Alternative splicing of HsfA2 mediates thermotolerance in tomato species

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1 Zusammenfassung

Die Tomatenpflanze (*Solanum lycopersicum*) ist weltweit eine der wichtigsten Nutzpflanzen und umso dramatischer ist die Tatsache, dass sowohl milder anhaltender Hitzestress (HS) als auch Hitzewellen eine große Bedrohung für die Tomatenernte darstellen. Die sogenannte Thermotoleranz ist in Pflanzen abhängig von der Aktivität der Hitzestress-Transkriptionsfaktoren (Hsfs), die als Hauptregulatoren Änderungen am Transkriptom unter erhöhten Temperaturen vornehmen können (Nover et al., 2001). In der Tomatenpflanze ist die Hitzestress-Antwort (HSR) abhängig von der Aktivität des Hauptregulators HsfA1a (Mishra et al., 2002). Im Gegenzug, initiiert die Aktivität von HsfA1a die Produktion von HsfA2, welches zwar nicht für den Einsatz der HSR benötigt wird aber für die erworbene Thermotoleranz essentiell ist (Fragkostefanakis et al., 2016) und wie bereits in *Arabidopsis thaliana* gezeigt, vermutlich das Stressgedächtnis aufrechterhält (Lämke et al., 2016). Während des Hitzestresses interagiert HsfA2 mit dem Hitzeschockprotein (HSP) Hsp17-CII unter Bildung großer cytosolischer Aggregate den sogenannten Hitzestressgranula (HSG), in denen HsfA2 temporär akkumuliert (Port et al., 2004). Bei wiederholten Hitzestress wird HsfA2 aus den Hitzestressgranula freigesetzt, um die Transkription stressinduzierter Gene zu initiieren (Fragkostefanakis et al., 2016).

Die Suppression von HsfA2 in transgenen Tomatenpflanzen vermindert die Fähigkeit der Tomatenkeimlinge sich von einer erworbenen Thermotoleranz-Behandlung zu erholen, suggerierend dass eine Anhäufung von HsfA2 nach einer Hitzestress-Akklimatisierung wesentlich ist, um die Hitzestressantwort zu stimulieren und somit die Toleranz gegen ein zweites stärkeres Stressereignis zu erhöhen. Im Gegenzug hat die HsfA2 Suppression keinen Effekt auf die basale Thermotoleranz, was darauf hindeutend, dass HsfA2 keine wichtige Rolle in der Hitzestressantwort auf einen direkten Stress spielt.

Alle bekannten eukaryotischen Hsfs besitzen ein Intron, das die N-terminale Region kodierend für die DNA-binde Domäne (DBD) umfasst. RT-PCR Analysen von Tomatenkeimlingen (*S. lycopersicum* cv Moneymaker), die unter verschiedenen Hitzestressbedingungen behandelt wurden, haben die Anwesenheit eines zweiten Introns am 3´-Ende des offenen Leserasters bis zur 3´-UTR, dargelegt. Bei den Keimlingen, die für eine Stunde bei Temperaturen zwischen 25-50°C behandelt wurden, wurden sechs Transkriptvarianten identifiziert, während eine zusätzliche Variante in der Erholungsphase nach dem Hitzestress detektiert wurde. Diese Resultate zeigen, dass alternatives Spleißen (AS) von Hsf2A hochdynamisch und sensibel auf Temperaturschwankungen die weniger als 2,5°C betragen, reagiert.

Diese sieben Transkriptvarianten wurden in drei Gruppen klassifiziert (I-III). HsfA2-Iα, -β und –γ kodieren für Volllängen-Proteine und werden als Resultat der Beibehaltung des Introns oder der

Nutzung unterschiedlicher 5´ Donor-Seiten generiert. Unter den HsfA2-I kodierendenTranskripten, ist HsfA2-Iα das am häufigsten vorkommende Transkript bei Temperaturen zwischen 40 und 47,5°C, während HsfA2-Iγ überwiegend bei Temperaturen zwischen 30 und 37,5°C vorkommt. Die Regulation und Aktivität der HsfA2-I Isoform ist ausgiebig als die Isoform erforscht worden, die in der erworbenen Thermotoleranz involviert ist (Scharf et al., 1998; Hahn et al., 2011; Chan-Schaminet et al., 2009).

Die drei hochmolekularen Transkripte HsfA2-IIIα, -β und -γ, werden aufgrund der Beibehaltung eines Introns oder durch partielles Spleißen, dass durch die Anwesenheit eines 82 nt langen Miniexon im Intron 1 verursacht wird, generiert. In jedem Falle, führt AS zur Bildung eines Vorläufigen Stoppcodons. HsfA2-III Transkripte werden zur Degradation über den Non-sense-mediated Decay (NMD) Weg geleitet, da eine Suppression des NMD durch den Translationsinhibitor Cycloheximid zu einer Akkumulation des A2-III Transkripts führt. Die Anwesenheit des A2-III Transkripts ist vermutlich verbunden mit der Verminderung oder der Suppression der Hitzestressantwort unter ernsthaften Stressbedingungen.

HsfA2-II wird durch das vollständige spleißen beider Introns generiert, welches zur Produktion eines verkürzten Proteins führt, dessen nukleares Exportsignal (NES) und ein Teil des zweiten am Carboxy-Terminus befindlichen Aktivierungsmotivs fehlt. HsfA2-Transkripte sind vorhanden bei Temperaturen zwischen 30 und 40°C, mit einem Maximum bei 37,5°C. Die Immunodetektion mit einem HsfA2-spezifischen Antikörper bestätigte die Anwesenheit von HsfA2-II, welches dennoch weniger abundant ist als HsfA2-I.

HsfA2-I zeigt ein nukleozytoplasmatisches Pendeln, welches auf die Anwesenheit eines NES und eines Nuklearen Lokalisationssignals (NLS) zurückzuführen ist, wobei die zytosolische Lokalisation bevorzugt auftritt (Scharf et al., 1998). Für eine effiziente nukleare Retention, benötigt HsfA2-I die Interaktion mit HsfA1a (Scharf et al., 1998). Das Fehlen der NES in HsfA2-II führt zur nuklearen Retention, wie GFP-HsfA2-II Fusionsplasmide exprimiert in Tomaten Mesophyll Protoplasten gezeigt haben.

HsfA1a und HsfA2-I haben eine geringe transkriptionelle Aktivität, dennoch führt die Co-Expression beider Faktoren zur Bildung eines Komplexes, welches die Transkription in einer synergistischen Weise stimuliert (Chan-Schaminet et al., 2009). Die Aktivität übertragen durch den HsfA1a-HsfA2-II-Komplex auf das Reporterkonstrukt *pGmHsp17-CI*::GUS war ähnlich zu der von HsfA1a-HsfA2-I, suggerierend, dass beide Komplexe die gleiche Kapazität für die Induktion von HS-bezogenen Genen haben. Interessanterweise, ist die Aktivität von HsfA2-II alleine signifikant höher als die von HsfA2-I. Dieses könnte der höheren Effizienz der nuklearen Retention von HsfA2-II im Vergleich zu HsfA2-I zugeschrieben werden, da bei Anwesenheit des nuklearen Exportinhibitors Leptomycin B (LMB), beide Isoformen die gleiche Aktivität

aufweisen. Diese Resultate konnten auch für die endogenen Proteine Hsp101 und Hsp17-CI erhalten werden. Zusätzlich ist die Aktivität von HsfA2-II unabhängig von HsfA1a, im Vergleich zu HsfA2-I, welches die Interaktion mit HsfA1a für die nukleare Retention benötigt.

Als Reaktion auf einen direkte HS Behandlung, interagiert HsfA2-I mit Hsp17.4-CII, welche verstärkt Hitzestressgranula bilden, die als große cytoplasmatische Aggregate vorübergehende Lagerstellen für HsfA2 darstellen (Port et al., 2004). Dieses Phänomen kann in nicht-gestressten Protoplasten reproduziert werden, wenn beide Proteine zur gleichen Zeit exprimiert werden. In der Gesamtheit, entgeht die HsfA2-II Isoform dem Repressoreffekt von Hsp17.4-CII und nur eine kleine Fraktion von HsfA2-II Co-lokalisiert mit Hsp17.4-CII in perinukleären Aggregaten. Der starke Repressoreffekt von Hsp17.4-CII auf HsfA2-I und nicht HsfA2-II wurde durch einen GUS-Reporter-Assay bestätigt, wobei Protoplasten entweder mit HsfA2-I oder HsfA2-II zusammen mit steigenden Konzentrationen eines Hsf17.4-CII kodierenden Plasmids transformiert wurden. Höhere Levels von Hsp17.4-CII hat die Aktivität von HsfA2-I komplett aufgehoben, während es HsfA2-II möglich war geringe Aktivitäten aufrechtzuerhalten. Hinzukommend, zeigt HsfA2-I eine höhere Proteinstabilität im Vergleich zu HsfA2-II, wie an relativen Proteinmengen in Protoplasten behandelt mit Cycloheximid für eine Dauer von 6 Std. gezeigt haben. Zusammenfassend zeigen diese Resultate, dass eine Exposition bei Temperaturen zwischen 30 und 40°C zur Produktion zweier Isoformen führt, wobei HsfA2-I dazu bestimmt ist langfristigen Schutz und den Erwerb von Thermotoleranz zu gewährleisten und HsfA2-II, welches sofort verfügbar die Stimulation der Transkription von HSR-zugehörigen Genen anregt.

Das zweite Intron ist in vielen Kultur- und Wildsorten der Tomate als auch in einigen anderen Nutzpflanzen der Familie der Solanaceen wie der Kartoffel (*Solanum tuberosum*) und der Paprika (*Capsicum annuum*) vorhanden. Interessanterweise, unterscheidet sich die Anzahl der Transkripte, die durch AS an Intron 2 generiert werden und die Effizienz des Spleißens signifikant unter den verschiedenen Tomatenspezies. AS in Keimlingen von *S. lycopersicum* Kultursorten als auch die nächsten Verwandten *S. galapagense* und *S. pimpinelifolium* zeigen HsfA2-Iα, HsfA2-Iγ und HsfA2-II Transkripte, wenn diese Temperaturen von 37,5°C ausgesetzt werden, wenngleich bei anderen Wildsorten darunter *S. peruvianum* und *S. pennellii* HsfA2-Iγ fehlt. Genauso variierte auch die Splicing-Effizienz, die anhand der Fraktion des HsfA2-II Transkripts untersucht wurde, wobei *S. lycopersicum* und *S. galapagense* eine Effizienz von etwa 50% zeigt, während die Wildsorten mit zwei Transkripten eine Splicing-Effizienz mit einem Maximum von 95% zeigen. Interessanterweise, zeigt die Wildsorte *S. pimpinelofolium* (LA1261), eine Effizienz ähnlich zu *S. lycopersicum,* während andere (LA2852) mehr Ähnlichkeit zu *S. peruvianum* haben.

Sequenzanalysen offenbarten die Anwesenheit von drei G>A single nucleotide polymorhisms (SNPs) im Intron, die direkt mit dem Splicing-Mustern von HsfA2 korrelieren. Das dritte SNP ist am GT Dinukleotid lokalisiert und dient als 5´-Splicing Seite für HsfA2-Iγ, womit die Abwesenheit

dieser Variante in den vielen Wildsorten erklärt wäre. Interessanterweise, hat LA2852 von den zwei untersuchten *S. pimpinelifolium* Wildsorten kein spezifisches Polymorphismus, während LA1261 nur das zweite SNP (GAG) beinhaltet, das die Produktion von A2-II erhöht. Der Beitrag von jedem SNP zu dem Splicing-Muster von HsfA2 wurde anhand eines Minigen Splicing-Assays untersucht. Das Austauschen einer oder mehrerer G´s zu A´s in dem *S. lycopersicum* (SolycA2) Minigen (bestehend aus GFP fusioniert mit einem HsfA2-Fragment), resultierte in einer höheren Splicing-Effizienz. Im Gegenzug, wird der Austausch von mindestens zwei A´s zu G´s benötigt, um die Splicing-Effizienz von HsfA2 in *S. peruvianum* (SoperA2) Minigen erheblich zu inhibieren. Schlussfolgernd, ist die Splicing-Effizienz von HsfA2 abhängig von drei SNPs und unabhängig von dem genetischen Hintergrund, da das *S. peruvianum* Minigen eine erhöhte Splicing-Effizienz in *S. lycopersicum* Protoplasten zeigt.

Um zu untersuchen, ob die Polymorphismen in Verbindung stehen mit unterschiedlichen Thermotoleranzen in verschiedenen Tomatenspezies, nutzen wir einen Keimling-Hypokotyl-Assay. Junge Keimlinge sind homogenes Material und erlauben einen Hochdurchsatz-Thermotoleranz-Screening und eine Einkreisung der hohen Variation in Entwicklung und Phänotyp unter den Tomatenspezies. Die Keimlinge von 22 Sorten (Genotypen), einschließlich mehrerer Kultursorten, die von Tomato Genetics Resource Center (TGRC) als Thermotolerant charakterisiert wurden, wurden bei 37,5°C exponiert und anschließend für 90 min bei 50°C behandelt. Normalerweise wirkt sich eine direkte Hitzebehandlung bei 50°C letal auf alle Genotypen aus, und deren Überleben ist abhängig von Schutzmechanismen die währen der 37,5°C Vorbehandlung aktiviert werden. Die Gruppe der Genotypen mit einem HsfA2 Allel (AAA oder GAG) zeigten eine höhere Spleiß-Effizienz, welches als Fraktion von HsfA2-II Transkripten und durch erhöhte Thermotoleranz bestimmt wurde im Vergleich zu Genotypen ohne Polymorphismen. Im Gegenzug, beobachteten wir eine hohe Korrelation zwischen Spleiß-Effizienz und Thermotoleranz unter den 22 analysierten Genotypen, darauf hinweisend, dass HsfA2 Spleißen einen positiven Effekt auf den Schutz gegen eine starken HS-Behandlung ausübt.

Der positive Effekt des erhöhten HsfA2 prä-mRNA Spleißens wurde daraufhin durch eine Introgressionlinie (IL8-1), welches das *S. pennellii* (LA716) HsfA2 in *S. lycopersicum* cv M82 als genetischen Hintergrund enthält, bestätigt. IL8-1 akkumulierte höhere Levels von Hsp17.7C-CI und legte eine erhöhte Keimlingsthermotoleranz dar.

Bemerkenswerterweise, obwohl *A. thaliana* wie andere Brassicaceen ein einzelnes HsfA2 kodierendes Gen besitzt, dass kein zweites Intron besitzt, kodieren viele Monokotyledone wie Reis (*O. sativa*), Mohrenhirse (*S. bicolar*) und Mais (*Z. mays*) für mehr als ein HsfA2 Ortholog. Interessanterweise, besitzen diese Spezies HsfA2 Orthologe die ein NES haben während andere keines besitzen. Wir vermuten, dass die funktionelle Diversifikation, dass durch alternatives

Spleißen von HsfA2 in Solanaceen erzielt wurde, durch Genduplikation in anderen Pflanzenfamilien ersetz wurde.

Wir zielten darauf ab den Spleißfaktor zu finden, der das Spleißen des Intron 2 in HsfA2 vermittelt. Zu diesem Zweck, konzentrierten wir uns auf die Familie der Serin/Arginin-reichen Proteine (SR-Proteine), welche als zentrale Regulatoren des Alternativen Spleißens bekannt sind. Unter Verwendung von Transkriptomdaten, konnten wir einige SR-Proteine kodierende Gene identifizieren, die in Keimlingen unterschiedlich exprimiert wurden, während andere durch Hitzestress induziert wurden. Interessanterweise, zeigten RT-PCR Analysen, dass die Mehrheit der SR-Proteine bereits unter Kontrollbedingungen umfangreichem alternativen Spleiß-Prozessen unterzogen werden, die unter Stressbedingungen verändert vorliegen. In manchen Fällen führte dieses zu niedrigen bzw. hohen Transkriptleveln, die für das funktionelle Volllängen Protein kodieren.

Sieben SR-Proteine als da sind RS2Z36, RSZ40, SC24, SC30a, SC30b, SCL29 und SR32, wurden ausgewählt und C-Terminal mit einem His-Tag fusioniert. Zunehmende Mengen der GFP-SR-Protein-Fusionsplasmide wurden mit SolycHsfA2 und SoperHsfA2 Minigenen in Protoplasten Co-transformiert. Die Untersuchung erfolgte durch Analysen des veränderten Splicing-Musters in entsprechenden Rationen zwischen GFP-A2-I und GFP-A2-II, unter Verwendung eines α-GFP Antikörpers. Wir identifizierten zwei SR-Proteine, namentlich SCL29 und RS2Z36, die eine signifikante Zunahme der GFP-A2-I Isoform speziell im *S. lycopersicum* Minigen zeigen, wenngleich kein Effekt in *S. peruvianum* erkennbar ist. Mittels RT-PCR haben wir den inhibitorischen Effekt von RS2Z36 auf das Spleißen der Solyc Minigene bestätigen können, während SCL29 keinen Effekt hervorbrachte, welches möglicherweise darauf hinweist, dass SCL29 im Post-Splicing Prozess involviert ist.

Insgesamt ist in dieser gegenwärtigen Studie gezeigt worden, dass HsfA2 Spleißen durch die Aktivität von einem SR-Protein vermittelt wird, nämlich RS2Z36. Die Fähigkeit von RS2Z36 an die prä-mRNA von *S. lycopersicum* zu binden oder damit zu assoziieren führt zur Inhibition des Spleißens, welches die Bildung eines HsfA2-I Isoforms favorisiert und daher für die erworbene Thermotoleranz und für die Erholung nach wiederholten Zyklen von Hitzestress wichtig ist. Eine ähnliche Strategie wurde für *S. galapangese* und für manche *S. pimpinelifolium* Genotypen angenommen. Im Gegensatz dazu, produzieren die wilden Verwandten der Tomatenpflanze, wie *S. peruvianum* und *S. pennellii*, welche in rauerer Umgebung einheimisch sind, höhere Levels von HsfA2-II, dass die direkte Stressantwort durch gesteigerte Synthese von Hsps und anderen HS-induzierten Genen, initiieren kann. Der Unterschied in der Aktivität von RS2Z36 im HsfA2-Intron der kultivierten und wilden Spezies ist auf die drei Polymorphismen im Intron zurückzuführen, welche die Bindung bzw. die Assoziation der SR Proteine mit der prä-mRNA erlauben oder inhibieren. Das polymorphe HsfA2 kann in genetischen Programen genutzt

werden um das Tomaten-Keimplasma zu verbessern, da die Introgressionslinie, welches den genetischen Hintergrund von *S. lycopersicum* cv M82 und den HsfA2 Lokus von *S. pennellii* trägt, die Thermotoleranz *S. lycopersicum* cv M82 verbesserte.

2 Abstract

Heat stress transcription factors (Hsfs) play essential role in heat stress response and thermotolerance by controlling the transcriptional activation of heat stress response (HSR) genes including molecular chaperones. Plant Hsf families show a striking multiplicity, with more than 20 members in the many plant species. Among Hsfs, HsfA1s act as the master regulators of heat stress (HS) response and HsfA2 becomes one of the most abundant Hsfs during HS. Using transgenic plans with suppressed expression of HsfA2 we have shown that this Hsf is involved in acquired thermotolerance of *S. lycopersicum* cv Moneymaker as HsfA2 is required for high expression and maintenance of increased levels of Hsps during repeated cycles of HS treatment.

Interestingly, HsfA2 undergoes temperature-dependent alternative splicing (AS) which results in the generation of seven transcript variants. Three of these transcripts (HsfA2-Iα-γ), generated due to alternative splicing of a second, newly identified intron encode for the full length protein involved in acquired thermotolerance. Another 3 transcripts (HsfA2-IIIα-γ) are generated due to alternative splicing in intron 1, leading in all cases to a premature termination codon and targeting of these transcripts for degradation via the non-sense mRNA decay mechanism (NMD).

Interestingly, excision of intron 2, results into the generation of a second previously unreported protein isoform, annotated as HsfA2-II. HsfA2-II shows similar transcriptional activity to the full-length protein HsfA2-I in the presence of HsfA1a but lacks the nuclear export signal (NES) required for nucleocytoplasmic shuttling which allows efficient nuclear retention and stimulation of transcription of HS-induced genes. Furthermore, stability assays showed that HsfA2-II exhibits lower protein stability compared to HsfA2-I.

The presence of a second intron and the generation of a second protein isoform we identified in other Solanaceae species as well. Remarkably, we observed major differences in the splicing efficiency of HsfA2 intron 2 among different tomato species. Several wild tomato accessions exhibit higher splicing efficiency that favors the generation of HsfA2-II, while in these species the splice variant HsfA2-Iγ is absent. This natural variation in splicing efficiency specifically occurring at temperatures around 37.5^{o}C is associated with the presence of 3 intronic polymorphisms. In the case of wild species these polymorphisms seemingly restrict the binding of RS2Z36, identified as a putative splicing silencer for HsfA2 intron 2.

Tomato accessions with the polymorphic “wild” HsfA2 show enhanced thermotolerance against a direct severe heat stress incident due to the stronger increase of Hsps and other stress induced genes. Introgression of the “wild” *S. pennellii* HsfA2 locus into the cultivar M82, resulted in enhanced seedling thermotolerance highlighting the potential use of the polymorphic HsfA2 for breeding.

We conclude that alterations in the splicing efficiency of HsfA2 have contributed to the adaption of tomato species to different environments and these differences might be directly related to natural variation in their thermotolerance.

