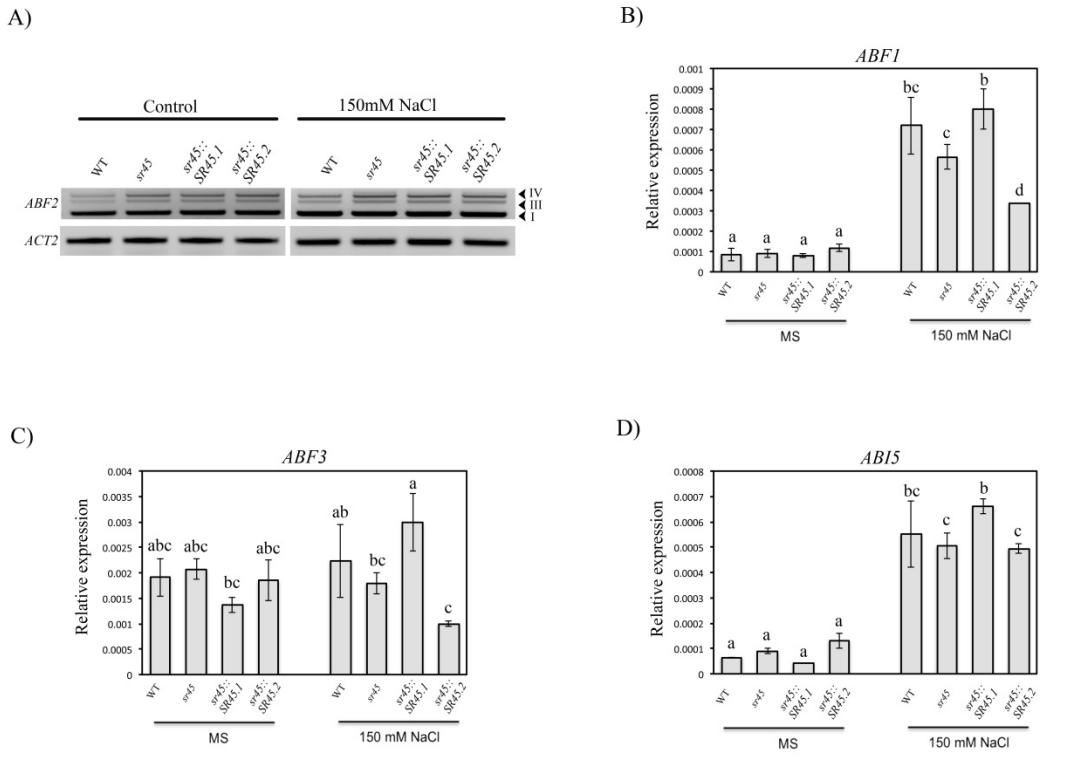


Figure 31: **Expression and alternative splicing analysis of ABA signaling pathway genes in response to salt stress**. (A) RT-PCR analysis of *ABF2* in WT, *sr45* and the two complemented lines under normal and NaCl treated conditions, Roman numerals indicate spliced isoforms. *ACT2* was used as a control for loading. B, C and D show qPCR analysis of some ABA pathway genes in WT, *sr45* and transgenic plants. Statistical analysis in B, C and D was performed as described before and experiment was repeated three times with similar results.

DISCUSSION

In *Arabidopsis* over 60% of intron-containing genes are alternatively spliced to produce two or more splice variants from a gene (Filichkin et al., 2010). Recent studies indicate that alternative splicing likely increases proteome diversity and also provides new ways to regulate gene expression through nonsense-mediated decay and miRNAs (Schoenberg and Maquat, 2012; Yang et al., 2012). Although alternative splicing generates multiple mRNA isoforms from a gene, the functions of most of the splice variants in plants are not known. SR45, an SR like protein, was first isolated as an interacting protein of U1-70K, one of the U1snSNP specific proteins, and later shown to interact with several spliceosomal proteins (Golovkin and Reddy, 1999; Reddy, 2007). Interestingly, pre-mRNAs of *SR45* are alternatively spliced to generate two mRNA isoforms that differ by a 21-nucleotide sequence (Palusa et al., 2007; Zhang and Mount, 2009). Phenotypic analysis of a T-DNA insertion mutant of *SR45* revealed many developmental defects both during vegetative and reproductive phases of plant development (Ali et al., 2007). Complementation of mutant phenotype with individual isoforms uncovered distinct biological functions for each isoform in plant growth and development where the long isoform (*SR45.1*) complemented the flower phenotype while the short isoform (*SR45.2*) complemented the root growth (Zhang and Mount, 2009). In addition to developmental defects, *sr45* was found to be hypersensitive to glucose and abscisic acid (ABA) (Carvalho et al., 2010). Either one of the two isoforms complemented the glucose hypersensitive phenotype, suggesting functional overlap between isoforms in glucose signaling. SR45 is known to interact with U1-70K, SC35-like (SCL33), AFC2 kinase, U2AF35, and *SR30* intron (Ali et al., 2003; Ali and Reddy, 2006; Day et al., 2012; Golovkin and Reddy, 1999) and therefore might regulate the splicing of other genes. In fact, the alternative splicing of pre-mRNAs of several SR genes is altered in *sr45*, suggesting

SR45 modulates alternative splicing either directly or indirectly (Ali et al., 2007; Zhang and Mount, 2009). Since alternative splicing is implicated in reprogramming of the transcriptome in response to stresses (Ali and Reddy, 2008; Duque, 2011; Iida et al., 2004) and SR45 appears to be an important regulator of alternative splicing, here I investigated the role of SR45 in abiotic stress responses using the mutant and complemented lines expressing either the long or short mRNA isoforms. Here I show that a loss-of-function mutant of *Arabidopsis* SR45 enhances sensitivity to heat and salt stresses at different developmental stages and that the expression and splicing of transcription factors and different stress responsive genes are altered in the mutant. Furthermore, the long isoform is found to be necessary for normal splicing and expression of these genes suggesting that only the long isoform functions in both stresses.

SR45 is a Positive Regulator of Thermotolerance in *Arabidopsis* at Different Developmental Stages

My results show that the *SR45* loss of function mutant displayed heat sensitivity phenotypes at two different temperatures (38ºC and 45ºC) and at different developmental stages (seeds, seedling and 25 day old leaves). Heat-treated seeds of *sr45* showed inhibition of primary root growth. Similarly, exposure of 7-day-old seedlings of *sr45* also showed inhibition in root growth. In addition, enhanced bleaching of leaves in response to heat was observed in the mutant (Fig 10B, 12A and 13A). To my knowledge this is first study that shows that SR45 is required for abiotic stress tolerance. I also found that the *SR45* long isoform was able to complement *sr45* heat stress phenotypes at all tested developmental stages. These results indicate that only one of the splice variants (the long isoform) plays an important role in thermotolerance at different developmental stages.

Studies have shown that thermotolerance in plants is a highly complex process and involves a network of multiple regulatory pathways (Mittler et al., 2012). Among these regulatory networks are HSFs which regulate the expression of a set of proteins that are important for the survival of plants at high temperature (Saidi et al., 2011). HsfA2 is one of the essential regulatory components of the *Arabidopsis* HSFs signaling network (Ikeda et al., 2011; Nishizawa-Yokoi et al., 2011). Previous studies on alternative splicing of *HsfA2* and its functions in thermotolerance (Liu et al., 2013) have led us to investigate the role of SR45 in *HsfA2* expression and splicing.

One possible mechanism by which SR45 regulates thermotolerance is by modulating the splicing and/or the expression of genes involved in heat stress response such as heat shock transcription factors and heat shock genes. Expression and alternative splicing of heat shock transcription factors as well as heat shock proteins is induced in response to heat treatment. Interestingly, the expression and splicing of several HSFs and their targets are reduced in *sr45* (Figs 14, 15, 16 and 19) and these changes are rescued to the wild type level in lines complemented with the long isoform. *HsfA2* is one of the HSFs that showed altered expression and splicing. It has been shown previously that *HsfA2* is a central component of the *Arabidopsis* HSF signaling network (Ikeda et al., 2011; Nishizawa-Yokoi et al., 2011). The use of a cryptic 5' splice site in the sole intron produces a short isoform (*HsfA2-III*) that accumulates in response to high temperature (Liu et al., 2013). The short HsfA2 isoform (*HsfA2-III*) positively autoregulates the HsfA2 transcription in response to heat stress (Liu et al., 2013). Since SR45 is a splicing factor and it is known to interact with other splicing factors to modulate splice site selection it is possible that SR45 regulates alternative splicing and expression of *HsfA2*. To determine whether SR45 is involved in *HsfA2* regulation I performed RT-PCR expression and

splicing analyses in control and heat-treated seedlings (Fig 14). The expression of *HsfA2* was reduced in *sr45* under control and heat stress conditions. These results show that the heat sensitivity of *sr45* might be related to or partially related to the misregulation of *HsfA2*. Apart from *HsfA2*, the expression and splicing of several other heat shock transcription factors, which play essential roles in heat stress and other abiotic stresses, was also found to be misregulated in the mutant (Fig 19). Among the other HSFs that are altered in *sr45* are *HsfA1a, HsfA1b* and *HsfA1e*. It has been shown that HsfA2 hetero-oligomerizes with HsfA1 to form a complex with much higher activity than the homodimers (Scharf et al., 1998). Hence, misregulation of these transcription factors is likely to alter expression of their target genes.