3 Abbreviations

aa	Amino acid	IDD	INDETERMINATE DOMAIN
ABI	ABSCISIC ACID-INSENSITIVE	IL	Introgression line
AD	Activation domain	IPCC	Intergovernmental panel on climate change
AF	Chlorophyll autofluorescense	IR	Intron retention
AHA	Activator motif	KO	Knock out
APX3	Ascorbate peroxidase 3	MACE	Massive analysis of cDNA ends
AS	Alternative splicing	MBF	Multiprotein bridging factor
ATT	Acquired thermotolerance	MU	4-methylumbelliferone
BIFC	Bimolecular fluorescence complementation	MUG	4-methylumbelliferone β-glucuronide
BF	Bright field	NES	Nuclear export signal
BT	Basal thermotolerance	NLS	Nuclear localization sequence
CBP	CREB binding protein	NMD	Non-sense mediated decay
CDS	Coding sequence	NS	No significant difference
CTD	C-terminal domain	OD	Oligomerization domain or HR-A/B region
CHX	Cycloheximide	OE	Overexpression
CLSM	Confocal laser scanning microscope	OL	Overlay
CS	Cosuppression	PCR	Polymerase chain reaction
kDa	Kilo Dalton	PTC	Premature termination codon
DBD	DNA binding domain	QK	Quadruple knockout
DREB	Dehydration-responsive element binding protein	RD	Repressor domain
DNA	Deoxyribonucleic acid	RNA	Ribonucleic acid
cDNA	Complementary DNA	mRNA	Messenger RNA
ES	Exon skipping	RNAi	RNA interference
EST	Expressed sequence tag	RNAPol	RNA polymerase
eYFP	Enhanced yellow fluorescent protein	pre-mRNA	Precursor mRNA
FtsH	Filamentation temperature sensitive	ROF	Rotamase FKBP
GFP	Green fluorescent protein	RRM	RNA recognition motif
GUS	β-Glucuronidase	RS	Arginine/Serine-rich domain
HAC1	Histone acetyltransferase of the CBP family 1	RT-PCR	Reverse transcription PCR
HA-tag	Hemagglutinin-tag	qRT-PCR	Quantitative real time PCR
HS	Heat stress	RFU	Relative fluorescence units
HSE	Heat stress element	SNP	Single nucleotide polymorphism
Hsf	Heat stress transcription factor	TF	Transcription factor
HSG	Heat stress granule	TMD	Transmembrane domain
Hsp	Heat shock protein	TPM	Transcripts per million
HSR	Heat shock response	UTR	Untranslated region
sHsp	Small heat shock protein	WT	Wild type
LmB	LeptomycinB	YFP	Yellow fluorescent protein

4 Introduction

4.1 Heat stress response in plants

An increasing body of evidence during the last years has proven the ongoing global climate change and its impact on various biological processes (Bokszczanin & Fragkostefanakis, 2013; Pauls et al., 2013; Walther et al., 2002). Climate changes include, among others, the more frequent occurrence of extreme phenomena like heavy rainfall and floods, extended drought periods, the gradual increase of annual temperatures and increase in temperature anomalies including heat waves. Particularly, the average temperature is predicted to increase by 2-5°C till the end of 21st century (Thuc et al., 2012). The current warming trends around the world have brought disastrous outcomes on the agricultural crop production (IPCC, 2012). Presently, an increase of temperature by 3-4°C could lead to a loss of crop yield by 15%-35% in Africa and Asia and by 25-35% in the Middle East (Bita & Gerats, 2013; R. Ortiz et al., 2008). A current model predicts that climate change is most likely to cause a considerable loss on cereal production in Southeast Asia and Southern Africa if no strategy to cope with this challenge will be developed (Bita et al, 2013; Fischer et al., 2010).

Since plants are sessile organisms that cannot escape environmental stresses such as heat or cold, they have evolved specialized protection systems to decrease the damage caused by the various abiotic stresses. Heat is one of the major and most severe forms of abiotic stress that plants can experience in nature. HS typically occurs when plants are exposed to temperatures 5-15^{o}C above their optimum for growth and development (Fragkostefanakis et al., 2014). This can have adverse effects on general plant growth and disturb the majority of developmental processes (Bokszczanin & Fragkostefanakis, 2013). Most notable is the more prominent effect of high temperatures on reproductive development (Young et al., 2004; Zinn et al., 2010). Only a few degrees above the optimum during microsporogenesis can cause male sterility and subsequently severe yield loss (Mittler et al., 2010; Zinn et al., 2010). Therefore, for plant biologists and geneticists, dissecting the basis of HSR and thermotolerance is an utmost goal towards the development of strategies that will improve the performance of the currently used germplasm (Mittleret al., 2010).

Exposure of plants to high temperatures leads to the reorganization of metabolic activities and cellular structures either aiming to minimize the destructive effects of the stress or promote cell death in case of unresolved stress (Wise et al., 2004). HS has a major impact on proteome integrity, as under high temperatures many proteins are unable to achieve their native structure leading to the accumulation of unfolded proteins and proteotoxicity (Gidalevitz et al., 2011; Wang et al., 2004). Maintenance of protein homeostasis is a key step for cell survival under stress conditions and this is mainly achieved by the synthesis of molecular chaperones such as

heat shock proteins (Hsps) to further expand the capacity of the constitutively existing cellular chaperone system (Gidalevitz et al., 2011; Wang et al., 2004) .

Hsps are important players contributing to cellular homeostasis under both optimal and unfavorable growth conditions (Wang et al., 2004). The main function of these proteins is either assisting protein refolding, or dissolving protein aggregates during recovery from stress (Boston et al., 1996; Nakamoto & Vígh, 2007; Taipale et al., 2010; Wang et al., 2004). There are five major classes of plant Hsps: the small Hsp (sHsp) family, the chaperonins (GroEL/Hsp60); the Hsp100 (Clp); the Hsp70 (DnaK) and the Hsp90 family (Wang et al., 2004). Hsps are strongly induced by a range of environmental insults, including high temperature stress, drought stress, osmotic, cold and oxidative stress (Vierling, 1991; Waters et al., 1996), indicating that Hsps play a pivotal role in protecting plants against stress and in the re-establishment of cellular homeostasis. The role of Hsps in HSR and thermotolerance has been investigated in transgenic overexpression plants or knock-out/down mutants (Grover et al.,2013). Enhanced thermotolerance was observed in the plastidial Hsp21 overexpression (OE) lines of tomato (Neta-Sharir et al., 2005) and in Hsp70-1 OE lines of tobacco (Cho & Choi, 2009). Antisense inhibition and *KO* of Hsp101 in *A. thaliana* leads to a severely compromised capacity of the plants to acquire heat tolerance after mild pretreatments (Hong & Vierling, 2001; Queitsch et al., 2000).

Apart from Hsps, other proteins also contribute to thermotolerance. For example, ubiquitin ligases, dehydrins, thioredoxins, glutathione S-transferases, dehydroascorbate reductases and cytosolic Cu/Zn-superoxide dismutase were identified to be induced during HSR and required for protection against oxidative damage and dehydration (Ferreira et al., 2006; Herouart et al., 1994; Lee et al., 2007; Ortiz & Cardemil, 2001). Furthermore, proteins involved in energy metabolism might play important roles during HSR and thermotolerance. β-amylase which is involved in starch degradation was up-regulated while glucose-1-phosphate adenyltransferase involved in starch synthesis was down-regulated by HS (Majoul et al.,2004).

4.2 The definition of thermotolerance in plants

Plants have evolved different strategies to cope with various HS phenomena in nature. Basal thermotolerance (BTT) is based on the inherent ability of plants to respond to and survive a direct strong temperature stress that exceeds the optimum for growth (Bokszczanin & Fragkostefanakis, 2013). The limits of BTT can be extended when prior to the severe stress, plants are pre-exposed to non-lethal, moderate high temperatures which allow priming of HSR (Bokszczanin et al., 2013; Larkindale et al., 2007; Mesihovic et al., 2016). This type of thermotolerance is called acquired thermotolerance (ATT) and can also be induced by a slow and continuous rise in temperature (Larkindale & Vierling, 2007). Such a gradual temperature

increase best mimics natural conditions, for example during a hot summer day (Mesihovic et al., 2016; Mittler et al., 2012).

The pre-exposure of plants to moderate HS leads to the synthesis of HS-related proteins which are involved in cellular protection and prompted tissue repair in case of an upcoming severe heat stress (Larkindale & Vierling, 2007). The pre-treatment protects the important processes essential for transcription and mRNA stability and therefore causes the generation of distinct transcriptome signatures in comparison to plants that have not been pre-acclimated, highlighting the differences between the basis of BTT and ATT (Larkindale & Vierling, 2007). For example, comparison of wheat plants pre-treated with moderate heat stress, or directly exposed to a strong stress led to the identification of 1314 transcripts showing differential expression profiles (Qin et al., 2008). The transcriptional activator MBF1c (multiprotein bridging factor 1c) accumulates rapidly upon heat stress and has been shown to positively regulate BTT in *Arabidopsis thaliana* (Suzuki et al., 2008). Interestingly, the activity of some Hsfs and Hsps has been specifically-related to ATT. The heat stress induced HsfA2, extends the duration of acquired-thermotolerance in tomato and *A. thaliana* but has no function in BTT (Charng et al., 2007; Fragkostefanakis et al., 2016). In addition, plastid Hsp21 and metalloprotease FtsH6 jointly regulate ATT in *A. thaliana* (Sedaghatmehr et al., 2016).

4.3 Transcriptional regulation of heat stress response by Hsfs

4.3.1 The plant Hsf family

The accumulation of Hsps and the induction of other HS-related genes is mainly under the control of Hsfs (Scharf et al., 2012; von Koskull-Döring et al., 2007). Despite the fact that the basic characteristics of the HSR are highly conserved among eukaryotes, plants have developed a more complex cellular response network to cope with high temperatures. This is reflected in the number of Hsf encoding genes by plant genomes, as for example, *A. thaliana* Hsf family includes 21 members, tomato (*Solanum lycopersicum*) 27, pepper (*Capsicum annuum*) 25 and soybean (*Glycine max*) 52 (Scharf et al., 2012; Guo et al., 2016). Notably, 56 Hsf-encoding genes have been reported so far in wheat (*Triticum aestivum*) (Xue et al., 2014). In contrast, the human genome encodes for four Hsfs while yeast and *Drosophila* have only one (Nover& Scharf, 1997; Nover et al., 1996; Scharf et al., 2012; Voellmy, 2004). The higher complexity of Hsfs in plants has been proposed to be due to gene and whole-genome duplications during evolution (Proost et al., 2011; Scharf et al., 2012).

4.3.2 Domain structure of plant Hsfs

Hsfs have a modular structure typical to other transcription factors (Baniwal et al., 2004; Scharf et al., 2012). Generally, the basic structure of Hsfs is comprised by an N-terminal DNA-binding domain (DBD), followed by an oligomerization domain (OD), nuclear import signal (NLS) and export signal (NES), and C-terminal activator motifs present in class A Hsfs (von Koskull-Döring et al., 2000; Heerklotz et al., 2001; Nover et al., 1996). The domain structures of three best studied tomato Hsfs (HsfA1a, HsfB1 and HsfA2) are shown in Figure 1.

The DBD is highly structured and consisted of three helical bundles and a β-sheet with four antiparallel strands (Damberger et al., 1994; Schultheiss et al., 1996). The DBD binds through the central helix-turn-helix motif to heat stress elements (HSE) typically found in promoters of HS-induced genes (Santoro et al.,1998). HSEs are repetitive palindromic motifs with a consensus sequence 5'-AGAAnnTTCT3' (Pelham, 1982).

A flexible linker with variable length connects the DBD with the OD or HR-A/B. Similar to DBD, OD is common to all Hsfs (Scharf et al., 2012; Scharf et al., 1990) and mediates the oligomerization of Hsfs through a coiled coil domain which is comprised of heptad repeats of hydrophobic amino acids (Peteranderl et al., 1999). Based on the length of the linker between the A and B part of HR-A/B, plant Hsfs are categorized in three classes: A, B and C (Nover et al., 2001; Scharf et al., 2012; von Koskull-Döring et al., 2007). Linkers of class A Hsfs are comprised of 21 amino acid residues, of class C Hsfs 7 residues, while the HR-A/B regions of plant class B Hsfs is compact similar to non-plant Hsfs (Nover et al., 1996). The structural variability in the OD domain leads to distinct coordinated effect through the formation of specific hetero-oligomeric Hsf complexes (Baniwal et al., 2004; Chan-Schaminet et al., 2009).

The NLS of Hsfs is consist of monopartite or bipartite clusters of basic amino acid residues C-terminal of the OD (Lyck et al., 1997). The nuclear import of Hsfs is mediated by the NLS. The NES at the C-terminal end of many Hsfs is formed by several repeats of leucine residues (Heerklotz et al., 2001). Unlike the NLS sequence, not all plant Hsfs have NES. The presence of both signals mediates the intracellular distribution and activity of Hsfs which depends on the balance of nuclear import and export (Heerklotz et al., 2001; Lyck et al., 1997).

The short activator peptide motifs (AHA motifs) located in the C-terminal domains (CTD) mediate the transcriptional activator function of Hsfs. They are comprised of aromatic (W, F, Y), large hydrophobic (L, I, V) and acidic (E, D) amino acid residues (von Koskull-Döring et al., 2000; Kotak et al.,2004). AHA motifs only exist in class A Hsfs and combination of AHA motifs and their adjacent NES represent a signature region in the C-terminus of several class A Hsfs (Kotak et al., 2004; Nover et al., 2001). Notably, HsfA8 lacks AHA motifs (Kotak et al., 2004) while the activator function of HsfA3 is mediated by a pattern of tryptophan residues (Bharti et al., 2000).

Class B Hsfs which lack the activator function, are characterized by the presence of a characteristic LFGV peptide motif located at the C-terminus, which confers repressor function (Czarnecka-Verner et al., 2004; Ikeda & Ohme-Takagi, 2009).

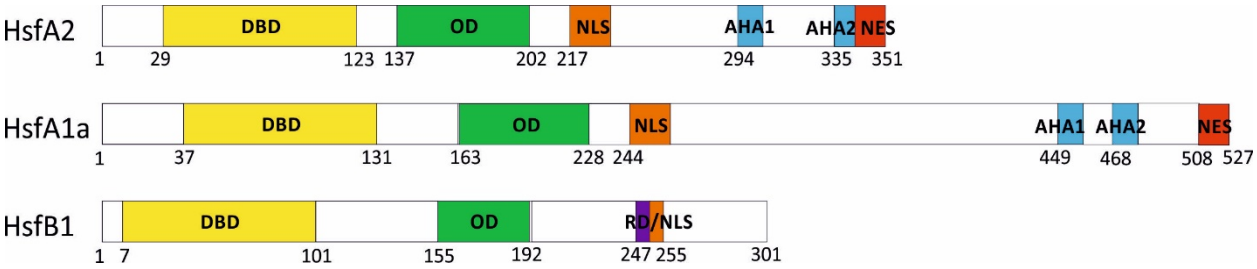

Figure 1. Basic structure of tomato HsfA1a, HsfA2 and HsfB1.
DBD, DNA binding domain; OD, oligomerization domain (HR-A/B region); NLS, nuclear localization signal; NES, nuclear export signal; AHA, activator motifs; RD, tetrapeptide motif -LFGV- as core of repressor domain. (Adapted from *Scharf et al. 2012*).

4.3.3 Functional diversification of plant Hsfs

4.3.3.1 HsfA1a is the master regulator of heat stress response in tomato

For tomato it has been shown that HsfA1a has a unique function as the master regulator of HSR, as transgenic plants with suppressed HsfA1a levels cannot to induce HSR as indicated by the failure to accumulate stress induced chaperones and Hsfs, such as HsfA2 (Mishra et al., 2002). Consequently, repression of HSR leads to hypersensitivity and plant death after exposure to 45^{o}C for 1 hour (Mishra et al., 2002). On the other side, the ectopic expression of HsfA1a leads to a stronger HSR and higher survival rate compared to wild type plants under severe heat stress conditions, as shown in the same study.

Similar to tomato, *A. thaliana* has a four member HsfA1 subfamily (HsfA1a, b, d and e) however no single master regulator function has been appointed for one of the Arabidopsis HsfA1s as single knockout mutants did not show reduced thermotolerance (Liu et al., 2011; Liu & Charng, 2013; Yoshida et al., 2011). Instead, BTT and ATT were practically abolished in the quadruple knockout mutant (*QK*) but varied in triple *KO* at different developmental stages which indicates that the comparable role of master regulator is shared among the four paralogues.

HsfA2 has been identified to be one of the most HS-inducible Hsf at the protein level in tomato and Arabidopsis. HsfA2 has high activator potential for transcription of Hsp genes and is essential for survival and recovery from long term or repeated cycles of heat stress. In this respect, *Arabidopsis thaliana* HsfA2 *KO* mutant lines have a decreased capacity to acquire

thermotolerance (Charng et al., 2007). Furthermore, HsfA2 is an important player in the maintenance of transcriptional memory by regulating the sustained accumulation of histone H3 lysine 4 methylation which serves as a signature of transcriptionally active loci (Lämke et al., 2016).

In tomato, a series of proteins influence many aspects of HsfA2 including solubility, intracellular distribution and activator function (Fig. 2). The long term effects of HsfA2 can be attributed to the remarkable stability of the protein which is subjected to a seemingly unique regulatory process involving interactions with class CI and CII small cytosolic Hsps. More specifically, Hsp17.4-CII interacts with the C-terminal activator domain of HsfA2 leading the repression of activity of HsfA2 (Fig. 2). During heat stress the two proteins are recruited to large cytosolic aggregates, called heat stress granules (HSGs; Mishra et al., 2002; Scharf et al., 1998). Re-solubilization of HSGs and release of HsfA2 is mediated by interaction with class CI sHsps, typically occurring when stress is prolonged (Port et al., 2004). The release of HsfA2 allows its interaction with HsfA1a to form a highly active activator complex (Fig. 2) (Port et al., 2004). In this respect, HSGs have been characterized as temporary deposition sites of HsfA2.

OE of Arabidopsis HsfA2 enhanced thermotolerance in *QK* (*hsfA1a, b, d, e*) mutant plants, which indicates that HsfA2 can sustain its activity and play an important role in HSR in the absence of HsfA1s, and it is tempting to speculate that interactions between HsfA2 and other Hsfs may be favoured in the *QK* mutant (Guo et al., 2016; Liu & Charng, 2013). Similarly, ectopic expression of rice (*Oryza sativa*) HsfA2a and wheat (*Triticum aestivum*) HsfA2d in Arabidopsis increased thermotolerance highlighting the importance of this factor for the HSR in different species (Chauhan et al., 2013; Yokotani et al., 2008).

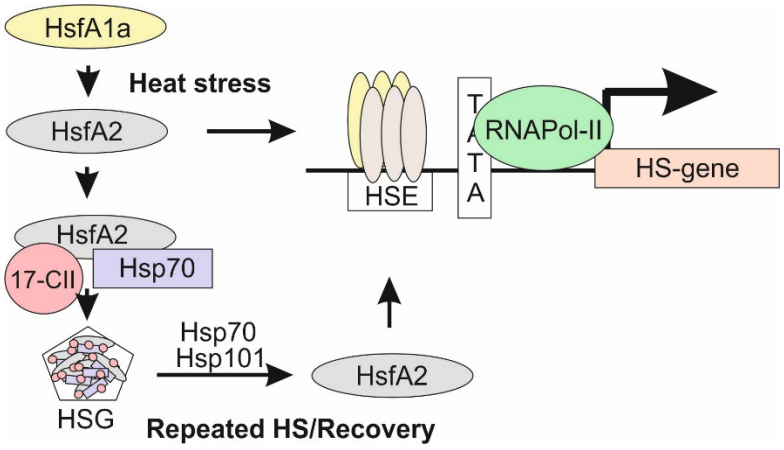

Figure 2. Regulation of HsfA2 abundance and activity by HsfA1a and interaction with molecular chaperones.
HsfA1a induces the expression of HsfA2 during HS, while interaction of the two proteins leads to the efficient nuclear retention of the former. In addition, interaction of HsfA2 with Hsp17.4-CII during HS leads to the

accumulation of both in large protein aggregates called HSGs, from which they are released during recovery from HS.

4.3.3.2 Contribution of other Hsfs in thermotolerance

Beyond the essential function of HsfA1s and HsfA2 for BTT and ATT several studies have highlighted the importance of other Hsfs in various aspects of HSR and thermotolerance, as indicated by the results of genetic studies utilizing Hsf mutants or transgenic plants. For example, OE of *Arabidopsis* HsfA6b displayed enhanced BTT and ATT, indicating that HsfA6b positively regulates thermotolerance (Huang et al., 2016). Furthermore, OE of *TaHsfA6f* in transgenic plants led to higher expression levels of Hsps and a number of other heat stress protection genes, and displayed enhanced thermotolerance compared to wild type (Xue et al., 2015). In contrast to the expression profiles of other HS-induced Hsfs (Hsfs A2, A7a, A7b, B1, B2a and B2b), *A. thaliana* HsfA3 is detectable in the late phase of HS and declines rapidly in early recovery phase. The T-DNA insertion mutant (*hsfA3*) and RNA interference lines (*hsfA3 RNAi*) displayed reduced thermotolerance at different developmental stages which could be attributed to lower levels of Hsp101 as well as sHsps in both lines (Schramm et al., 2008).

In contrast to class A Hsfs, class B Hsfs have no transcriptional activity due to the lack of an activator domain (Scharf et al., 2012). However, tomato HsfB1 has the unique feature to act as a co-activator of HsfA1a by assembling into an enhanceosome-like complex resulting in strong synergistic transcriptional activation in plant cells (Bharti et al., 2004). The co-activator function depends on the recruitment of plant CREB binding protein (CBP) orthologue histone acetyl transferase *HAC1* (Bharti et al., 2004). In contrast, *A. thaliana* HsfB1 cannot act as the co-activator because the essential histone-like motif GRGKMMK with the invariant Lys residue (underlined) in tomato HsfB1 is substituted to GSRMTETK in Arabidopsis HsfB1 (Bharti et al., 2004). Instead, *A. thaliana* HsfB1 and HsfB2b have repressor activities in transient assays which is caused by the 11 amino acid carboxyl terminal motif (GEGLKLFGVWL) acting as a repressor domain (Miho Ikeda & Ohme-Takagi, 2009). Arabidopsis HsfB1 and HsfB2b were characterized as transcriptional repressors of the HS-inducible Hsfs and Hsps including HsfA2 and HsfA7a. In turn, the *hsfb1* and *hsfb2b* double mutants show increased thermotolerance compared to wild type when seedlings were exposed to a 42^{o}C stress (Ikeda et al., 2011). Interestingly, the double mutants exhibited decreased acquired thermotolerance (Ikeda et al., 2011). This lower acquired thermotolerance in *hsfb1 hsfb2b* plants might be caused by the impaired activities of HsfA1s. These results indicate that HsfB1 and HsfB2b may suppress the expression of genes coding for Hsps that interfere with the nuclear migration of HsfA1 and consequently, promote the activity of HsfA1s (Ikeda et al., 2011).