In addition to the effect of SR45 on expression and splicing of *HSFs*, I analyzed expression of other stress-related, non-chaperone encoding genes like *GOLS1* and *APX2* and heat shock proteins which are known to be downstream genes of HsfA2. Expression of several of these is also affected in the *sr45* mutant (Fig 15 and 16). The altered expression of these genes is also rescued to the wild type level in the long isoform-complemented line, suggesting the importance of this splice variant in thermotolerance. This is consistent with the phenotype of the transgenic plant complemented with the *SR45* long isoform. Both the morphological and molecular level phenotypes of the mutant are restored to wild type by expressing only the long isoform. These findings may explain the basis for heat sensitivity phenotypes of *sr45*. Together, these results indicate that SR45 mediates heat stress response by regulating expression level and alternative splicing of HSFs and HSPs. In this regard, it is worth mentioning that expression of *HsfA2* together with chaperones like *Hsp90, Hsp70, and Hsp17* protect maturing and germinating pollen from heat damage (Frank et al., 2009; Giorno et al., 2010; Zinn et al., 2010).

It has been reported that *HsfA2* undergoes post-transcriptional regulation at the splicing level in response to heat stress (Liu et al., 2013). Through a cryptic splice site in its first and only intron a new isoform is produced only under heat stress. My results show that expression and splicing of *HsfA2* is altered in *sr45*, suggesting a role of SR45 in *HsfA2* regulation. This raises an important question, how does SR45 regulate *HsfA2* splicing? SR45 is a splicing factor with an RNA recognition motifs (RRM) and it has been reported to bind to an intronic region of *SR30* RNA (Day et al., 2012). Therefore, one possible mechanism by which SR45 regulates *HsfA2* is by direct binding to its intron and modulating splice site choice. Using EMSA with an *in vitro* transcribed RNA of the *HsfA2* intron and purified recombinant SR45 protein, I have shown that SR45 binds specifically to the *HsfA2* intron (Fig 18), suggesting that the regulation of alternative splicing of the *HsfA2* intron might be mediated by SR45. The fact that SR45 interacts with several spliceosomal proteins such as U170K, U2AF35 and several SR proteins suggests a possible regulation of AS of this intron in response to heat stress by recruiting other spliceosomal proteins. It is possible that SR45 binds to the intronic region of *HsfA2* and recruits U1 snRNP to the 5' splice site either through direct interaction with one of the U1snRNP proteins or with other SR proteins that interact with U1 snRNP. There is prior evidence that SR45 interacts with U170K, one of the U1 snRNP proteins and with SR33 protein (Golovkin and Reddy, 1999, Day et al., 2012). In addition, SR45 also interacts with U2AF35, which plays an important role in 3' splice site selection. Hence, SR45 has the capability of affecting splice site choice both at 5' and 3' splice sites. Altered splicing of *HsfA2* pre-mRNAs in loss-of-function mutant of *SR45* supports this assumption. Since only the long isoform of SR45 rescues the mutant phenotype but not the short isoform, I predict that the extra eight residues present in the long isoform are important for binding, splice site selection and regulated splicing. Binding analysis with

truncated RNA probes and mutant versions of the long isoform in which isoform-specific residues are changed will help map the region in RNA that binds and also identify key residues in the long isoform that are important for this interaction. Based on these results the short isoform is not expected to bind to this intron, but this needs to be confirmed experimentally.

The Long Isoform of SR45 Confers Salt Tolerance

Previously, it has been shown that the glucose and ABA signaling pathways are impaired in *sr45* (Carvalho et al., 2010) and my studies, as discussed above, have shown an important role for SR45 in heat stress response at different developmental stages. Analysis of publicly available RNA seq data from control and stressed tissues has shown that *SR45* expression is induced under several abiotic stresses (Fig 7). These observations suggest SR45 may have a role in other abiotic stresses. To determine whether SR45 is involved in salt stress response, I investigated the role of both splicing variants of *SR45* in salt tolerance and intracellular ions homeostasis. Remarkably, loss-of-function of SR45 enhanced sensitivity to NaCl and others ions including K^+, Li^+, and Cs^+. This sensitivity phenotype varied depending on the developmental stage. As in heat stress tolerance complementation of *sr45* with the long isoform rescued the salt hypersensitive phenotype, suggesting that the SR45 long isoform has an essential positive role in salt tolerance as well as in ion homeostasis. It is interesting that in both the tested abiotic stresses only the long form is required. Additional stress studies with these lines are needed to see if the short isoform has any role in other abiotic stresses.