4.3.3.3 Hsfs are involved in plant growth and development

The control of Hsf activity is not only important for plant thermotolerance but also for the return of the stressed plants to a physiological state upon recovery as Hsf networks can interfere with various growth and developmental processes. For example, constitutive OE of HsfA2 in *Arabidopsis thaliana* caused induction of HS-induced genes under non-stress conditions, dwarfism, but also enhanced callus formation suggesting interference with cell proliferation (Ogawa et al., 2007). Furthermore, the expression of several HS-induced genes is mediated by HsfA2 during pollen development to prime sensitive stages against possible stressful conditions (Fragkostefanakis et al., 2016). Consistent with this, tomato anther had a stronger accumulation of HsfA2 than other flower tissues and both short and prolonged heat stress caused further induction of HsfA2, indicating that HsfA2 may be required for the activation of protection mechanisms in the tomato anther during heat stress (Giorno et al., 2010).

Manipulation of levels of other Hsfs have also yielded phenotypic and developmental alterations. OE of *A. thaliana* HsfB4 resulted in altered root development and early duplication of endodermis cells (Begum et al., 2013). HsfA9 was identified as the only Hsf among 21 members of the Arabidopsis Hsf family that is exclusively expressed in the late stage of seed development and its level decreases rapidly during seed imbibition. This specific expression of HsfA9 is regulated by the seed–specific transcription factor ABSCISIC ACID-INSENSITIVE3 (ABI3) through activation of the HsfA9 promotor (Kotak et al., 2007). More interestingly, expression of several Hsps is HsfA9-dependent in vegetative tissues under non-stress conditions (Kotak et al., 2007). Similarly, the stronger accumulation of Hsps and improved seed longevity were observed by overexpressing sunflower HsfA9 in tobacco seeds (Prieto-Dapena et al., 2006). Collectively, these results highlight the importance of Hsfs beyond their classical stress functions which consequently requires a tight control for regulation of their activity in order to balance stress response and development.

4.3.4 The crosstalk among Hsfs, Hsps and co-chaperones

Hsfs and Hsps act as central regulators in the HSR. Hsfs serve as the terminal component of signal transduction and mediate the expression of Hsps (Kotak et al., 2007). In turn, the activity of Hsfs in plants but also in other organisms is mediated by interactions with molecular chaperones (Akerfelt et al., 2010; Hahn et al., 2011). In the absence of stress that causes proteotoxicity, human Hsf1 is maintained in an inactive state bound by Hsp70 (Akerfelt et al., 2010). Under stress conditions that enhance the accumulation of unfolded proteins, Hsp70 is released from the complex with Hsf1 to assist folding and protection of misfolded proteins. This allows the trimerization of Hsf1, nuclear import and binding to HSEs of HS-induced genes

(Abravaya et al., 1992). Hsp90 controls activity of Hsf1 in a similar way (Ali et al., 1998; Zou et al., 1998).

In plants, the regulatory network between Hsfs and chaperones has been extensively studied in the tomato system and particularly for HsfA1a, HsfA2 and HsfB1 (Hahn et al.,2011). Remarkably, individual Hsfs seem to be subjected to distinct regulatory mechanisms, which involve different chaperones (Hahn et al., 2011). The constitutively expressed tomato HsfA1a remains inactive in complex with Hsp70 and Hsp90 under control conditions (Hahn et al. 2011). HsfB1 protein levels are maintained at very low levels due to their interaction with Hsp90, which leads to HsfB1 degradation via the 26S proteasome machinery (Hahn et al., 2011; Röth et al., 2016). During the early phase of heat stress, HsfA1a is released from the complex with the chaperones, and HsfB1 is stabilized, resulting in the rapid transcriptional activation of HS-responsive genes including HsfA2. During the attenuation phase of HSR, interaction of HsfA1a with Hsp70 and interaction of HsfB1 with Hsp90 lead to inactivation of HsfA1a and accelerated degradation of HsfB1, resulting in the strong attenuation of transcription of HS-responsive genes.

The activity of HsfA2 during the early phase of the stress response is controlled by interactions with members of the sHsp family (Port et al., 2004). The specific physical interaction and functional interplay between HsfA2 and Hsp17.4-CII leads to the formation of HSGs. In the presence of Hsp17-CI, and probably with contribution of Hsp70 and Hsp101, HsfA2 is released from the inactive storage complex (Port et al., 2004; Scharf et al., 2012). In *Arabidopsis* it has been reported that the regulation of HsfA2 depends on two large FK506-binding proteins (FKBPs): AtFKBP62 (ROF1) and AtFKBP65 (ROF2) which are co-chaperones of Hsp90 (Meiri et al., 2010). ROF1 binds Hsp90 and they form a cytosolic regulatory complex under normal conditions. Under HS conditions and in the presence of HsfA2, the ROF1-Hsp90 complex is imported into the nucleus (Meiri & Breiman, 2009). The HS-inducible ROF2 which is homologous to ROF1, interacts with ROF1 and they form heterodimers in the complex ROF1-Hsp90-HsfA2 and in that way repress the activity of HsfA2 (Meiri et al., 2010). To date, it is not clear whether the same regulatory mechanism described in *A. thaliana* is also present in tomato.

4.4 Post-transcriptional regulation by alternative splicing and its relation to HSR

4.4.1 Constitutive and alternative pre-mRNA splicing

Precursor-mRNA (pre-mRNA) splicing is a process that removes introns from pre-mRNAs and joins exons to generate functional messenger RNAs (mRNAs) (Reddy, 2007). This process takes place co-transcriptionally in the spliceosome, a large multicomponent megadalton complex (Jurica & Moore, 2003). The spliceosome is comprised of 5 small nuclear ribonucleoproteins

(snRNPs) and around 300 proteins (Jurica & Moore, 2003). There are two types of spliceosomes: the major U2-type spliceosome and the minor U12-type spliceosome. The former, consisting of U1, U2, U4, U5, and U6 snRNPs, facilitates the removal of introns containing canonical (GT-AG) splice sites. The later catalyzes the splicing of U12-type introns which represent less than 1% in Arabidopsis and humans (Reddy, 2007; Sharp & Burge, 1997). In addition to U-snRNPs, the spliceosome contains several heterogeneous nuclear ribonucleoprotein particles (hnRNPs), DExD/H-box, and Serine/Arginine-rich (SR) proteins (Wang & Brendel, 2004). Despite the fact that plant spliceosome complexes have not been isolated, identification and analysis of *A. thaliana* genes encoding orthologous spliceosomal proteins from non-plant systems has shown the presence and functional similarity of many of the proteins found in metazoans (Reddy, 2007; Wang & Brendel, 2004). In total, 74 snRNPs genes and 395 genes encoding splicoesomal and spliceosome-associated proteins have been identified in *A. thaliana* (Wang & Brendel, 2004). Most animal spliceosomal proteins are conserved in plants (Lorkovic et al.,2005; Wang & Brendel, 2004), indicating that plant and animal spliceosomes most likely have similar constituents.

Alternative splicing (AS) occurs when during eukaryotic pre-mRNA processing, the spliceosome selects alternate 5´-donor and/or 3´-acceptor splice sites, which leads to the generation of two or more mature mRNAs. AS expands the transcriptome and proteome complexity and is considered as a key step for the evolution of organisms (Filichkin et al., 2015). AS influences many aspects of mRNA metabolism, including mRNA degradation through the RNA surveillance pathway called NMD, mRNA export and translation activity (Reddy, 2007). Intron retention (IR) frequently results in the generation of nonsense mRNA due to the formation of a premature termination codon (PTC) (Filichkin & Mockler, 2012; Filichkin et al., 2010; Reddy et al., 2013; Staiger & Brown, 2013; Syed et al., 2012). mRNAs harboring PTCs can be deleterious for the cell and are therefore rapidly degraded by the NMD machinery (Lareau et al., 2007; Lewis et al., 2003). AS also plays an important role in regulating the complexity of proteins, as it can lead to the generation of new protein isoforms with distinct functions (Black, 2003). A frame shift in the coding region can cause the exclusion of functional domains and/or the generation of new ones which can alter protein functions, including cellular localization, protein stability, activity and interaction with other proteins (Reddy & Shad Ali, 2011; Stamm et al., 2005).

In plants, AS is a largely unexplored area of gene expression, as until recently this phenomenon used to be considered rare. However, in the recent years, high-throughput sequencing-based analyses have revealed that up to around 60% of intron-containing genes in flowering plants undergo AS, which is by far more prevalent than previously predicted (Marquez et al., 2012). The complexity of AS differs among tissues, cell types and developmental stages and is significantly altered when plants are exposed to stress conditions (Marquez et al., 2012). IR is

the most frequent event of AS in land plants based on the wide-transcriptome analysis of different AS events in *A. thaliana* and several other plants (Baek et al., 2008; Filichkin et al., 2010; Keller et al., 2016;Marquez et al., 2012; Ner-Gaon et al., 2004; Syed et al., 2012; Wang & Brendel, 2006). Instead, the predominant AS type is exon skipping (ES) and IR is least prevalent in metazoans, indicating that the mechanisms involved in splice site recognition likely differ in metazoans and plants (Reddy, 2007). In addition, global analysis of gene structure has demonstrated that plant and animal genes display several differences in the architecture including the average number of introns/exons, the average size of introns/exons and the composition of introns. For example, In *A. thaliana* the average gene is 2.4 kb long with about 5 exons and 4 introns (Reddy, 2007). In human, the average gene is 28kb long with 8.8 exons and 7.8 introns (Lander et al., 2001). The average length of introns in plants is ~173 nucleotides, which is much shorter compared to introns with ~3000 nucleotides in human genes (Reddy, 2007).

4.4.2 Regulation of Hsfs by alternative splicing

A. thaliana HsfA2 is regulated by AS at the post-transcriptional level. Under stress conditions, AS generates *HsfA2-II* transcript harboring a 31-bp mini-exon within the conserved intron in the DBD-coding region. *HsfA2-II* transcript contains a PTC and is degraded by NMD (Sugio et al., 2009). This mechanism can adjust the level of functional HsfA2 protein to adapt to different HS conditions (Sugio et al., 2009). Furthermore, under severe HS (45^{o}C), another splice variant, HsfA2 III which encodes for a 14 kDa protein is generated in expense of HsfA2-II (Liu et al., 2013). The truncated HsfA2-III protein contains the Hsf helix-turn-helix DNA binding motif and is localized in the nucleus. Interestingly, this shorter protein can directly bind the HsfA2 promoter HSEs to stimulate the expression of HsfA2 gene (Liu et al., 2013). Similarly, the temperature-dependent AS in the conserved intron spanning the DBD coding region has been detected in AtHsfA4c*,* AtHsfA7b, AtHsfB1 and AtHsfB2, as well as in other HS-induced transcription factors including DREB2B (DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2B) (Liu et al., 2013). However, whether the splice variants of DREB2B are degraded through the NMD pathway or whether they can encode a truncated protein and regulate their own expression in a way similar to HsfA2-III is not known. In rice, tissue-specific and temperature-dependent AS of HsfA2 generates six splice variants, with only one coding for the functional protein (Wang et al., 2013). AS of OsDREB2B which is a temperature- and drought-responsive gene produces two transcript variants. Under non-stress conditions, a PTC-containing transcript (*OsDREB2B-1*) which results from the inclusion of a 53-bp miniexon, is dominantly expressed. Under high temperature (42°C), exon skipping leads to the generation of another isoform (*OsDREB2B-2*) encoding for the full length protein (Matsukura et al., 2010). More interestingly, this post-transcriptional regulation of DREB2B in response to stress was also observed in wheat, barley

and maize, suggesting a highly conserved regulatory mechanism (Egawa et al., 2006; Qin et al., 2007; Xue & Loveridge, 2004).

4.4.3 Plant Serine/Arginine-rich proteins

SR proteins belong to a family of highly conserved phosphoproteins, playing a key role in both constitutive and alternative splicing of pre-mRNA (Long & Caceres, 2009). Members of this family contain a modular basic domain structure (Fig. 3) comprised of one or two RNA recognition motifs (RRMs) at the N-terminus and a C-terminal arginine/serine-rich (RS) domain (Barta et al., 2010; Manley & Krainer, 2010). The RRMs bind specific RNA sequences (*cis*-acting elements which act as either splicing enhancer or suppressor) in pre-mRNA, whereas the RS domain functions as a protein-protein interaction module to recruit other proteins and in some cases contacts the pre-mRNA branch point (Zhu & Krainer, 2000). Furthermore, the RS domain is believed to be involved in nuclear localization and nucleocytoplasmic shuttling (Reddy & Shad Ali, 2011). Plants possess a higher number of genes encoding for SR proteins compared to other eukaryotes, with a total of 24 in rice (Iida & Go, 2006), and 17 in *A. thaliana* (Barta et al., 2010) whilst there are only seven SR proteins in *C. elegans* (Longman et al., 2000) and 12 in humans (Manley & Krainer, 2010). Whole-genome and extensive segmental duplications in the evolution of the plant kingdom most likely resulted in larger and more diverse SR proteins of plants (Barta et al., 2010; Duque, 2011).

A. thaliana SR proteins are grouped into six subfamilies (Fig. 3) based on phylogenetic analysis (Barta et al., 2010). Three of these subfamilies (SR, SC, and RSZ) have orthologues in mammalian systems while the others (SCL, RS2Z and RS) are plant-specific (Barta et al., 2010; Duque, 2011; Reddy & Shad Ali, 2011). The existence of plant-specific subfamilies of SRs might have evolved from the difference between plants and animals in intron/exon architecture and plant specific cis-elements involved in pre-mRNA splicing (Reddy & Shad Ali, 2011).

A striking feature of plant genes encoding splicing components, especially SR proteins, is that they often undergo AS themselves and subjected to NMD pathway as versatile means to regulate their abundance (Reddy, 2007; Wang & Brendel, 2006). In Arabidopsis, it has been shown that 14 of the 18 genes undergo AS and generate more than 90 transcripts, indicating a six-fold increase in transcripts by AS (Palusa, et al., 2007). Only four SRs (RSZ21, RSZ22, RSZ22a and SCL28) produce a single pre-mRNA, three of which belong to the RSZ subfamily. In contrast, the members of the RSZ subfamily in mammalian cells undergo AS (Cavaloc et al., 1994; Lareau et al., 2007), suggesting AS in this family is not conserved between plants and animals. Most of these splice variants possess a PTC and may produce either nonfunctional or truncated

proteins with altered functions. However, analysis of the splicing patterns of all SR pre-mRNAs in a mutant that lacked up-frameshift 3 (UPF3), one of the core components of the NMD machinery, has demonstrated that almost only half of these PTC-containing transcripts are targeted to degradation by NMD (Palusa & Reddy, 2010). PTC-containing splice variants escaping NMD might encode for protein isoforms with distinct functions.

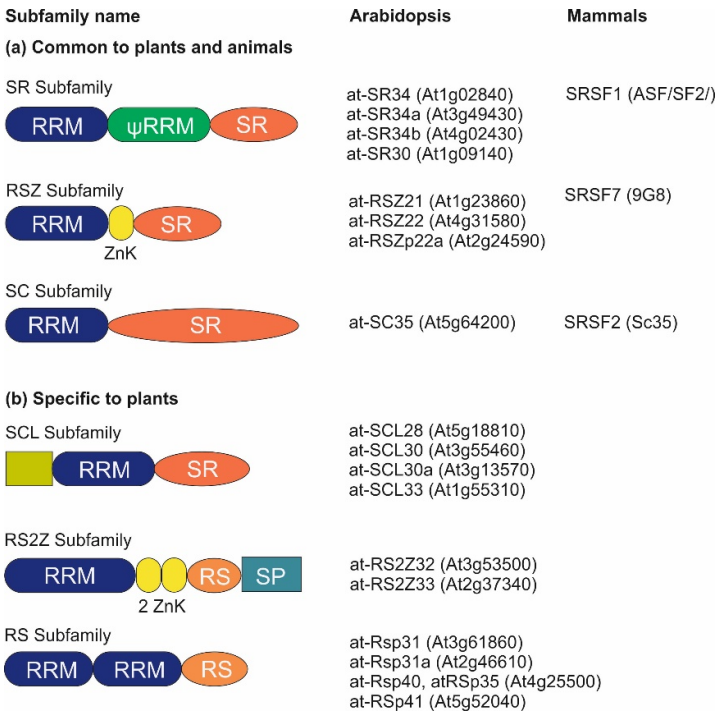

Figure 3. Schematic diagram of plant SR protein subfamilies with their conserved functional domains. At the right of the diagram showing the domain structure the corresponding Arabidopsis SR proteins (gene ID) and their human orthologues are indicated. In addition to three human SR protein that are shown here, there are other 9 SR proteins that have no orthologues in flowering plants. There is a highly conserved motif (SWQDLKD) existing in the second RRM domain in the SR subfamily (orthologues of mammalian SRSF1/SF/ASF). The SR proteins belonging to the RSZ subfamily (orthologues of mammalian SRSF7/9G8) possess one Zn knuckle. The members of the SC subfamily are consisting of only one RRM domain and an RS domain. Plant-specific SCL subfamily members contain an N-terminal charged extension followed by an RRM domain and one RS domain. The proteins of the plant-specific RS2Z subfamily are characterized by two Zn knuckles and a C-terminal SP-rich region. The plant-specific RS subfamily proteins have two RRM domain followed by one RS domain. (Adapted from *Reddy & Ali, 2011; Barta et al., 2010*).

4.4.4 Function of plant SR proteins in splicing, development, and stress responses

In the basis of what is known in animals, it is thought that SR proteins can either promote or inhibit splicing by direct interactions with exonic and intronic splicing regulators, snRNPs and other RNA binding proteins (Reddy, 2007). In addition, SR proteins have divergent functions and

are involved in mRNA nuclear export, mRNA stability, translation, and genome maintenance, thereby having a broader role in regulation of gene expression (Zhong et al.,2009). In relation to their function SR proteins play a role in regulating various developmental processes. For example, the splicing pattern of *Arabidopsis* SR genes is tissue- and developmental stage-specific (Palusa et al., 2007). Analysis of *Arabidopsis* SR30 OE lines have shown a bushy phenotype (Lopato et al., 1999). The *KO* mutants of Arabidopsis splicing factor 1 (SF1) conferred early flowering and abnormal sensitivity to abscisic acid (Jang et al., 2014). Loss-of-function *sr45* mutants showed late flowering, reduced root growth, and abnormal floral organs (Ali et al., 2007).

Several studies have demonstrated that SR proteins are involved in plant stress responses (Duque, 2011; Palusa et al., 2007; Reddy &Ali, 2011). The splicing analysis of Arabidopsis SR proteins has shown that the splicing pattern of several SR proteins changed strikingly under various abiotic stress conditions (Filichkin et al., 2010; Lazar & Goodman, 2000; Palusa et al., 2007; Tanabe et al., 2007). Stress-induced changes in SR protein gene products could in turn influence the splicing of downstream targets resulting in adaptive transcriptome changes in response to environmental stresses (Duque, 2011; Reddy, 2007). In addition, the phosphorylation status and the subcellular localization of plant SR proteins are mediated by stress signals (Ali& Reddy, 2003; Forment et al2002; Rausin et al.,2010; Savaldi-Goldstein et al., 2000; Tillemans et al., 2006).

4.4.5 Regulation of SR proteins under heat stress conditions

Temperature changes can influence the activity and abundance of the spliceosome components, as well as the speed of RNA Polymerase II and RNA structure thereby affecting both, constitutive and alternative splicing (Hiller et al, 2007; Ip et al., 2011; Staiger & Brown, 2013). Changes in the splicing profile of downstream targets can lead to adaptive transcriptome changes in response to high temperatures (Filichkin et al., 2010; Lazar &Goodman, 2000; Palusa et al., 2007). The abundance of AtSR30 transcript encoding for the full-length SR30 protein increases when plants are exposed to higher temperatures and high light, whereas an unproductive PTC-containing transcript declines at elevated temperature, indicating that AS plays an important role in regulating the level of functional protein (Filichkin et al., 2010). Similarly, heat and drought stress increase the proportion of exon-skipped transcripts encoding the full length SR45a protein (Gulledge et al., 2012). In addition, it is also known that stress signals, especially heat stress, can have an effect on both, phosphorylation status and subcellular localization of plant SRs and splicing related proteins (Ali et al., 2003; De La Fuente Van Bentem et al., 2008; Koroleva et al., 2009; Rausin et al., 2010; Tillemans et al., 2006; Tillemans et al., 2005). For example, SR45 is localized in enlarged nuclear speckles under heat stress conditions, whereas cold treatment causes a redistribution of the SR45 to the

nucleoplasmic pool (Ali et al., 2003). The intra-nuclear distribution of SR45 in response to temperature is regulated by protein phosphorylation (Ali et al., 2003). Phosphorylation also affects the function of splicing factor protein isoforms. For example, two protein isoforms of SR45 in *Arabidopsis* differ by an eight amino acid segment which contains a putative phosphorylation site. These two isoforms have very different functions on plant development (Zhang & Mount, 2009). Overexpressing SR45.1 isoform can rescue the flower petal phenotype, but not the root growth phenotype of *sr45* mutant, while overexpressing the other isoform (SR45.2) can complement root growth but not floral morphology (Zhang & Mount, 2009).

5 Materials and Methods

5.1 Plant material

Seeds of tomato wild and cultivated accessions were provided by the Tomato Genetics Resource Center (TGRC). The seeds of the introgression lines were a gift from Dr. Arnaud Bovy (Wageningen University, Netherlands). The transgenic HsfA2 plants were provided by Dr. Klaus-Dieter Scharf. Sense (A2S) and antisense (A2AS) transgenic plants aiming for overexpression and suppression of HsfA2 (Solyc08g062960), were generated as described by Fragkostefanakis et al. (2016) using the *Agrobacterium tumefaciens* strain GV3101 (Pmp90) (Koncz & Schell, 1986). In A2S and A2AS transgenic plants the T-DNA cassette with the sense or antisense HsfA2 coding sequence (CDS) was expressed under the control of CaMV 35S promoter, respectively. The sense orientation of the CDS results in overexpression of HsfA2, while the antisense of CDS leads to RNAi-induced knock down of the endogenous HsfA2.

Tomato (*Solanum lycopersicum cv. Moneymaker*) plants were grown on gelrite-solidified Murashige and Skoog (MS) medium supplemented with 20 g l^{-1} sucrose for isolation of protoplast. Tomato wild species were grown in a glasshouse under a 16/8-h day/night cycle (25-20°C). Details of the tomato accessions or cultivars used in this study are shown in Table 1.

5.2 Stress treatments

Tomato young seedlings or different tissues were exposed to various HS treatments according to designated experiments. Stress treatments on leaves were done either by exposing whole plants to HS in a growth chamber or by incubating petri dishes with detached young leaves kept on wet filter paper under controlled temperature conditions in the water bath. Seedlings were treated in petri dishes in similar manner like detached leaves. For cyclohexamide (CHX) treatment, 1 week old seedlings were exposed to 45°C for 1 hour and then treated with the translation and NMD-inhibitor cyclohexamide (CHX) [100µg ml^{-1}] or ddH_2O (control) for 3 hours in 50 ml flasks under constant gentle shaking. Samples were harvested at the desired time points and stored at -80°C until further processing.

Table 1: Tomato accessions or cultivars used in this study.

Name of accession	Species
Moneymaker	*Solanum lycopersicum*
Red setter	*Solanum lycopersicum*
LA1994	*Solanum lycopersicum*
LA2375	*Solanum lycopersicum*
LA2661	*Solanum lycopersicum*
LA2662	*Solanum lycopersicum*
LA3120	Solanum lycopersicum
LA3320	*Solanum lycopersicum*
LA3847	*Solanum lycopersicum*
LA1993	*Solanum pimpinellifolium*
LA1261	*Solanum pimpinellifolium*
LA2852	*Solanum pimpinellifolium*
LA1409	*Solanum galapagense*
LA0436	*Solanum galapagense*
LA1273	*Solanum pennellii*
LA1926	*Solanum pennellii*
LA1777	*Solanum habrochaites*
LA1353	*Solanum habrochaites*
LA2695	*Solanum chmielewskii*
NO911	*Solanum chmielewskii*
LA2157	*Solanum arcanum*
LA2172	*Solanum arcanum*
LA1326	*Solanum neorickii*
LA735	*Solanum neorickii*
LA2133	*Solanum neorickii*
LA1932	*Solanum chilense*
LA1969	*Solanum chilense*
R.58/480 selection	*Solanum chilense*
LA1935	*Solanum peruvianum*
LA1955	*Solanum peruvianum*
NO2333	*Solanum peruvianum*
M82	*Solanum lycopersicum*
LA716	*Solanum pennellii*
LA3509	*IL8-1 (M82 × LA716)*
LA1274	*Solanum corneliomulleri*

IL: introgression line

5.3 Thermotolerance assay for tomato seedlings

A thermotolerance assay based on the relative hypocotyl elongation assay as shown for *A. thaliana* (Queitsch et al., 2000) was established and optimized for tomato seedlings. Tomato seeds were washed by 70% ethanol for 1 minute, followed by a quick wash with sterile water. Next, seeds were surface sterilized by a 15 min wash with 15% sodium hypochloride and 4 consecutive washes with sterile water. Sterilized seeds were put on wet paper towels in petri dishes and allowed to germinate in dark at 25°C. Four-days-old etiolated seedlings were exposed to different heat stress regimes in basal or acquired thermotolerance assays. The stress treatment was performed for seedlings sealed in petri dishes placed in water baths with controlled temperature. Seedlings were photographed before the heat stress treatment and then every 24 hours for 6 days. Hypocotyl length was determined using ImageJ.

5.4 RNA Extraction

Plant tissues were homogenized with a TissueLyser MM300 (Qiagen/Retsch). Total RNA was isolated from leaves and seedlings using the E.Z.N.A. Plant RNA kit (Omega Bio-Tek) according to the manufacturer's instructions. Total RNA was extracted from protoplast with Trizol reagent (ThermoFisher Scientific). One milliliter of Trizol reagent was added to 400,000 protoplasts. After vigorous vortex samples were incubated for 5 minutes at room temperature and mixed with 200 µl chloroform. Following centrifugation (12,000 × g for 15 min at 4°C) the upper phase was transferred into a new tube and then 500 µl of 100% isopropanol was added, followed by an incubation at room temperature for 10 minutes. Subsequently, samples were centrifuged at 12,000 × g for 10 min at 4°C and the RNA pellet was washed, air-dried and resuspended in DEPC-treated RNase-free ddH_2O. One microgram of RNA was treated for 45 minutes at 37°C with DNase I (Applichem) for digestion of genomic and plasmid DNA.

5.5 cDNA synthesis

One microgram of total RNA was used for cDNA synthesis with Revert Aid reverse transcriptase (Thermo Fisher Scientific) following the manufacture's protocol. The RNA was mixed with 1 µl oligo-dTVN oligonucleotide (T_{24}VN) and RNase-free water at a final volume of 11 µl. The sample was incubated at 70°C for 5 minutes to relax RNA second structures and kept on ice for another 5 minutes. A 9 µl aliquot of reaction mix for Reverse transcription (RT) containing 1µl RevertAid reverse transcriptase (ThermoFisher Scientific, 200 U), 4 µl of 5X RT-buffer, 2 µl dNTP mix (10 µM) and 2 µl RNase-free water was added and then incubated at 42°C for 1 hour. Subsequently, the reactions were incubated at 70°C for 15 minutes to deactivate the enzyme and kept at -20^{o}C till further use. All cDNA templates were checked for contaminating genomic DNA by using RT-controls containing all RT reagents except reverse transcriptase. No DNA contamination was detected.

5.6 RT-PCR

cDNA samples were used for polymerase chain reaction (PCR) with specific primers for different genes. Primers were designed using Primer3 software (Untergasser et al., 2012). The primers are listed in Supplemental Table S1. Equal loading and normalization of samples were inspected by RT-PCR with EF1a (*Solyc06g005060*) as housekeeping gene. The RT-PCR analysis of nine Hsf genes and housekeeping gene (EF1a*: Solyc06g005060*) was performed in a total volume of 20 µl with 55°C to 62°C annealing temperature. All RT-PCR reactions were performed using Taq polymerase.

5.7 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was employed to determine the relative transcript levels of selected genes. qRT-PCR was done using PerfeCTa SYBR® Green FastMix (Quanta Biosciencies, Gaithersburg, MD, USA) on a Stratagene Mx3000P cycler (Agilent Technologies, Palo Alto, CA, USA). The qRT-PCR reaction (total 10 µl) contained the template (cDNA corresponding to 5 ng of total RNA), gene-specific primers (0.3µM each primer; Supplemental Table S2) and SYBR Green Mix. Thermal cycling profile was 95°C/3 min followed by 95°C/15 s, 60°C/30 s, and 72°C/30 s for 40 cycles. Data were calculated according to the standard method (Livak & Schmittgen, 2001). Ubiquitin (*Solyc07g064130*) and EF1α (*Solyc06g005060*) genes were included in the reactions as an internal control to normalize the variations in the amounts of cDNA samples. All qRT-PCR reactions were performed in biological triplicates using RNA samples isolated from three independent experiments.

5.8 Genomic DNA extraction

60-100 µg of plant tissues were homogenized with a TissueLyser MM300 (Qiagen/Retsch). 1300 µl of extraction buffer (100 mM Tris-HCI pH 6.0; 700mM NaCI; 50 mM EDTA pH 8.0; 10 µg µl-1 RNase) was added on the tissue, followed by a 15 min incubation at 65°C. Subsequently, samples were cooled at room temperature for 1 min, supplemented with 650 µl chloroform/isoamyl alcohol (24:1) and shaken gently for 5 min. Next, samples were centrifuged at 14,000 rpm for 2 min at room temperature, and the upper phase was transferred to a new tube and treated with 10 µl RNase A at 37°C for 10 min. Samples were precipitated with 700 µl isopropanol, gDNA was pelleted by centrifugation, washed with 70% ethanol, air dried and resuspended in 100 µl ddH2O.

5.9 Construction of minigenes splicing reporters

Minigene reporters contain a genomic segment of the target gene, including the intron of interest, flanked by 100-200 nt of the adjacent exons. In here, the minigene segment of HsfA2 is consisted of 184 nt 3´-end of exon 2, 298 nt of intron 2 and 135 nt 5´-end of exon 3. The HsfA2 minigene is fused in frame to the carboxyl-terminal of green fluorescent protein (GFP). The

genomic segment was generated by PCR amplification with high-fidelity Pfu polymerase, using genomic DNA of *S. lycopersicum* or *S. peruvianum* as a template. These two fragments were cloned between Acc651 and XbaI sites of pRTdS vector (Table 2).

A single forward primer with mutated nucleotide(s) (Table 2) for corresponding construct was designed to introduce the mutations of interest to generate minigenes mutants. Introduction of the minigenes carrying mutations of interest into the parental minigenes was done by employing site-directed mutagenesis.

5.10 QuickChange site-directed mutagenesis

Site-directed mutagenesis was performed using QuickChange mutagenesis PCR. For the introduction of the mutations an oligonucleotide primer (Sigma-Aldrich) that carries the desired mutations was phosphorylated with T4-polynucleotide kinase (PNK) in a 20 µl reaction according to the manufacturer´s protocol (ThermoFisher) at 37°C for 30 minutes, followed by a 70°C incubation for 15 min. Subsequently, a standard PCR was employed with the following modifications: 5 ng plasmid DNA as template, 5 pmol the phosphorylated primer, 0.625 mM NAD^+ and 5 U Ampligase (Biozym) and Pfu polymerase in a 20 µl reaction. Annealing temperature of PCR was chosen based on the primer specific melting temperature and the elongation time was adjusted according to the plasmid size (1 min per 0.5 kb). The PCR was performed for 25 cycles. The PCR products were treated overnight with 0.5 µl DpnI at 37°C to remove the template plasmid DNA. Half of mixture was directly transformed into chemically competent *E.coli* DH5α. The fidelity of the minigenes was confirmed by sequencing (GATC Biotech).

5.11 Plasmid constructs

The constructs used in this thesis were produced according to the standard cloning procedures (Hahn et al., 2011; Röth et al., 2016; Tripp et al., 2009). Oligonucleotides used for vector construction are listed in Table 2.

Table 2: Information of plasmid constructs used in this study.

Name of construct	Cloned gene	Origin (template)	Direction	Sequence (5’ to 3’)	Restriction site
pRT-GFP-HsfA2-I	Solyc08g062960	pRTdsGFP-HsfB1	forward	TTAGGTACCGAGGATGTAGTGAAAGTGAAGG	Acc65I
			reverse	TAATCTAGACTAAAGGAAACCAAGCTGATCCACAAGG	XbaI
pRT-GFP-HsfA2-II	Solyc08g062960	pRTdsGFP-HsfB1	forward	TTAGGTACCGAGGATGTAGTGAAAGTGAAGG	Acc65I
			reverse	TTTTCTAGATTAACCCCATTCAGGTGTTTTCAC	XbaI
pSAT1-HsfA1a-	Solyc08g	pSAT1-cEYFP-N1	forward	CATCTCGAGATGGAGCCGAATTCTTATGG	Acc65I

cEYFP	005170	1071	reverse	GTCGGTACCAGATCATATGTTTTTG TTG	XhoI
pSAT1-HsfA2-II-cEYFP	Solyc08g 062960	pSAT1-cEYFP-N1 1071	forward	CATCTCGAGATGGAGGATGTAGTG AAAGT	Acc65I
			reverse	GTCGGTACCAACCCCATTCAGGTG TTTTC	XhoI
pSAT1-HsfA2-II-nEYFP	Solyc08g 062960	pSAT1-nEYFP-N1 1075	forward	CATCTCGAGATGGAGGATGTAGTG AAAGT	Acc65I
			reverse	GTCGGTACCAACCCCATTCAGGTG TTTTC	XhoI
pSAT1-HsfA2-I-nEYFP	Solyc08g 062960	pSAT1-nEYFP-N1 1075	forward	CATCTCGAGATGGAGGATGTAGTG AAAGT	Acc65I
			reverse	GTCGGTACCAAAGGAAACCAAGCT GATC	XhoI
pRT-GFP-A2 minigene-lyco	Solyc08g 062960	pRTdsGFP-HsfB1	forward	TTAGGTACCAGGCCGGATTCTGTT GTGAC	Acc65I
			reverse	GCGTCTAGAGGACATTCCTCCAGA CTAAC	XbaI
pRT-GFP-A2 minigene-peru	Solyc08g 062960	pRTdsGFP-HsfB1	forward	TTAGGTACCAGGCCGGATTCTGTT GTGAC	Acc65I
			reverse	GCGTCTAGAGGACATTCCTCCAGA CTAAC	XbaI
pRT-GFP-A2-mut-mini-lyco	Solyc08g 062960	pRT-GFP-A2 minigene-lyco	forward	TGGTTTCCTTTAGAGATCACTCTTG TGTTCC	no
pRT-GFP-A2-mut-mini-peru	Solyc08g 062960	pRT-GFP-SpA2 mg-peru	forward	TGGTTTCCTTTAGAGGTCACTCTTG TGTTCC	no
pRT-GFP-A2-m1-mini-lyco	Solyc08g 062960	pRT-GFP-A2-mut- mini-lyco	forward	ATTACAAGACCTTGTAGATCAGCTT GGTTTC	no
pRT-GFP-A2-m1-mini-peru	Solyc08g 062960	pRT-GFP-A2-mut-mini-peru	forward	ATTACAAGACCTTGTGGATCAACTT GGTTTC	no
pRT-GFP-A2-m2-mini-lyco	Solyc08g 062960	pRT-GFP-A2-mut- mini-lyco	forward	AGACCTTGTGGATCAACTTGGTTTC CTTTAG	no
pRT-GFP-A2-m2-mini-peru	Solyc08g 062960	pRT-GFP-A2-mut-mini-peru	forward	AGACCTTGTAGATCAGCTTGGTTTC CTTTAG	no
pRT-GFP-A2-m3-mini-lyco	Solyc08g 062960	pRT-GFP-A2-mut- mini-lyco	forward	ATTACAAGACCTTGTAGATCAACTT GGTTTCCTTTAG	no
pRT-GFP-A2-m3-mini-peru	Solyc08g 062960	pRT-GFP-A2-mut-mini-peru	forward	ATTACAAGACCTTGTGGATCAGCTT GGTTTCCTTTAG	no
pRT-A2-mCherry	Solyc08g 062960	pRTdsHsp17.2CII-mCherry	forward	GCTGGTACCATGGAGGATGTAGTG AAAGTG	KpnI
			reverse	GGCACTAGTGTAAAGGAAACCAAG CTGATCCAC	SpeI
pRT-A2-II-mCherry	Solyc08g 062960	pRTdsHsp17.2CII-mCherry	forward	GCTGGTACCATGGAGGATGTAGTG AAAGTG	KpnI
			reverse	GGCACTAGTGTAACCCCATTCAGGT GTTTTC	SpeI

Note: letters underlined in the oligonucleotide sequence show the mutations of interest.

Table 3: HA-tagged-SR protein expression constructs.

Name of constructs	Gene name	Direction	Primer sequence	Restriction site
pRT-35S-3HA-RS2Z36	Solyc09g005980	forward	ACCGGTACCTCCTCGTTATGATGATCGT	Acc65I
		reverse	ACCTCTAGAGCGCAAGTTTCAAGGTGACT	XbaI
pRT-35S-3HA-RSZ21	Solyc08g006430	forward	ACCGGTACCTTCAAGAGTGTACGTTGGA	Acc65I
		reverse	ACCACTAGTAACTTCTCCGTTCAGCTC	BcuI
pRT-35S-3HA-SC24	Solyc10g009330	forward	ACCGGTACCTAAATCTGGGTATGCTTTCG	Acc65I
		reverse	ACCTCTAGAGCTCAAGACCTCCGAACAGG	XbaI
pRT-35S-3HA-SC30a	Solyc04g074040	forward	ACCGGTACCTTCTCACTTCGGTAGATCAGG	Acc65I
		reverse	ACCACTAGTTTAGGAATGTCCTGATAC	BcuI
pRT-35S-3HA-SC30b	Solyc01g105140	forward	ACCGGTACCTTCGCACTTCGGGAGAACTG	Acc651
		reverse	ACCTCTAGAGCCTAATCCTCATCAACACG	XbaI
pRT-35S-3HA-SCL29	Solyc01g005820	forward	ACCGGTACCTAGGAGGAGAAGTTACAGC	Acc651
		reverse	ACCTCTAGAGCTGGATCATGGTGAGTAGG	XbaI
pRT-35S-3HA-SR32	Solyc03g082380	forward	ACCGGTACCTAGTGGCCGCTTTTCACG	Acc651
		reverse	ACCACTAGTATTAGCCACTGTTTGATC	XbaI

The constructs were provided by Dr. Sotirios Fragkostefanakis.

The expression constructs of pRT-HsfA2, pRT-HsfA1a were described previously (Chan-Schaminet et al., 2009). The pRTdsAtEnp1-mCherry construct was kindly provided by Dr. Benjamin L. Weis (Weis et al., 2014). The expression construct of pRT-Hsp17.4 CII-mCherry has been previously described (Port et al., 2004).

5.12 Plasmid midi-preparation

In order to isolate plasmid DNA of high-quality and yield required for efficient protoplast transformation, plasmid midi-preparation (Mishra et al., 2002) was employed. In short, plasmid DNA was transformed in *E.coli* DH5α. The bacterial culture was harvested by centrifugation and the pellet was resuspended in 3 ml of lysis buffer (50 mM Tris/HCl pH8.0; 50mM EDTA pH8.0; 15% [w/v] sucrose). Seven milliliters of fresh 0.2 M NaOH/1% SDS buffer was added, mixed gently and kept at room temperature for 10 minutes. Next, 3.5 ml of (3M potassium acetate, 11.5% (v/v) glacial acetic acid) was added, shaken vigorously and left for 20 minutes on ice. After centrifugation (20 minutes at 5,000 rpm at 4°C), the supernatant was filtered through cotton and 7 ml of isopropanol was added for precipitation, followed by incubation at -20°C for at least 30 minutes. Following a centrifugation (11,000 rpm for 20 minutes at 4°C), and removal of the supernatant the pellet was kept, dried and resuspended by adding 500 µl of TE buffer (10 mM Tris/HCl pH 7.6; 1 mM EDTA pH 8.0) and mixed with an equal volume of 5 M LiCl/50 mM MOPS buffer. Samples were centrifuged (8,000 rpm for 10 minutes at 4°C), then the supernatant was mixed with 0.1 volume of 3M NaAc (pH5.2) and 2.5 volume pure ethanol and put at -20°C for at least 30 minutes. After centrifugation (11000 rpm for 20 minutes at 4°C) the dried pellet was resuspended in 300 µl TE buffer, supplemented with 10µl of RNase A (500 units/ml in

5mMTris/HCl pH8.0; Roth) and RNase T1 (500 units/ml in 5mM Tris/HCl pH8.0; ThermoFisher). The mix was incubated for 30 minutes at 37°C, followed by addition of 30µl of 10× Proteinase K and incubation at 37°C for 15 minutes. After addition of an equal volume of a phenol/chloroform/isoamylalcohol mixture (25:24:1), the samples were centrifuged (2 minutes at 14,000 rpm at room temperature), and the upper phase was transferred to a new tube and mixed with an equal volume of chloroform, vortexed and centrifuged again. The supernatant was transferred to a new Eppendorf tube and precipitated with ethanol and sodium acetate. Finally, following centrifugation, the pellet was washed with 70% ethanol, air dried and resuspended in TE buffer.

5.13 TA cloning

One-step cloning strategy (TOPO TA Cloning kit, Invitrogen) was employed to directly insert *Taq*-polymerase-amplified PCR products or *Pfu*-polymerase-amplified PCR products after adding 3' A-overhangs by incubation with Taq on the PCR products into a plasmid vector. Firstly, PCR products were produced and then gel purification was performed by using E.Z.N.A Gel Extraction Kit (OMEGA Bio-Tek Inc., Doraville, GA, USA) according to the manufacturer's protocol. The TA cloning reactions were prepared and transformed into *DH5α* competent cells. After that, 6-10 white or light blue colonies were picked for colony PCR using M13 primers (Supplemental Table S1) or miniprep.

5.14 DNA sequencing

Sequencing reactions (5 µl of plasmid DNA and 5 µl of 5 µm primer) were prepared for sequencing (GATC Biotech, Konstanz, Germany). Sequence alignments were done in Clone Manager 9 or by Multalin software (multalin.toulouse.inra.fr/multalin/).

5.15 Protoplast isolation and transformation

Intact tomato mesophyll protoplasts were isolated and transformed as previously described (Mishra et al., 2002). In short, leaves of 6-7 week-old tomato wild-type (*Solanum lycopersicum* cv. Moneymaker) or A1CS2 transgenic plants grown under sterile conditions, were gently sliced with a sterile surgical scalpel. The sliced leaves were incubated overnight in dark at room temperature in an enzyme solution (K3M-S solution (0.4 M mannitol, 24.7 mM KNO_3, 1.01 mM $MgSO_4 \times 7H_2O$, 1.09 mM $NaH_2PO_4 \times H_2O$, 1.01 mM $(NH4)_2SO_4$, 6.12 mM $CaCl_2 \times 2H_2O$, 0.56 mM m-inosid, 3 mM NH_4NO_3, 5 ml L^{-1} of 9.2 g L^{-1} $FeSO_4$EDTA [Sigma-Aldrich], 1×Trace elements [500 ml 100×stock: 37.5 mg KJ, 500mg $MnSO_4 \times H_2O$, 100 mg $ZnSO_4 \times H_2O$, 150 mg H_3BO_3, 12.5 mg $Na_2MoO_4 \times 2H_2O$, 1.25 mg $CoCl_2 \times 6H_2O$, 1.25 mg $CuSO_4$), 5.57 mM NES, 0.89 mM BA, 29.65 mM vitamin B_1, 4.86 mM vitamin B6, 8.12 mM nicotinamide, 5mM MES; PH5.7-5.8)] containing 0.25% cellulase and 0.1% macerozyme [Duchefa]. Following the separation of the protoplasts from the leaf tissue, the protoplast-containing solution was centrifuged at 470 rpm for 7 min at

room temperature to separate the cells from the debris. The upper 2/3 of centrifuged protoplasts was washed twice in W5 solution (125 mM $CaCl_2$, 154 mM NaCl, 0.54 mM KCl, 0.56 mM glucose, 0.5 mM MES, pH 5.6-5.8) with centrifugation for 10 min at 670 rmp and finally resuspended in K3M (K3M-S with 3% [w/v] sucrose) solution to a final concentration of 10^6 protoplasts per milliliter. For transformation, 10^5 protoplasts were mixed with 20 µg total plasmid DNA in a 2 ml tube and added the same amount of PEG solution (25% PEG 6000, 0.45 M mannitol, 0.1 M $Ca(NO_3)_2 \times 4H_2O$, pH 6.0 (KOH)) for 25 min. One milliliter of K3M was added to stop the reaction. Protoplasts containing transformed with plasmid DNA were incubated for the appropriate time based on the experiment at room temperature in the presence of light to allow the expression of the protein of interest. Protoplasts were harvested (centrifugation at maximum speed for 5 mins at 4°C and supernatant removal by aspiration).