It is known that high salts in the medium can lead to passive entry of salt ions into the cell and its accumulation in the cytoplasm, which can inhibit the activity of enzymes (Munns and Tester, 2008) and then growth retardation. Biochemical analysis of ions indicated that *sr45*

accumulated less Na^+, K^+, Ca^{2+}, and Mg^{2+} (Fig 24). Hence, the hypersensitivity phenotype of *sr45* is not due to increased accumulation of salt. Some other mechanism of SR45-mediated salt tolerance may be at play or accumulation of ions is restricted to specific cell types or tissues leading to growth retardation. Biochemical analysis of salt responsive genes in wild type, *sr45* and the complemented lines under control and salt stress conditions indicate that the expression and splicing of *SOS* genes (*SOS1, SOS2, SOS3, and SOS4*) is affected in *sr45* and restored to the wild type level in the long isoform-complemented line (Figs 25 and 26). It is generally assumed that Na^+ ion induces Ca^{2+} signaling leading to activation of SOS3, which then interacts, with a protein kinase, SOS2, at the plasma membrane. The SOS3/SOS2 complex phosphorylates and activates ATP-dependent Na^+/H^+ exchanger that insures Na^+ exclusion from a plant cell via sodium exchange with protons at the expense of ATP (reviewed in (Kosová et al., 2013). SOS1 also controls Na^+ loading into xylem elements and therefore it regulates root to shoot transport (Shi et al., 2002a). In my analysis I used whole seedling for ion analysis and it would be of interest to quantify the ion content in separate tissues. SR45 might affect the ions translocation and distribution in the plant and therefore analysis of ions in different tissues might help elucidate the role of SR45 in ion homeostasis. Another gene in the SOS pathway, which is induced in response to salt stress and known to be alternately spliced, is *SOS4*. Interestingly, the expression and splicing of *SOS4* is affected in both *sr45* and the short isoform-complemented line (Fig 26). *SOS4* encodes a pyridoxal kinase and is important for root hair development (Shi et al., 2002b). Furthermore, I have found that *SOS4* expression and splicing is altered in *sr45* in response to other abiotic stresses. In response to heat stress the same isoform (isoform *III*) is induced in both wild type and the mutant complemented with the long isoform, suggesting that SR45 long isoform is important for SOS4 regulation, which in turn play a role in salt stress

tolerance.

Another set of genes that are differentially induced in response to desiccation, cold and high salt stress are *RD29* (Response to Desiccation including *RD29A, RD29B*), *ADH1* and *DREB2* (Yamaguchi-Shinozaki and Shinozaki, 1993). Not only are *RD29* genes induced in response to abiotic stresses, but they are also used as marker genes for stress tolerance (Cheong et al., 2010) and when they are introduced in combination with DREB1A/B into different plants they improve resistance to different stresses (Jia et al., 2012). To investigate if SR45 is involved in the regulation of these genes, I tested the expression of these genes in response to salinity. qPCR analysis indicated that the SR45 long isoform is needed for normal expression of these genes. How does SR45 regulate the expression of these genes? The promoter of *RD29* genes (*A* and *B*) includes two *cis*-acting elements, DRE (Dehydration-Responsive Element) and ABREs (ABA-Responsive Element) (Kiyosue et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). RD29A is regulated in both ABA-dependent and ABA-independent pathways while RD29B is regulated in the ABA-dependent pathway only. Previous studies have shown that SR45 is involved in ABA accumulation in response to glucose signaling (Carvalho et al., 2010). So it is possible that SR45 regulates expression of *RD29* genes by controlling the accumulation of ABA in response to stress and impacting the ABA signaling pathway. To test this hypothesis I have analyzed the expression of several ABA signaling genes including *ABF1, ABF2, ABF3*, and *ABI5* by qPCR using RNA isolated from control and salt-treated seedlings. The expression of these genes and alternative splicing of *ABF2* are misregulated in the mutant (Fig 31). Similar to other stress-responsive genes the *SR45* long isoform complements the expression of these genes to the wild type level, suggesting that the SR45 long isoform widely regulates the expression and alternative splicing of salt tolerance related genes and is important for abiotic stress tolerance.

In conclusion, in addition to developmental processes, SR45 regulates the response to two abiotic stresses in an isoform-dependent manner where the long isoform is needed for heat and salinity stress tolerance. The long form confers heat and salt tolerance by controlling the expression and/or splicing of sets of transcription factors and stress response genes. Based on the results it would be interesting to test the affect of other abiotic and biotic stresses on SR45 and the role of splice variants in those stresses. Since the long isoform confers salt tolerance, over-expression of this specific isoform in the wild type background may increase salt tolerance. Identification of global mRNA targets of the long and short isoform will provide further clues to the roles of *SR45* mRNA isoforms and a mechanistic basis for their action.

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