Nuclear export was inhibited by Leptomycin B (LMB; final concentration 22 ng ml^{-1}), as described before (Heerklotz et al., 2001; Kudo et al., 1998; Port et al., 2004). LMB was added in protoplast samples 3 hours before harvesting.

5.16 Protein extraction

Frozen seedlings or leaf tissue (50-100 mg) was homogenized and subsequently was used for protein extraction. Protein extraction was done using the high salt buffer (Port et al., 2004) (20 mM Tris/HCl pH 7.8; 500 mM NaCl ; 25 mM KCl; 5 mM $MgCl_2$; 30 mM EDTA; 0.5% Nonidet-P40; 0.2% sarcosyl; 5% sucrose; 5% glycerol; 14.2 mM β-MCE; proteinase inhibitor cocktail: Pefabloc (in 10 mM Hepes pH7.5) [10 µg ml^{-1}]; Pepstain A (in ethanol or isopropanol) [1µg ml^{-1}]; leupeptin [2µg ml^{-1}]; aprotinin [2 µg ml^{-1}]; TLCK [50 µg ml^{-1}]; TPCK (in Ethanol) [20µg ml^{-1}]; benzamidine [150µg ml^{-1}]). The addition of high salt buffer in the homogenized tissue was followed by mild sonication (Bandelin Sonopuls HD70, amplitude 35%; cycle 20). For protein extraction from protoplast, pellets of 10^5 protoplasts were resuspended in 60µl high salt buffer and vortexed vigorously. Subsequently, the samples were centrifuged for 5 min at 14.000 rmp at 4°C. The supernatant was transferred into a new Eppendorf tube and mixed with an equal volume of 2×SDS loading buffer (160 mM Tris/HCl pH 6.8; 6.4% SDS; 0.32 g ml^{-1} glycerol; 400 mM DTT, bromophenol blue) and boiled for 5 minutes.

5.17 Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of were performed as described before (Hahn et al., 2011). Samples were separated by SDS-PAGE (Laemmli, 1970) and the percentage of which was chosen based on the size of the analyzed proteins. Equal amounts (10-20 µg) of protein were loaded onto a mini (8.6×6.7 cm) format SDS-PAGE gel along with molecular weight marker (Unstained Protein Molecular Weight Marker, Thermo). After protein separation by gel electrophoresis, SDS-gels were transferred to an

Amersham Protran 0.45 µm nitrocellulose blotting membrane (GE Healthcare), and protein transfer was achieved using the semidry blotting method (40 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol; 1mA cm^{-2} of transferred area for 75 minutes). After blotting the membrane was stained with Ponceau S (0.4% [w/v] Ponceau S, 3% [v/v] TCA, 1% [v/v] acetic acid). The large subunit of RubisCO was used to confirm equal protein loading across the samples. Blots were blocked in 5% [w/v] non-fat milk in PBS (140 mM NaCl, 2.6 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4) and incubated with the appropriate primary antibody (Supplemental Table S3) overnight at 4°C or 1-2 hours at room temperature. Secondary antibodies conjugated to horseradish peroxidase (Sigma-Aldrich) were used for detection of the primary antibody which was achieved by using enhanced chemiluminescence method (ECL kit, Perkin-Elmer Life Sciences).

5.18 β-Glucuronidase (GUS) activity reporter assay

Transient GUS reporter assays were performed with tomato mesophyll protoplasts to estimate the transcriptional activity of transcription factors on the Hsf-dependent pGmhsp17.3BCI::GUS reporter construct in a quantitative and fluorimetrical manner. GUS activity was measured by monitoring the cleavage of the β-glucuronidase substrate 4-methylumbelliferyl β-glucuronide (MUG) (Jefferson et al., 1987; Gallagher, 1992). When transcription factors with activator potential bind to the promoter harboring the reporter gene GUS, the β-glucuronidase is activated and cleaves the MUG to 4-methylumbelliferone (MU). Once the fluorescent moiety MU is free in solution, it has a peak excitation of 365 nm (UV) and a peak emission of 455 nm (blue) and therefore can be analyzed photo metrically (Gallagher, 1992). 50.000 protoplasts were co-transformed with 0.5 µg plasmid DNA carrying the appropriate Hsf expression construct and 1 µg GUS reporter plasmid DNA. Total DNA amount was adjusted to 10µg by adding pRT-Neo construct. After harvesting, 50 µl of GUS buffer containing β-mercaptoethanol (50mM $NaPO_4$, pH7.0; 10mM $EDTA(Na)_2$ pH 8.0; 0.1% N-Laurylsarcosine-Na-salt (v/v), 0.1% Triton X-100 (v/v); 14.3 mM fresh β-mercaptoethanol) was added to resuspend the protoplast pellets. Samples were vigorously vortexed and frozen in liquid nitrogen. Lysis of the protoplasts was achieved by 3 cycles of freeze-thaw and vigorous vortexing. Following lysis, the samples were centrifuged at maximum speed for 5 mins at 4°C. 25µl of supernatant was transferred to a microtiter plate with 96 wells on ice and mixed with 25 µl of MUG solution (0.44 mg ml^{-1} in GUS-buffer in dark). The 96-well plate was packed in aluminum foil and incubated at 37°C. Measurements were taken for several time points. MU-fluorescence was measured in the "Fluostar" fluorometer (BMG LabTechnologies GmbH) at 365nm excitation and 460nm emission wavelength. Background fluorescence was subtracted and values were calculated relative to samples transformed only with GUS reporter and Neo. Neo is a plasmid encoding for the Neomycin resistance gene, which is used as mock plasmid DNA. The rest of the triplicates were pooled and used for Western blot analysis.

5.19 Microscopy analysis

The intracellular localization GFP- and mCherry-fusion proteins expressed in tomato leaf mesophyll protoplasts was examined under a Leica SP5 confocal laser scanning microscopy (CLSM). GFP and mCherry were excited at 488nm and 561nm, respectively. Chlorophyll autofluorescence was excited together with GFP measurements. The emission of GFP was recorded at 490-548nm, of mCherry at 570-656nm and of chlorophyll at 665-735 nm. Crosstalk between different channels was excluded by sequential excitation.

5.20 MACE (Massive analysis of cDNA ends)

Total RNA isolated from young tomato (*cv. Moneymaker*) seedlings under both control and HSR (39°C and 45°C) using the E.Z.N.A Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer instructions. Massive analysis of cDNA ends (MACE, Zawada et al., 2014) analysis was performed as described previously (Fragkostefanakis et al., 2015). Only the results for SR protein-encoding genes are presented here. Bioinformatic analysis was done by Dr. Stefan Simm.

5.21 Gene numbers

Sequences of tomato Hsfs gene and SR protein genes can be found in the SGN database (https://solgenomics.net/). Gene IDs of these genes are given in Table 4. The IDs of HsfA2 orthologues, and selected housekeeping genes of Solanaceae species were shown in Table 5.

Table 4: *S. lycopersicum* Hsf, Hsp and SR protein genes used in the thesis.

Gene name	Accession number		
HsfA2	Solyc08g062960	RS29	Solyc01g096180
HsfA1a	Solyc08g005170	SC24	Solyc10g009330
HsfA1b	Solyc03g097120	RS40	Solyc03g026240
HsfA1c	Solyc08g076590	RS41	Solyc11g072340
HsfA3	Solyc09g009100	RSZ38	Solyc11g072340
HsfA4c	Solyc02g072000	RS2Z38	Solyc05g054920
HsfA6b	Solyc06g053960/50	SCL29	Solyc01g005820
HsfA7	Solyc09g065660	SC30a	Solyc04g074040
HsfB1	Solyc02g090820	SC30b	Solyc01g105140
HsfB2a	Solyc03g026020	SCL19	Solyc01g080660
Hsp17.7C-CI	Solyc06g076520	SR46	Solyc10g005590
Hsp101	Solyc03g115230	RS2Z36	Solyc09g005980
SR30	Solyc01g099810	Putative SR protein	Solyc03g093350
SR34	Solyc09g075090	SR28	Solyc06g009060
SR32	Solyc03g082380		

Table 5: Information on HsfA2 orthologues and housekeeping genes of Solanaceae species

Name	Species	Gene ID
*Pt*HsfA2	*Solanum tuberosum*	*PGSC0003DMG400008223*
*Ca*HsfA2	*Capsicum.annuum*	*CA08g05000*
*Nb*HsfA2	*Nicotiana benthamiana*	*Nb101Scf01777g02001.1*
PhHsfA2	*Petunia hybrida*	*Peaxi162Scf00327g00413*
*Sl*HsfA2	*Solanum lycopersicum*	*Solyc08g062960*
*Pt*EF1a	*Solanum tuberosum*	*PGSC0003DMG400008117*
*Ca*UBI3	*Capsicum annuum*	*AY486137.1*
*Nb*EF1a	*Nicotiana benthamiana*	*NbS00021090g0020.1*
*Ph*GAPDH	*Petunia hybrida*	*SGNU209515*
*Sl*EF1a	*Solanum lycopersicum*	*Solyc06g005060*
*Sp*EF1a	*Solanum peruvianum*	*Solyc06g005060*

6 Results

6.1 The role of HsfA2 in heat stress response and thermotolerance

HsfA2 is one of the best studied Hsfs in various species (Charng et al., 2007; Fragkostefanakis et al., 2016; Lämke et al., 2016; Liu & Charng, 2013). The use of *KO* mutants in *A. thaliana* has shown that HsfA2 is not involved in basal thermotolerance, but is important for acquired thermotolerance and maintenance of stress memory (Charng et al., 2007; Lämke et al., 2016). In *A. thaliana* the constitute expression of HsfA2 in the quadruple HsfA1a/b/d/e *KO* mutant was able to induce expression of Hsps and restore thermotolerance (Liu & Charng, 2013). The molecular details on the regulation of tomato HsfA2 during HSR have been very well described. However, little is known for the function of HsfA2 and its relevance for thermotolerance in tomato (*Solanum lycopersicum*).

The contribution of HsfA2 in BTT and ATT was examined using the hypocotyl elongation assay on 4-days-old dark-grown HsfA2 transgenic (*A2AS-3*, *-7* and *-15*) and wild type tomato seedlings. First the suppression of HsfA2 in the three transgenic lines was confirmed by qPCR analysis (Fig. 4). HsfA2 was suppressed in control, heat stressed (1 hour at 39°C) and recovery seedlings (1.5 hour at 25°C following HS). The levels of Hsp101 and Hsp17.7C-CI were also monitored by qPCR in the same samples, but showed no difference between the wild type and transgenic seedlings, suggesting that HsfA2 is not involved or is not essential for their induction in response to a direct stress treatment.

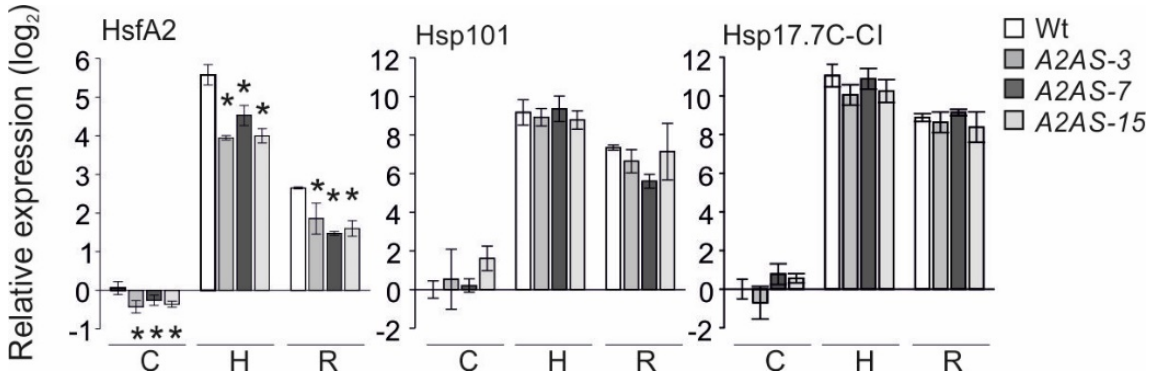

Figure 4. Confirmation of A2AS transgenic plants with reduced HsfA2 level by using qRT-PCR in seedlings.
Relative gene expression ($2^{-\Delta\Delta Ct}$) of HsfA2, Hsp17.7C-CI, and Hsp101 in wild-type and *A2AS* transgenic seedlings kept at 25˚C (control, sample C) or exposed to 1 h HS at 39˚C (sample H), which was followed by recovery for 1.5 h at 25˚C (sample R). The Ct value for each gene was normalized to Ct values for EF1a and UBI housekeeping genes and to expression in wild-type control. Vertical bars are the average ±SD of three replicates.

The seedlings of the transgenic lines were inspected for BTT and ATT and compared to wild type. For BTT, the seedlings were heat-stressed at 39, 42, or 45°C stress for 90 min or kept at

25^{o}C as control (Fig. 5A). After HS treatments, the hypocotyl elongation of HsfA2 transgenic seedlings were compared to that of the wild type. A moderate decrease in the hypocotyl elongation rate of the wild type and transgenic seedlings was observed after 90 min 39°C treatment, and this reduction was even stronger in seedlings exposed to 45°C (Fig. 5A). In all cases, the reduction in hypocotyl elongation was similar for wild type and transgenic seedlings suggesting that HsfA2 is not required for basal thermotolerance (Fig. 5A) which is in agreement with the similar expression levels of Hsp101 and Hsp17.7C-CI in all lines (Fig. 4).

The role of HsfA2 in short- and long-term ATT was also investigated. Four-days-old tomato seedlings grown in dark were pre-conditioned at 37.5°C for 90 min, allowed to recover for 3, 24 or 48 hours at 25°C, and then challenged by a severe HS, at 47.5°C for 90 min (Fig. 5B). Following the treatment the seedlings were allowed to recover at 25°C in dark for 5 days during which the hypocotyl length was recorded. The pretreatment (37.5°C) had no effect on hypocotyl elongation rate compared to the untreated seedlings (25^{o}C) for each line. Interestingly, all three transgenic lines showed reduced thermotolerance in the treatments with 3 or 24 hours recovery (Fig. 5B) but no difference with wild type for longer-term acquired thermotolerance (48 hours recovery, data not shown). Therefore, HsfA2 is important for acquisition of thermotolerance, which can be maintained at least for up to 24 hours from the pre-treatment. Instead, the loss of HsfA2 effect on ATT after 48 h recovery, marks the time limit for maintenance of the protection.

Based on an immunoblot analysis, the 37.5^{o}C pretreatment led to a strong accumulation of HsfA2 protein (Fig. 5C). More importantly, the HsfA2 protein was still detectable after 48 h of recovery in wild-type seedlings however at much lower levels than in the 24 hour recovery sample (Fig. 5C). After a direct and severe HS treatment at 47.5°C for 90 min, no accumulation of HsfA2 or Hsp17-Cl protein was detected in both wild-type and A2AS seedlings (Fig. 5C). Furthermore, a slight reduction on Hsp101 and a significant decrease of Hsp17-Cl protein levels were observed in the short- and long-term ATT assays.

The results of seedling thermotolerance from all three A2AS lines in both short- and long-termATT, show a correlation between the lower levels of HS-induced proteins (HsfA2, Hsf17-Cl and Hsp101) and the significant reduction on the growth rate compared to wild type (Figs. 5B and C). Collectively, the accumulation of HsfA2 by a mild HS is able to improve the ability of young seedlings to induce Hsp synthesis at higher temperatures and therefore to survive under otherwise lethal HS conditions.

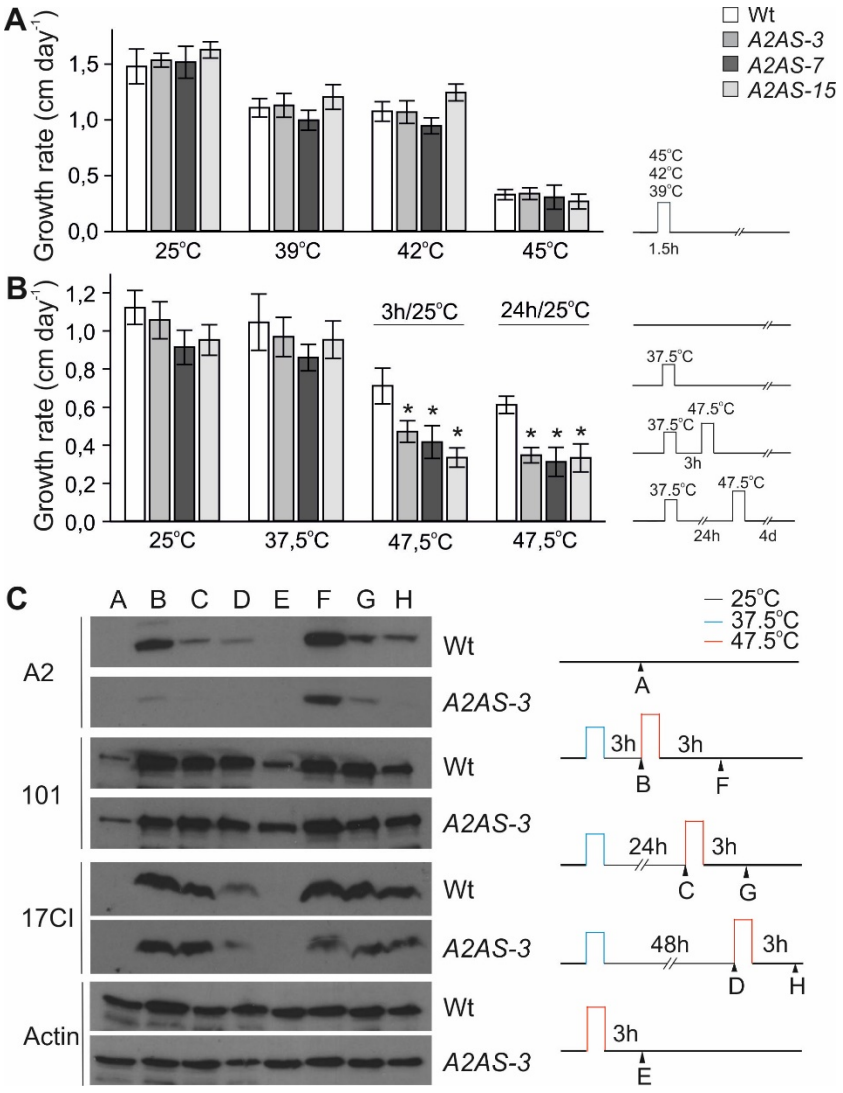

Figure 5. BTT and ATT of young seedlings of wild-type and HsfA2 (*A2AS*) transgenic plants.
(A) Four-day-old etiolated tomato wild-type, *A2AS-3, -7 and -15* seedlings were kept at 25°C or exposed to 39, 42 and 45°C for 90 min. After HS treatments hypocotyl length of each seedling was recorded for 5 days and then hypocotyl growth rate was calculated. (B) Same seedlings as in (A) were first conditioned at 37.5°C for 90 min, and then allowed to recover at 25°C for 3 h or 24 h. Following recovery, these seedlings were subjected to a severe HS at 47.5°C for 90 min. Hypocotyl length during the following 5 days of recovery in dark was measured every day and growth rate was calculated. (C) Immunoblot analysis of protein levels of HsfA2, Hsp101 and Hsp17Cl detected with corresponding antibodies. 4-d-old young seedlings of tomato wild-type and *A2AS-3* were kept at 25°C (Sample A) or treated with a moderate HS at 37.5°C for 90 min then allowed to recover at 25°C for 3 h, 24 h or 48 h (Samples B, C and D) or directly exposed to 47.5°C for 90 min and then harvested after 3 h recovery (Sample E). Following 3 h, 24 h and 48 h recovery, a severe HS at 47.5°C for 90 min was applied and then allowed to recover at 25°C for 3 h (Samples F, G and H). In each line, 20 µg of total protein was loaded. Protein level of actin was shown to ensure equal loading. Pictographs in the right in (A), (B) and (C) indicated the applied HS regimes. For (A) and (B), each data point represents the mean of at least 10 seedlings for each genotype and treatment. Error bars represent SE of the mean of at least 10 seedlings. For (B), asterisk denotes significant difference ($P< 0.05$) compared to the wild-type for a given sample or stress treatment as shown by pairwise t-test analysis.

Similar analysis was done using an HsfA2 overexpression *A2S* line. *A2S* seedlings showed increased levels of HsfA2 transcript and protein confirming the ectopic expression of the gene under stress conditions and after 1.5 hour recovery (Fig. 6A). *A2S* seedlings showed higher Hsp101 and Hsp17.7C-CI expression compared to wild type after a 39°C treatment as shown by qRT-PCR (Fig. 6A) and immunoblot analysis for Hsp101 (Fig. 6B). Interestingly, despite the OE of HsfA2 in A2S seedlings already in non-stress samples at the transcript level, no HsfA2 protein could be detected using an antibody recognizing the C-terminus of HsfA2 (Fig. 6B). The ectopic expression of HsfA2 led to enhanced BTT after a 42°C or 45°C treatment compared to wild type (Fig. 6C), suggesting that increased levels of HsfA2 improve the ability of seedlings to overcome an acute heat stress.

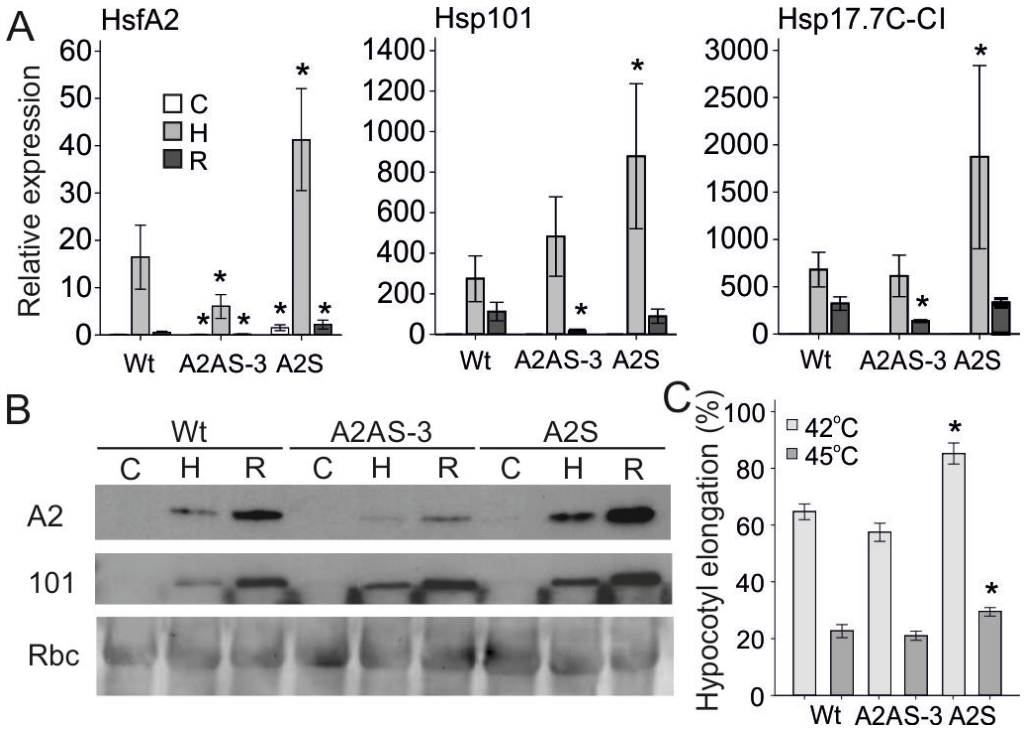

Figure 6. Thermotolerance of etiolated tomato seedlings of wild type and HsfA2 transgenic plants.

(A) Relative gene expression ($2^{-\Delta\Delta Ct}$) of HsfA2 (endogenous and transgene), Hsp17.7C-CI and Hsp101 in wild type and transgenic tomato seedlings (control, sample C) exposed to 1 hour heat stress at 39°C (H) followed by recovery for 1.5 hours (R). The Ct value for each gene was normalized to Ct values for EF1α and UBI housekeeping genes and to expression in wild type control. Vertical bars are the average ± SE of three replicates. (B) Equal amounts (20 µg) of total protein from seedlings treated as in (A) were probed with antibodies specific for HsfA2 (α-pep 6) and Hsp101, respectively. Rbc, Ponceau staining of Rubisco large subunit. (C) Four-day-old etiolated tomato wild type, *A2AS-3* and *A2S* seedlings were exposed to 42°C or 45°C for 90 min and hypocotyl length was measured every 24 hours for 4 days after the treatment. Values for hypocotyl length obtained for each genotype were normalized to the hypocotyl growth of seedlings at control conditions (25°C) to the same time. Each data point is the mean ± SE of three independent experiments with at least 10 seedlings each. Different letters at each point denote significant differences between means according to Duncan's multiple-range test at the 0.05 level.

6.2 Alternative splicing of HsfA2 pre-mRNA

6.2.1 HsfA2 undergoes alternative splicing in a temperature-dependent manner

Similar to other Hsfs, HsfA2 locus has been annotated with a single intron located within the conserved DBD-coding region (Scharf et al., 2012). HsfA2 modular structure is comprised of a N-terminal DBD, followed by the OD, a NLS, two short peptide motifs corresponding to activator domains and the C-terminal NES.

In order to investigate whether HsfA2 is alternatively spliced, RT-PCR analysis was employed using an exon 1-specific forward primer and a reverse primer that anneals to the 3´-untranslated region (UTR). Transcript analysis in cDNA samples derived from RNA isolated from young tomato seedlings exposed to temperatures ranging from 25 to 50°C for 1 hour revealed six transcripts corresponding to HsfA2 splice variants as confirmed by DNA sequencing (Fig. 7A). An additional transcript was detected in seedlings recovering from a 42.5°C stress (Fig. 8C). Generally, HsfA2 shows a significant induction in transcription level after a 30°C treatment, which peaks at 40°C (Fig. 7A). Further temperature increases cause suppression of expression which is completely abolished at 50°C. Interestingly, the levels of the splice variants show remarkable shifts during the different temperature treatments indicating that AS of HsfA2 is sensitive to temperature changes as small as 2.5°C.

All PCR products corresponding to HsfA2 transcripts were sequenced and models for putative protein isoforms were generated. The three lower molecular weight transcripts are generated due to AS of a second, newly identified intron (Fig. 7B). The 5´-splice site (5´-SS) of intron 2 lies within the second C-terminal activator motif (AHA2) and the 3´-SS in the UTR. The spliced variants were classified into 3 groups based on the corresponding deduced amino acid sequences. Groups I and II were generated due to AS of HsfA2 in intron 2 while group III due to AS in intron 1.

Intron retention of alternative 5’-donor selection site within the second intron downstream of the translation termination codon leads to the generation of three transcripts possessing variable 3’-UTR. These three transcripts designated in group HsfA2-I, are predicted to encode for the full length HsfA2 protein (Fig. 7B). They are generated either by intron 2 retention (HsfA2-Iα) or partial splicing due to selection of cryptic donor sites (HsfA2-Iβ and HsfA2-Iγ). The mRNA of these isoforms varies in abundance in response to different temperature treatments (Fig. 7A). At all samples examined, HsfA2-Iα and HsfA2-Iγ transcripts are much more abundant than HsfA2-Iβ, indicating that HsfA2-Iα and HsfA2-Iγ are the main contributors for HsfA2 protein synthesis under heat stress. Interestingly, HsfA2-Iγ is the major HsfA2-I variant in temperatures ranging from 30-37.5°C while HsfA2-Iα is more abundant at temperatures between 40 and 47.5°C.

The splicing of intron 2 leads to the generation of a truncated HsfA2 protein which lacks the NES domain and five of the 9 amino acids comprising AHA2 (Figs. 7B and C). HsfA2-II was detected in young tomato seedlings exposed to 30 to 37.5°C treatment for 1 hour and showed similar abundance to HsfA2-Iγ (Fig. 7A). The existence of the putative protein isoform was confirmed by immunodetection using a polyclonal antibody generated against recombinant HsfA2 (Fig.7D) (Scharf et al., 1998). The two isoforms were present in seedlings exposed to 37.5°C treatment for 1 hour and were both induced when heat stress was prolonged for an additional 1 hour. Both isoforms were detected in both 1 and 2 hours recovery after 1 hour 37.5°C HS. Interestingly, HsfA2-I levels were higher than HsfA2-II suggesting that additional control mechanisms following downstream of pre-mRNA splicing must exist.

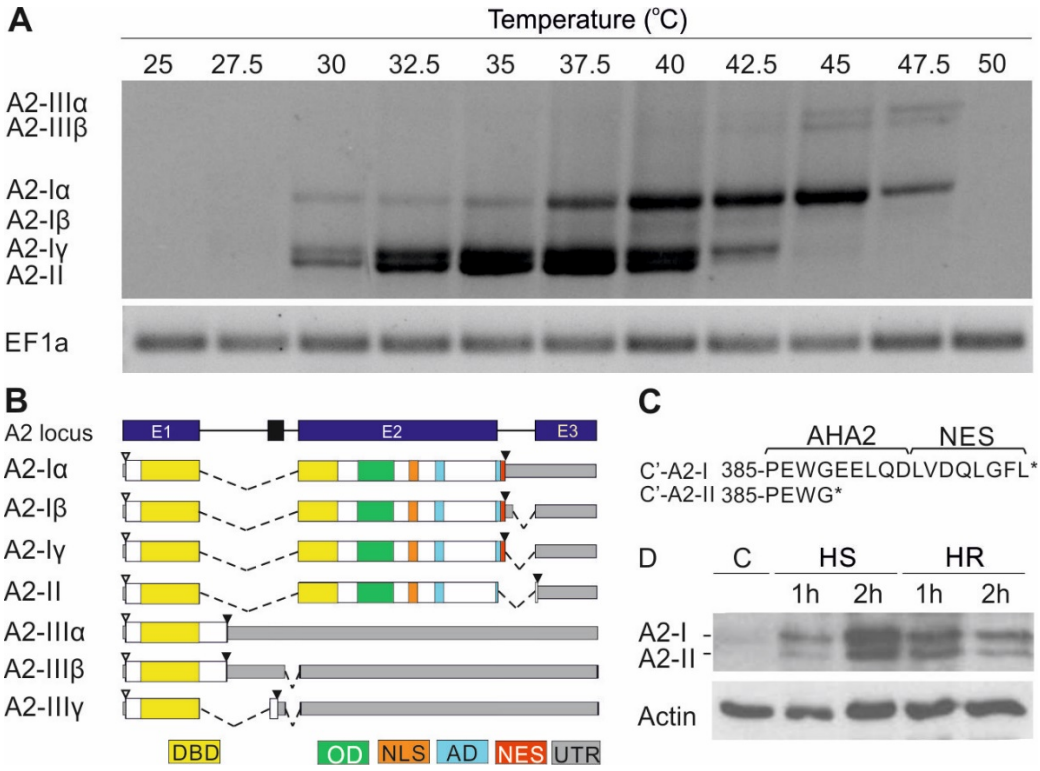

Figure 7. Temperature-dependent pre-mRNA splicing of tomato HsfA2.
(A) Identification of tomato HsfA2 splicing variants in tomato seedlings via RT-PCR exposure to the indicated temperatures for 1 h. The isoform HsfA2-IIIγ is generated only after prolonged HS (>3 h) and therefore not shown here. (B) Domain structure of seven HsfA2 isoforms. White arrowheads indicate the positions of start codons, while black arrowheads indicate the .positions of stop codons. White and gray boxes highlight coding sequences and 3´-UTR, respectively. Black box indicates mini-exon, blue boxes indicate exons. Black lines show introns. DBD: oligomerization domain, NLS and NES: nuclear localization and export signal, DBD: DNA-binding domain, AHA: activator motif. (C) Alignment of C-termini of tomato HsfA2-I and HsfA2-II protein sequences. (D) Immunoblot analysis of protein extract from seedlings treated at 37.5°C for indicated times and then allowed to recover at 25°C for 1 and 2 h from 1 h HS, respectively.

HsfA2-III transcripts appear in seedlings exposed to a severe heat stress (>45°C) or during recovery from a strong stress (42.5°C) (Figs 7A and 9C). They are generated due to intron 1 retention (HsfA2-IIIα), alternative selection of cryptic donor site (HsfA2-IIIβ) or inclusion of an 82nt long mini-exon (HsfA2-IIIγ) (Fig. 7B). All HsfA2-III variants are predicted to possess a PTC and a long 3'-UTR, indicating that they are subjected to degradation by the NMD pathway as shown for *A. thaliana* HsfA2-II (Liu et al., 2013). A putative protein isoform would contain only a truncated DBD with the essential helix 2-turn-helix 3 required for binding to HSEs (Fig. 8A). In contrast to Arabidopsis HsfA2-III, none of the HsfA2-III transcripts is predicted to encode for a protein with a leucine-rich carboxyl tail, although tomato HsfA2-III putative proteins are rich in hydrophobic amino acids (Liu et al. 2013; Fig. 8A).

NMD is a conserved RNA quality control system in eukaryotes that degrades mRNAs with a premature termination codon. Activation of NMD requires translation and can therefore be suppressed by translation inhibitors as cycloheximide (CHX) (Ramos et al., 2001). To test whether HsfA2-III transcripts are subject to NMD, the total HsfA2 and HsfA2-III RNAs level were determined by qRT-PCR using specific primers in seedlings treated with the CHX and compared to control (ddH_2O-treated) samples. Prior to addition of CHX or ddH_2O the seedlings were exposed to 45^{o}C to induce the accumulation of HsfA2-III transcripts. NMD inhibition resulted in an approximately 2-fold increase in the levels of HsfA2-III transcripts compared to the water-treated samples suggesting that these transcripts are targets for NMD (Fig. 8C). Such a clearance mechanism can contribute to the attenuation of HSR under severe conditions.

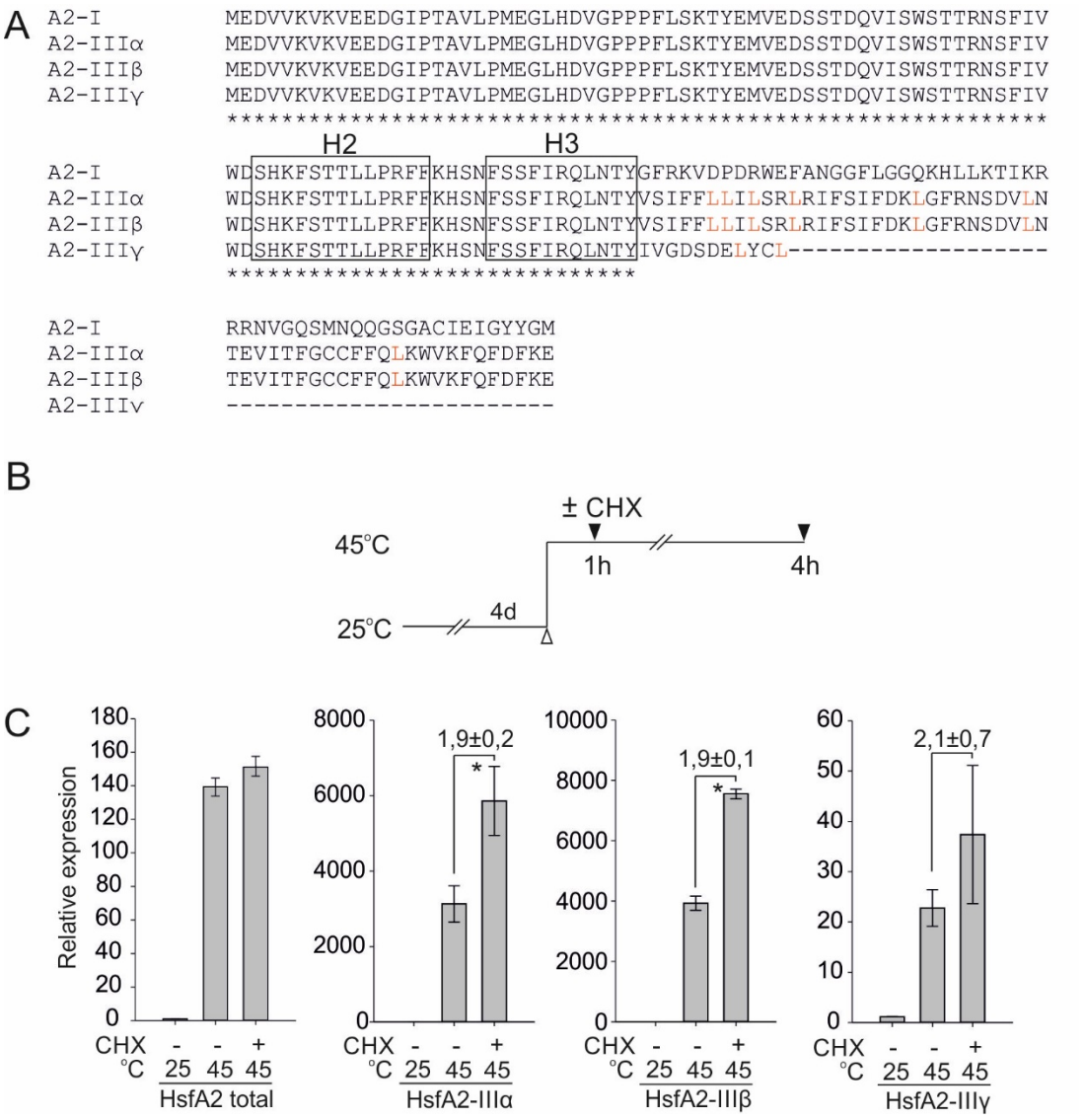

Figure 8. HsfA2-III isoforms are subjected to NMD.
(A) Alignment of putative amino acid sequences of tomato HsfA2-I and HsfA2-III isoforms. The boxed regions corresponding to the central helix-turn-helix motif (H2-T-H3). The amino acid leucine (L) at the leucine-rich motif of HsfA2-III isoforms was marked in red color. (B) and (C) Expression level of HsfA2-III transcripts were exampled by qRT-PCR. The data expressed as the values relative to the expression of EF1a; are the mean (±SD) of three biological and three technical replicates. Four day-old seedlings treated with the translation and NMD-inhibitor CHX (100µg/ml) or the same volume of ddH_2O as a control after one hour heat stress at 45°C. Asterisk indicates significant differences ($p<0.05$). The ratio between the treated and untreated samples is indicated.

The levels of HsfA2 transcript variants were further examined in seedlings exposed to long term heat stress conditions or prolonged recovery after a short, one hour heat stress treatment. For this, 7-day-old tomato seedlings were exposed to 37.5°C or 42.5°C for 1 to 4 h or for 1 h heat stress then allowed to recover at 25°C for 0.5 to 3 h. Samples were collected at designed time points (Fig. 9A, black arrowheads, white arrowheads and gray arrowheads). After RNA isolation

and cDNA synthesis, RT-PCR analysis was performed. Interestingly, each splicing variant has distinct expression profiles under different conditions.

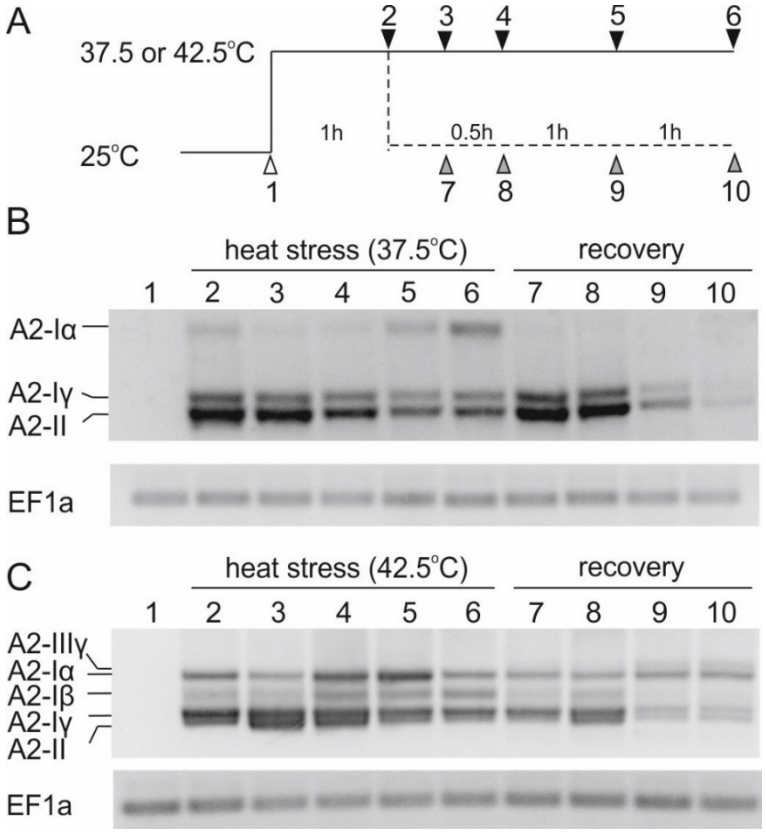

Figure 9. Splicing pattern of HsfA2 under long term heat stress and recovery conditions.

(A)The different regimes for HS treatment or recovery of seedlings are shown (Black line: shows long term HS treatment while gray line shows recovery after 1 hour HS). Black arrowheads indicate the time points of HS-treated sample harvesting, gray arrowheads indicate the time points of recovery sample harvesting, and white arrowhead indicates harvesting of control sample. (B) and (C) Young tomato seedlings exposed to 37.5 or 42.5°C for the time indicated (lines 2-6). Some of seedlings were recovered at 25°C for the indicated time points (lines 7-10) after 1 hour HS. The SolycEF1a gene served as an internal control.

HsfA2-Iα was gradually induced with the duration of heat stress treatment at both 37.5°C and 42.5°C (Fig. 9B and C). HsfA2-Iα was rapidly diminished when seedlings were returned to room temperature after 1 h exposure to 37.5°C (Fig. 9B) while it remained at constant levels during the recovery from 42.5°C stress (Fig. 9C). Unlike HsfA2-Iα, HsfA2-Iγ was gradually reduced during prolonged stress or recovery (Fig. 9B and C). Interestingly, HsfA2-II levels changed relatively similar to HsfA2-Iγ under these conditions (Figs. 9B and C). Furthermore, HsfA2-Iβ was detected after 1 h heat stress at 42.5°C and its expression was induced from 1 to 4 hours of stress and declined rapidly after 1 hour of recovery. Interestingly, HsfA2-Iβ was not detected under heat stress at 37.5°C (Fig. 9B and C). It is tempting to speculate that the diversity of expression profiles of HsfA2 splicing variants also results from differences in their stabilities under specific conditions, however this needs further experimental evidence.

6.2.2 Functional similarities and discrepancies between HsfA2 protein isoforms

6.2.2.1 Intracellular distribution

According to the prediction of amino acid sequence, HsfA2-II isoform lacks the entire NES motif and five out of nine amino acids of the AHA2 (Fig. 7B). The NES determines the intracellular distribution of Hsfs and the AHA motifs play a vital role in activation of transcription of HS-genes. HsfA2-I is a nucleocytoplasmic shuttling protein, requiring interaction with HsfA1a for efficient nuclear retention (Chan-Schaminet et al., 2009; Döring et al., 2000; Heerklotz et al., 2001; Scharf et al., 1998). To examine possible functional discrepancies between the two isoforms, the intracellular localization of these two isoforms fused to GFP were observed by CLSM.

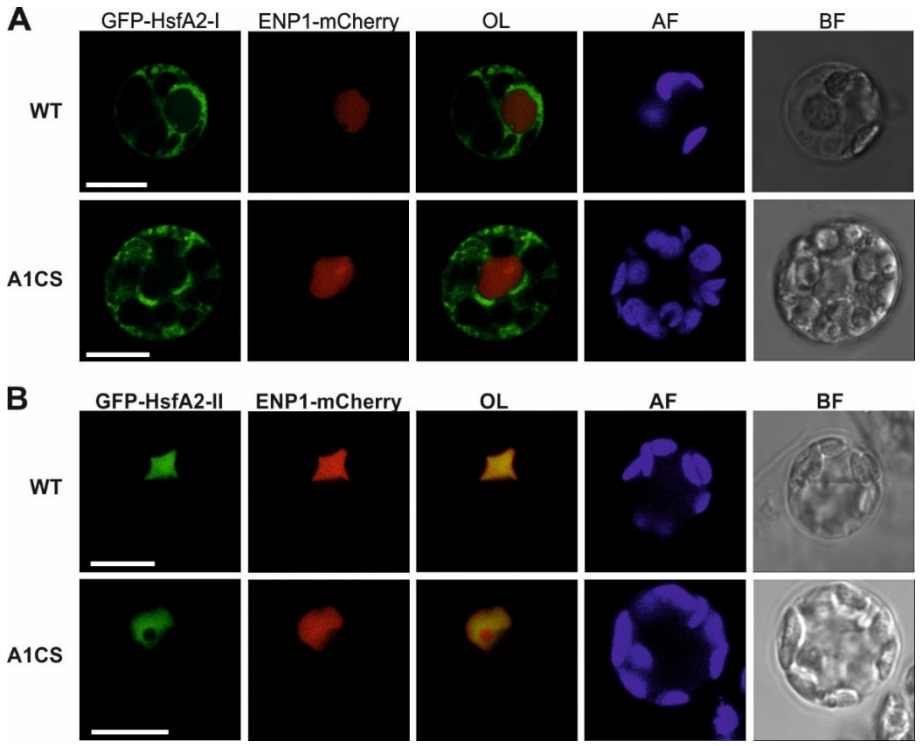

Figure 10. Localization of HsfA2 isoforms in tomato mesophyll protoplasts.
(A) Localization of N-terminally fused HsfA2-I with GFP in protoplasts from wild type and A1CS plants. Nuclear protein ENP1 used as a marker. (B) Protoplasts from WT and A1CS plants were transformed with equal amounts of Enp1-mCherry and GFP fused HsfA2-II. After 7 hours expression, GFP and mCherry fluorescence was analyzed by CLSM. OL=overlay, AF=autofluoresence, BF=brightfield. Scale bars indicate a length of 10 µm.

GFP-HsfA2-I and GFP-HsfA2-II fusion proteins were expressed in tomato mesophyll protoplast cells of wild type and HsfA1a knockdown transgenic plants (A1CS2, HsfA1a co-suppression; Mishra et al., 2002). The latter, allowed the investigation of the intracellular distribution of the two isoforms in the absence of the master regulator HsfA1a. GFP-HsfA2-I fusion protein showed a predominant cytosolic localization in both wild type and A1CS protoplasts (Fig. 10A) which can

be attributed to the presence of the NES which facilitates the efficient export from the nucleus (Fig. 10A) as suggested in previous reports (Heerklotz et al., 2001; Scharf et al., 1998). In contrast, due to the absence of the NES, GFP-HsfA2-II fusion was localized in the nucleus of protoplasts of both wild type and A1CS leaves (Fig. 10B), suggesting that this isoform can be efficiently retained in the nucleus in the absence of HsfA1a (Fig. 10A and B).

HsfA2-I oligomerization leads to the generation of trimeric homo-oligomeric complexes (Chan-Schaminet et al. 2009). As the two protein isoforms co-exist in stressed cells (Fig. 7D) we asked whether their nucleocytoplasmic distribution is affected in the presence of both. For this, mCherry was fused to the N-terminus of HsfA2-I isoform and the protein was expressed in protoplasts along with GFP-A2-II. Microscopic analysis revealed that the intracellular distribution of the two isoforms remained cytosolic and nuclear for HsfA2-I and II, respectively (Fig. 11) suggesting that probably the two proteins do not interact.

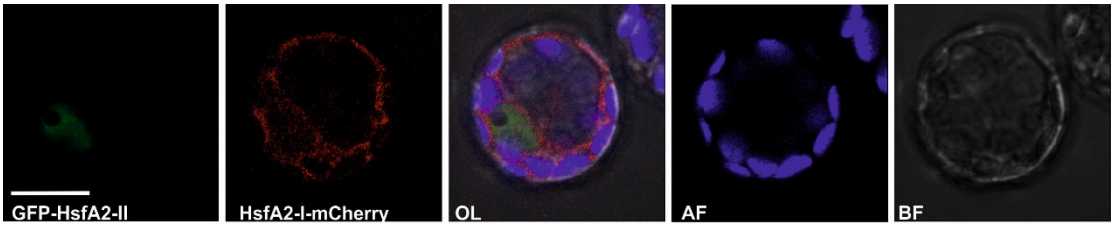

Figure 11. HsfA2 isoforms do not influence each other's subcellular localization.
Protoplasts were transformed with equal amounts of plasmid DNA encoding GFP-HsfA2-II and HsfA2-I-mCherry. Fluorescence was analyzed after 7-8 hours. Scale bars indicate a length of 10 µm.

6.2.2.2 Differences in the activity

Next, the activity of the two isoforms was investigated because the differences in intracellular distribution (Fig. 10) and the shorter AHA2 motif might influence the activity of HsfA2-II. The AHA2 is important for the synergistic activity between HsfA2-I and HsfA1a, as mutations of tryptophan and leucine residues in AHA2 abolish this function suggesting that AHA2 is essential for stimulation of transcriptional activity (Chan-Schaminet et al., 2009). The truncated HsfA2-II lacks the leucine residue, in this direction, the activity of the two isoforms was measured in transient reporter assays in tomato mesophyll protoplasts utilizing the Hsf-dependent *pGmhsp17.3BCI*::GUS reporter construct (Treuter et al., 1993). GUS activity was examined upon expression of the HsfA2 isoforms alone or in combination with HsfA1a (Fig. 12).
The activity of A2-II with partial AHA2 was approximately 3-fold higher compared to A2-I. To examine whether this difference is due to the difference in nuclear retention of the two isoforms, protoplasts expressing HsfA2-I or HsfA2-II were treated with the nuclear export inhibitor LMB. The addition of LMB resulted in similar activity between the two isoforms (Fig.

12) suggesting that the stronger activity of HsfA2-II can be attributed to its dominant nuclear localization. Moreover, HsfA2-I and HsfA2-II were able to enhance GUS activity to similar levels in the presence of HsfA1a, indicating that both isoforms can cooperate with HsfA1a, which leads to the stimulation of strong HSR. In agreement with this, the endogenous Hsp101 was induced at similar levels in cells co-expressing HsfA1a with either HsfA2-I or HsfA2-II (Fig. 12).

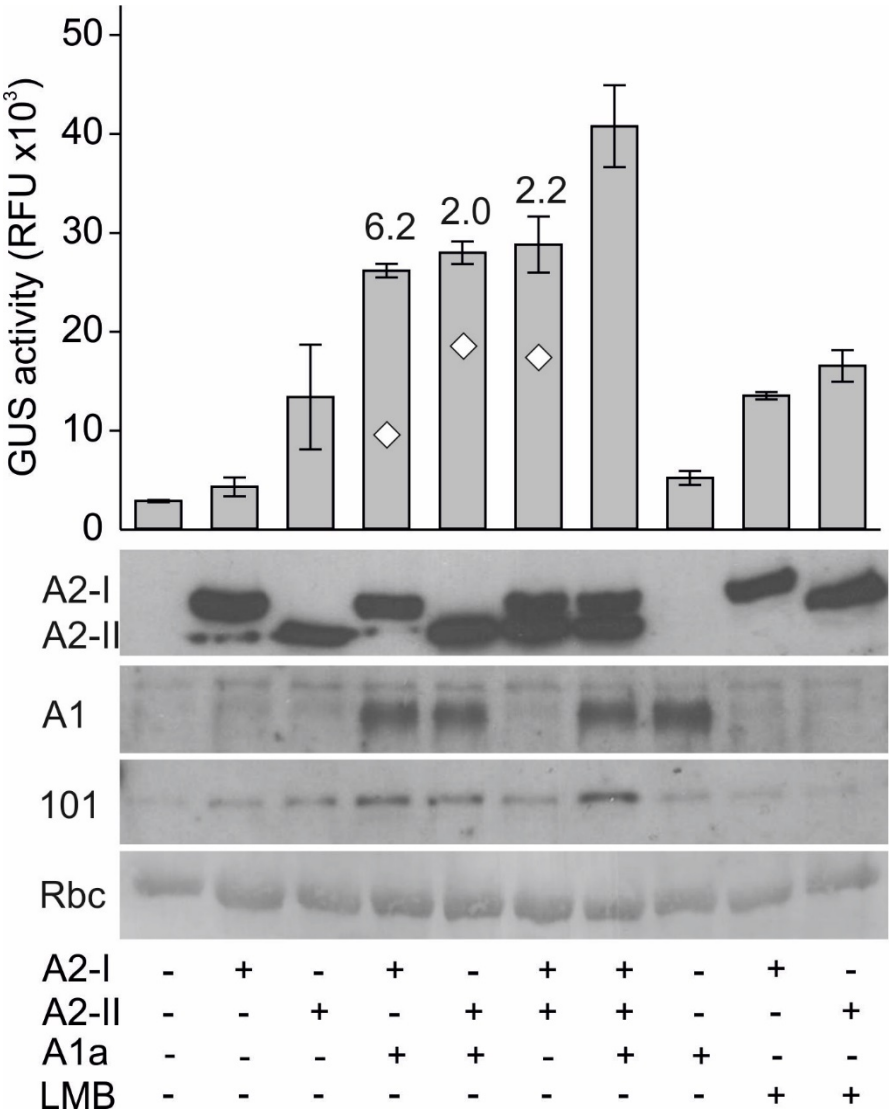

Figure 12. The activity of HsfA2-I or HsfA2-II.

Hsf activity was determined by expression of Hsf-dependent GUS reporter construct in tomato protoplasts. LMB was added 3 hours prior to harvesting. GUS reporter activities are presented as bars and diamonds indicate the ratio of GUS activities contributed by additive effects. The synergism of GUS activity for the corresponding Hsf combinations is given as numbers and was calculated as described previously (Chan-Schaminet et al., 2009). Expression controls for the transformed Hsf constructs determined by immunoblot analysis are shown. Error bars represent the SD of three independent experiments.

6.2.2.3 Protein stability and regulation of HsfA2 by Hsp17-CII

Based on the expression analysis of HsfA2 at different conditions, we observed that the transcriptional levels of HsfA2-II were almost equal to the levels of A2-I-encoding transcripts but the protein level of A2-II was significantly lower compared to A2-I (Fig. 7A and D). The inconsistency of expression profiles of these two isoforms raised the question, whether they have distinct protein stability. To examine this possibility, tomato protoplasts were transformed with equal amounts of either HsfA2-I or HsfA2-II expression plasmids. After 4 hours of expression CHX was added at a final concentration of 20 µg ml^{-1} (Röth et al., 2016). Samples were taken for analysis from 1 to 6 hours after the addition of CHX. The protein levels of the two isoforms were assessed by Western blot analysis and signal quantification using Image J after background subtraction (Abràmoff et al., 2004) and presented as relative to the point of translation arrest (t0). The two protein isoforms show remarkable difference in their turnover at steady state levels. HsfA2-I remained stable during the 6 hours of the treatment, whereas the level of HsfA2-II was reduced by approximately 35% within 1 hour and 60% within 2 hours of CHX treatment, having a half-life of 1.5 hour (Fig. 13A). The low protein stability for HsfA2-II indicates that this isoform does not play a role in long term acquired thermotolerance but might be involved in short term responses.

During heat stress, the functional state and intracellular distribution of tomato HsfA2 is influenced by a network of proteins including HsfA1a, Hsp17-CI, and Hsp17-Cll. Among them, Hsp17.4-CII has been identified as specific interaction partner of HsfA2, which results in the formation of insoluble cytosolic aggregates, called HSGs. The repressor function of Hsp17-CII on HsfA2 is dependent on the interaction with the C-terminal AHA2 of HsfA2 (Port et al., 2004). To investigate the influence of Hsp17.4-CII on the activation potential of HsfA2, we employed reporter assays in tomato protoplasts and co-expressed HsfA2 in presence of increasing amounts of Hsp17.4-CII-encoding plasmid (Fig. 13B). The activity of HsfA2-I was dramatically reduced in the presence of low levels of Hsp17.4-CII, while the activator potential of HsfA2-II was not influenced by the low amounts of Hsp17.4-CII. High levels of Hsp17.4-CII (2µg) completely suppressed the activity of HsfA2-I, but caused a 70% reduction in the activity of HsfA2-II. These results suggest that HsfA2-II can partially escape the repression control of Hsp17.4-CII. To further confirm this notion, we performed localization studies using GFP fusions of the two isoforms in the presence of mCherry-Hsp17.4-CII in protoplasts. As shown previously, in cells co-expressing HsfA2 and Hsp17.4-CII, both proteins were detected in cytosolic aggregates (Port et al., 20014). In contrast, in the presence of Hsp17.4-CII, a major fraction of GFP-HsfA2-II was able to translocate to the nucleus in even the absence of HsfA1a while the rest was found in more diffused perinuclear localization containing also Hsp17.4-CII (Fig. 13C).

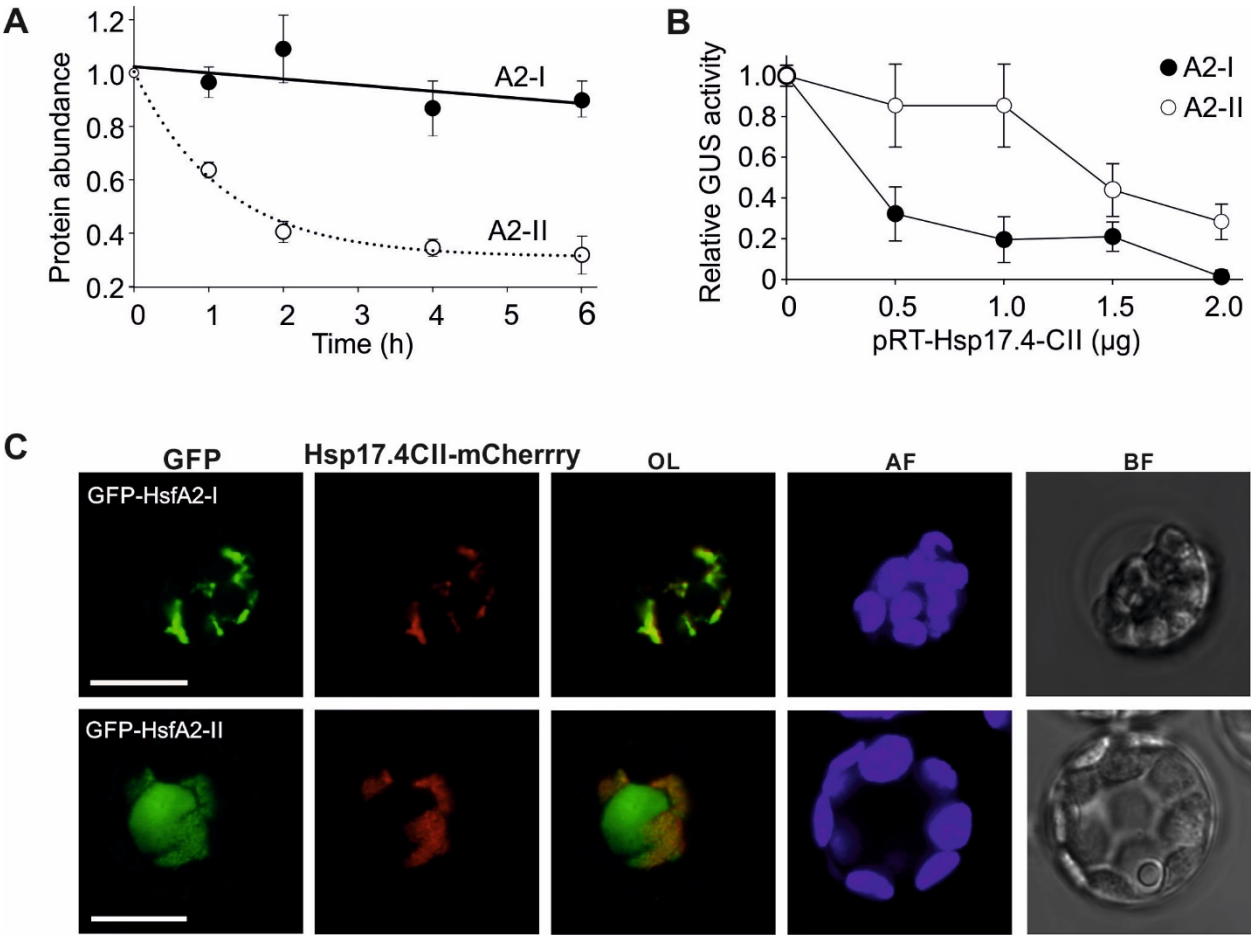

Figure 13. Protein stability HsfA2 isoforms and their interaction with Hsp17.4-CII.
(A) Protoplasts were transformed with 4 µg GFP-HsfA2-I or GFP-HsfA2-II plasmid DNA. After 4 hours protoplasts were supplemented with 20µg ml^{-1} cycloheximide. Samples were harvested at the indicated time points and subjected to SDS-PAGE and western blot analysis. Bands were quantified using ImageJ and signal intensities relative to T0 were calculated. (B) Activity of HsfA2 isoforms in the presence of different amounts of Hap17.4CII. The transcriptional activity of the two isoforms was monitored by expression of Hsf-dependent GUS reporter construct (PGmhsp17. 3B-CI::GUS) in tomato protoplast. Co-expression of the indicated amounts of Hsp17.4-CII plasmid and 2µg HsfA2-I (black circle) or HsfA2-II (gray circle) constructs, respectively. The GUS activity was measured in tomato protoplasts harvest around 7 hours after transformation. All measurements were done in triplicate. (C) Co-expression of HsfA2-I with equal amounts of Hsp17.4-CII-mCherry leads to their accumulation in HSG, while the majority of HsfA2-II can still translocate to the nucleus. Co-expression of GFP-HsfA2-I or GFP-HsfA2-II with equal amounts of Hsp17.4CII-mCherry in tomato mesophyll protoplasts from WT plants. After 6 hours expression the intracellular distribution of GFP and mCherry signal was analyzed by CLSM. Scale bars indicate a length of 10 µm.

Collectively, the functional comparison of the two isoforms indicate that efficient splicing of intron 2 at temperatures ranging from 32.5°C to 37.5°C results in the generation of HsfA2-II, which can immediately contribute to the strong accumulation of HS-induced genes. In turn, alternative splicing of intron 2 or full retention leads to the synthesis of HsfA2-I that is stored in HSGs during heat stress. During recovery HsfA2-I is released from HSGs but is maintained inactive in soluble complexes with class CI and CII sHsps until repeated cycles of heat stress

force again the interaction with HsfA1a to activate again the expression of HS-responsible genes, and enhance ATT (Fig. 14). To the best of our knowledge such functional diversification for a single Hsf by HS has not been previously reported.

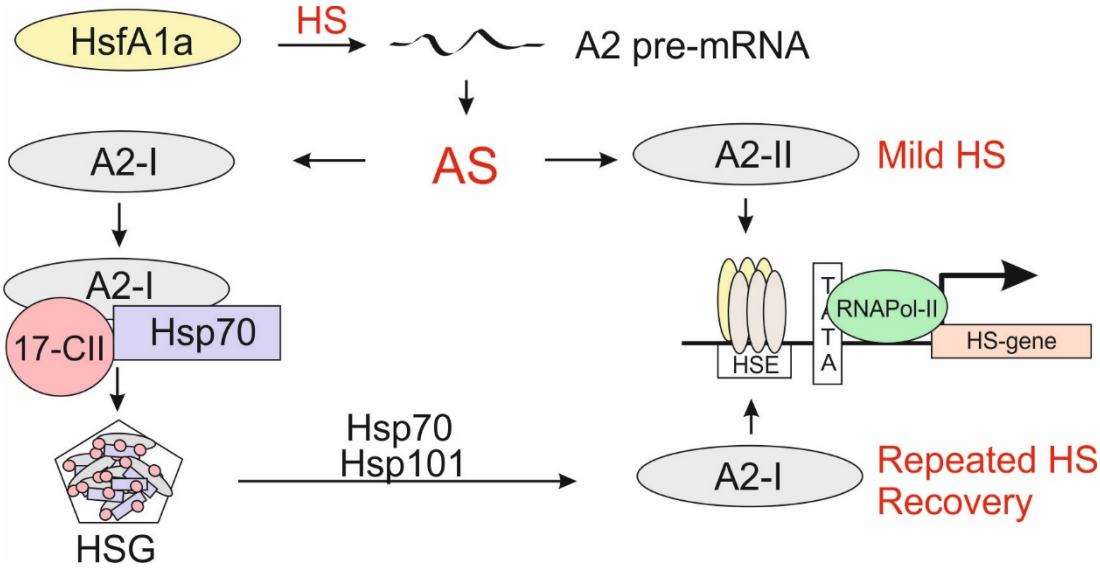

Figure 14. Proposed model for the contribution of two HsfA2 isoforms in HSR.
Under specific heat stress conditions AS of tomato HsfA2 allows to synthesize two isoforms (HsfA2-I and HsfA2-II). HsfA2-II localizes in the nuclear and can be immediately used to increase the activity of HS-induced genes, while HsfA2-I is stored in cytosolic HSGs in the presence of Hsp17.4-CII and is re-solubilized during recovery phase.

6.2.3 Intronic *cis*-elements determine the splicing efficiency of HsfA2

AS has been previously shown for HsfA2 intron 1 in *Arabidopsis thaliana* (Liu et al., 2013; Sugio et al., 2009), and rice OsHsfA2a (Wang et al., 2013) and OsHsfA2d (Cheng et al., 2015), but intron 2 has not been reported so far for any plant species to the best of our knowledge.

We asked whether this is a tomato specific event or HsfA2 orthologues in other Solanaceae species also undergo similar pre-mRNA regulation. In this direction, alternative splicing of HsfA2 was examined in potato (*S. tuberosum*), pepper (*Capsicum annuum*), tobacco (*Nicotiana benthamiana*), petunia (*Petunia hybrida*) and the wild tomato relative *Solanum peruvianum* young leaves harvested from three week old plants which were treated with 1 hour heat stress at 37.5°C or 42.5°C. Following RNA isolation and cDNA synthesis, HsfA2 splicing was evaluated by semi-quantitative RT-PCR using specific primers annealing to the exon 2 and the 3'-UTR (Supplemental table S1). Indeed, we found that HsfA2 in these species possess a second intron and undergoes alternative splicing in this intron 2 similar to the cultivated tomato (Fig. 15). Interestingly, with the exception of petunia, all other Solanaceae species had enhanced alternative splicing efficiency and generation of HsfA2-II transcripts in leaves exposed to 37.5°C

for 1 hour (Fig. 15). *S. peruvianum*, potato, tobacco and petunia had two isoforms at 37.5°C, while pepper produces 3 transcripts as shown for *S. lycopersicum* cv Moneymaker. In turn, a predominant intron retention was observed in leaves exposed to 1 hour heat stress at 42.5°C, (Fig. 15). In all cases, the deduced amino acid sequence of the putative isoforms yields two proteins, A2-I possessing an NES and A2-II with no NES, suggesting that AS of HsfA2 is a highly conserved mechanism within the Solanaceae family.

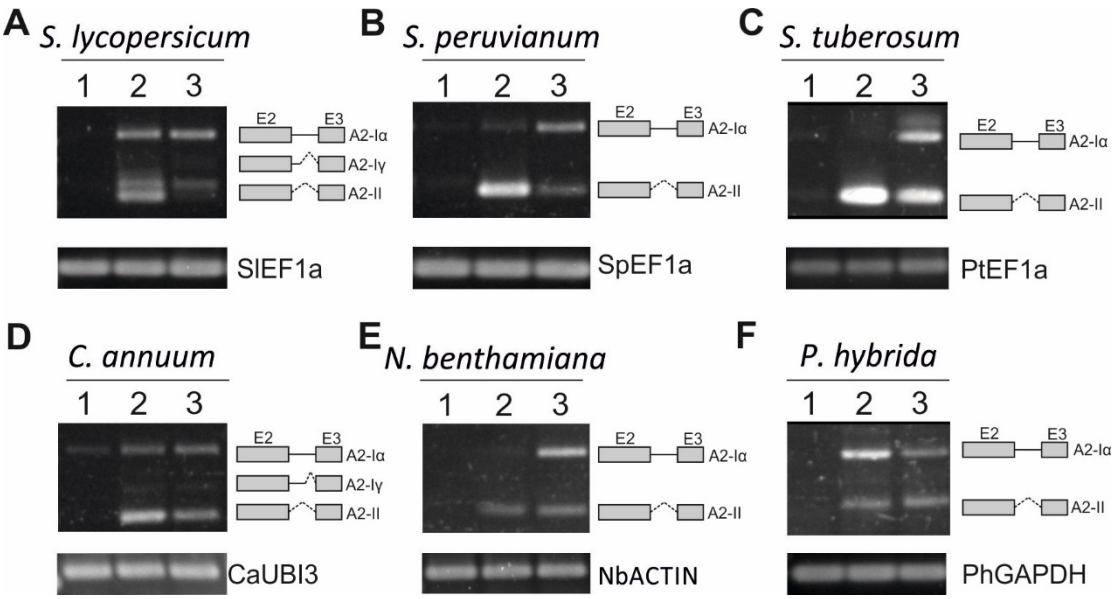

Figure 15. Alternative inclusion of intron 2 of HsfA2 is evolutionarily conserved among different Solanaceae species.

Splicing analysis of intron 2 of HsfA2 in Solanaceae family. Detached young leaves from 3-week-old plants of the indicated species were treated at 25°C (1), 37.5°C (2) or 42.5°C (3) for 1 h and then subjected to RT-PCR using an exon 2-specific forward primer and an exon 3-specific reverse primer. The schematic diagram at the right shows alternatively spliced mRNA isoforms. Gray boxes represent exons and black lines represent introns.

A closer inspection of the 37.5°C samples revealed interesting differences between *S. lycopersicum* HsfA2 (SolycHsfA2) and *S. peruvianum* (SoperHsfA2). SoperHsfA2 lacks the isoform A2-Iγ and shows significantly higher splicing efficiency and enhanced levels of HsfA2-II which accounts for more than 90% of the total HsfA2 transcripts at 37.5°C (Fig. 15A and B). The difference in splicing of the two orthologues might be due to the activity of trans-acting factors, e.g. components of the spliceosome machinery, or due to the presence of specific polymorphisms in *cis*-elements which might influence the splicing pattern and efficiency.

To address these questions, a minigene splicing assay was established. A minigene is consisted of a minimal gDNA fragment of a gene, having all sequence elements required for intron splicing and that is fused to a reporter gene, which in this case was GFP (Fig. 16A). The latter allows the

detection of minigene-specific transcripts using a GFP-specific primer while discriminating the effect on the endogenous HsfA2 gene. The segment of SolycHsfA2 or SoperHsfA2 is consisted of the 3´-end of exon 2 (184 nt), intron 2 (298 nt) and 135 nt of the 5´-end of exon 3. The GFP-minigene expression cassette was under the control of the CaMV 35S constitutive promoter. Following transformation and expression of the SolycHsfA2 and SoperHsfA2 minigenes in *S. lycopersicum* cv Moneymaker protoplasts, the cells were exposed to a 37.5^{o}C heat stress for 1 hour or kept at 25^{o}C as control samples (Fig. 16A and B). In non-stressed protoplasts, RT-PCR analysis revealed an approximately equal amount of HsfA2-I and HsfA2-II encoding transcripts for both SolycHsfA2 and SoperHsfA2 minigenes (Fig. 16B). Exposure to 37.5^{o}C heat stress for 1 hour inhibited splicing efficiency of SolycHsfA2 leading to increased levels of HsfA2-Iα compared to A2-II (Fig. 16B). In contrast, splicing was enhanced by the stress treatment in SoperHsfA2 minigene, which resulted in relative higher levels of A2-II, by large representing the splicing pattern in seedlings (Fig. 7A and 16B). This result indicates that the efficiency in pre-mRNA splicing of HsfA2 is controlled by intrinsic features present in the HsfA2 minigens and is not dependent on the genetic background of expression system.

This was further confirmed in *A. thaliana* Col protoplasts expressing the two minigenes. Following a 24 hour expression period, Arabidopsis protoplasts were exposed to 35°C or 37.5°C for 1 hour or kept at 25°C as control. In all samples, SolycHsfA2 minigene showed reduced splicing efficiency while splicing of SoperHsfA2 was enhanced (Fig. 16C). Because similar splicing patterns of SolycHsfA2 and SoperHsfA2 were detected in tomato and Arabidopsis, in some extent, this indicates that the splicing factors which are involved in the alternative splicing of HsfA2 are conserved between the two species. However, the splicing efficiency in unstressed *A. thaliana* protoplasts was similar to the stressed protoplasts, suggesting that in *A. thaliana* the mechanism that mediates temperature-dependent alternative splicing of HsfA2 intron is active under physiological conditions.

To find potential nucleotide polymorphisms that might be related to the differential pre-mRNA splicing of HsfA2, the sequence of the intron 2 of *S. lycopersicum* cv Moneymaker and *S. peruvianum* were aligned and inspected for variations. Seven nucleotide polymorphisms were found, from which a G>A transition (SL2.5:ch8_52380421) is located at the dinucleotide position of the A2-Iγ 5'-donor site. This was further supported by the minigene assay in *S. lycopersicum* mesophyll protoplasts (Fig. 16E). Exchange of G to A in Soper and SolycHsfA2 minigenes resulted in the loss or gain of A2-Iγ isoform (Fig. 16C and E). In other Solanaceae species, the same substitution (GT by AT) at the same position was detected, which is consistent with the absence of A2-Iγ (data not shown).

In the same manner, the contribution of the two adjacent SNPs was also examined. Exchange of all three Gs to As in GFP-SolycHsfA2 enhanced splicing efficiency to the levels of GFP-

SoperHsfA2. Accordingly, exchange of all three A to G in GFP-SoperHsfA2 resulted in inhibition of splicing. Interestingly, the HsfA2-minigene, containing GAG at the position ch8_52380477 (SNP1), ch8_52380471 (SNP2) and ch8_52380421 (SNP3), revealed higher splicing efficiency compared to the HsfA2-minigene with GGG (Fig. 16E). However, additional G>A exchange at SNP1 position induced intron retention indicating that three observed changes in splicing efficiency cannot be solely attributed to one of the three nucleotides. In support of this, A>G at SNP3 in SoperHsfA2 had no significant impact on splicing (Fig. 16E).

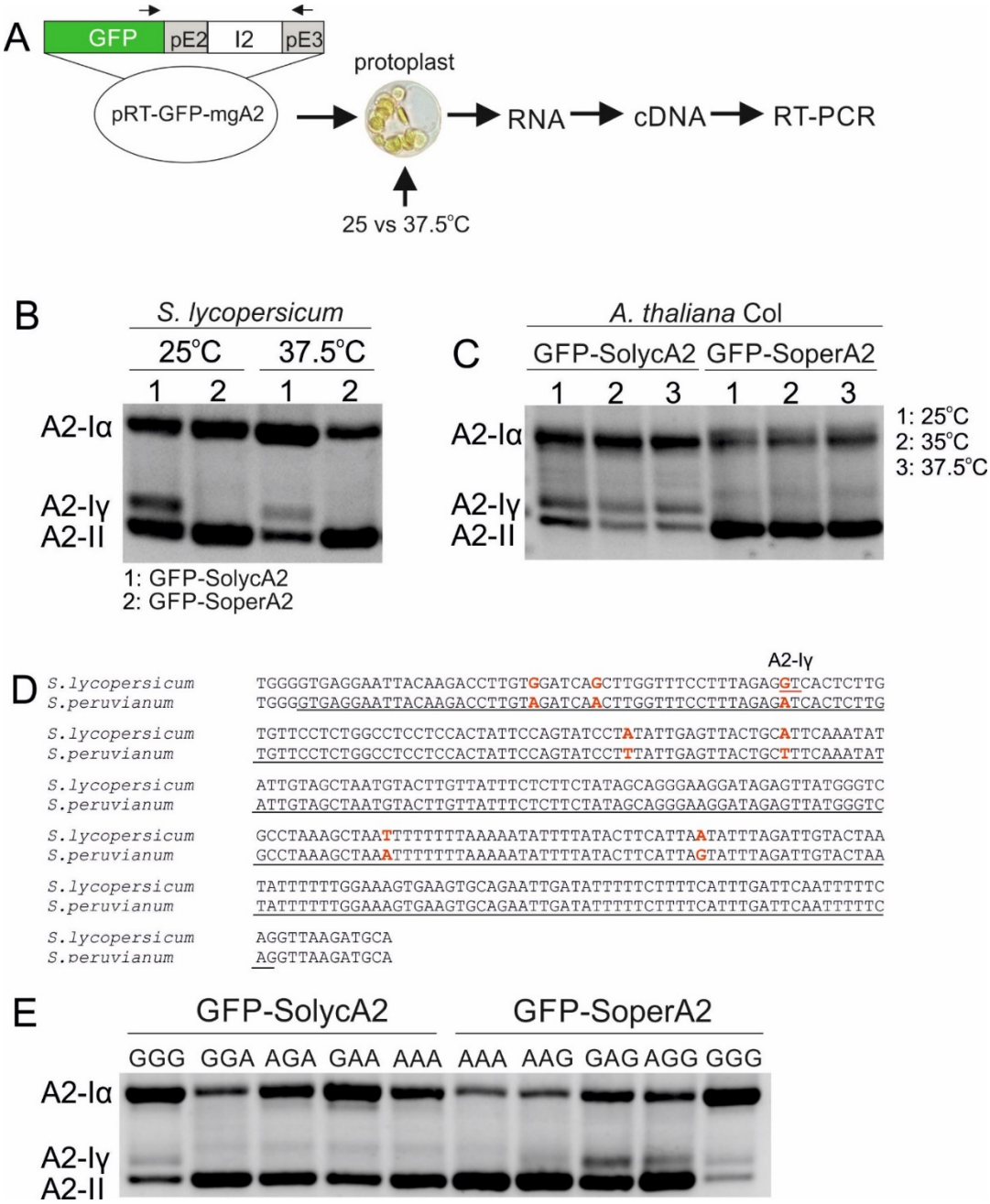

Figure 16. Three nucleotide polymorphisms in intron 2 determine the splicing efficiency of HsfA2.
Schematic representation of the pRT-GFP-HsfA2 minigene and the procedure of minigene assay in tomato mesophyll protoplasts. Green box represents the coding sequence of GFP, gray boxes represent exons and white box represents intron 2. Thin arrows above boxes indicate the primers used to amplify the spiced segment. (B) Splicing pattern of GFP-SolycA2 and GFP-SoperA2 in non-treated and 37.5°C treated tomato protoplasts. 4µg minigene plasmid was transformed in 10^5 tomato protoplasts and expressed for overnight. (C) Splicing analysis of GFP-SolycA2 and -SoperA2 in Arabidopsis (Col) protoplasts under different HS conditions. (D) Alignment of intron 2 sequences between *S. lycopersicum* and *S. peruvianum*. Red letters indicate nucleotide polymorphisms. The underlined letters in black and red represent the intron sequence and the donor site of A2-Iγ, respectively. (E) Agarose gel showing the PCR products from the minigenes (wild type and a series of mutated minigenes). Each minigene (4 µg) was transformed in tomato protoplasts and expressed overnight.

6.2.4 Relation of HsfA2 intronic polymorphisms and thermotolerance in different tomato accessions

The clear difference in the splicing profiles between the two tomato species (*S. lycopersicum* and *S. peruvianum*) prompted us to examine the presence of such polymorphisms in a larger set of accessions from cultivated and wild tomatoes. All examined *S. lycopersicum* cultivars and the close relative *S. galapagense* have G in all three SNP positions. In turn, all these accessions share similar splicing profiles with approximately 50% of total transcripts belonging to the HsfA2-II variant (Fig. 17A). On the other hand, all residual wild species tested so far with the exception of *S. pimpinelifolium* exhibited the splicing profile of *S. peruvianum*, with increased splicing efficiency at 37.5^{o}C (median 90% of HsfA2-II transcripts). Interestingly, from the two *S. pimpinelifolium* accessions examined, LA1261 had no polymorphism at these positions and behaved similar to *S. lycopersicum* while the second (LA2852) had a G>A substitution in SNP2. This led to increased splicing efficiency but also the presence of A2-Iγ (Fig. 17A and B).

Tomato wild species are considered as valuable donors for exotic genes for the improvement of cultivated tomato accessions, especially regarding the resistance in biotic factors or tolerance in abiotic stress factors (Foolad, 2007). We aimed to examine whether the variation in splicing efficiency of HsfA2 is related to the thermotolerance of these accessions. Due to the high phenotypic and developmental variance of tomato wild species, evaluation of thermotolerance is challenging. To avoid such interference, we used the hypocotyl elongation assay. Five-day-old etiolated seedlings were exposed to 37.5°C HS for 90 min to allow the accumulation of HsfA2-II, then directly subjected to severe HS treatment at 50°C for 90 min or kept at 25°C under dark condition. Hypocotyl length was recorded for 4 days and then the relative elongation rate was calculated. None of the accessions used in this study was able to survive the severe heat stress at 50°C without prior conditioning, indicating that the observed survival and recovery from the stress are dependent on the protection mechanisms activated during the pre-treatment. Based on the differences in splicing patterns of HsfA2 among wild species, the accessions were grouped into two sets, referred here as *S.lycopersicum* (GGG) and *S. peruvianum* groups (AAA, GAG). For *S. lycopersicum*, we utilized standard cultivars used in molecular biology studies such as Moneymaker, Ailsa Craig and M82, as well as accessions characterized as thermotolerant (i.e. Hotset, Nagcarlang (LA0436), LA1994 by the Tomato Genetics Resource Center (TGRC).

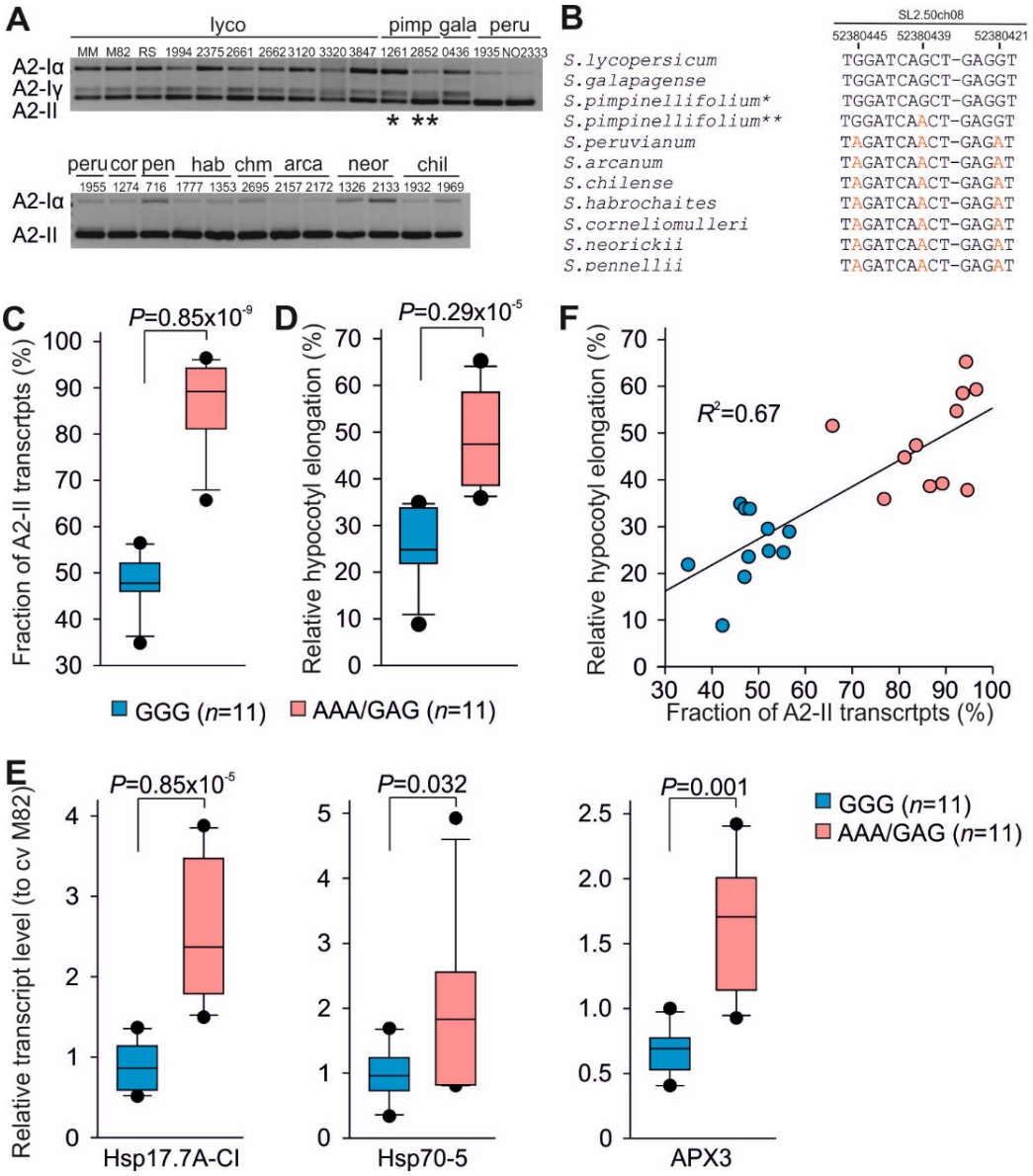

Figure 17. HsfA2 contributes to thermotolerance variation among tomato accessions.

(A) Splicing patterns of HsfA2 in tomato wild species. Numbers above agrose gel picture are the accession IDs. (B) The most conserved SNPs in intron 2 of HsfA2 among tomato wild species. Alignment of HsfA2 intron 2 sequences in tomato wild species (here only region of sequence harboring the three SNPs is shown). One asterisk represents the *S. pimpinellifolium* containing AAA in HsfA2 while two asterisks represent another *S. pimpinellifolium* harboring GAG in HsfA2. (C) Relative abundance of HsfA2-II in wild species harboring AAA or GAG in HsfA2 is much higher than that of the wild species containing GGG. (D) The wild species belonging to *S. peruvianum* group (AAA or GAG; red boxes) have higher thermotolerance than *S. lycopersicum* group (GGG; blue boxes). Relative hypocotyl elongation of each accession are presented as means ± standard deviations (n=11 per each group). (E) Relative transcript levels of Hsp17.7A-CI, Hsp70-5 and APX3 in wild species after 1 h HS treatment at 37.5°C. The Ct value was normalized to EF1a and UBI and to the expression of Hsp17.7A-CI in M82 at 37.5°C. Data are shown as boxplots. Student's t tests were used to generate the *P* values which are shown above boxplots. (F) There is a positive correlation between themotolerance and the fraction of HsfA2-II transcripts in wild tomato species. Each point represents an (x, y) pair. Blue points represent data points from *S. lycopersicum* (GGG) group, and read points

indicate data points from *S. peruvianum* group (AAA or GAG). The standardized correlation coefficient was represented by R^2.

Collectively the seedlings of the *S. lycopersicum* group (GGG) exhibited significantly lower thermotolerance compared to the *S. peruvianum* group ($P<0.29x10^{-5}$), while the correlation of thermotolerance and A2-II splicing was $r^2=0.67$, clearly indicating the relation between splicing efficiency and thermotolerance of seedlings (Fig. 17D and F). The accessions of the *S. peruvianum* group also showed higher accumulation of the HS-induced Hsp17.7A-CI, Hsp70-5 and APX3 transcripts (Fig. 17E). These results strongly suggest that higher splicing efficiency and increased synthesis of HsfA2-II is related to an increased stress response that lead to enhanced thermotolerance in response to a direct strong HS.

6.2.5 Introgression of *S. pennellii* HsfA2 into the cultivar M82 increases thermotolerance

Wild tomato species have been identified as donor for exotic genes to facilitate the genetic improvement of the cultivated tomato in terms of yield, quality and stress tolerance (Foolad, 2007). To examine whether HsfA2 from wild species can be used for the improvement of thermotolerance of cultivated tomato, we utilized an introgression line (IL8-1, LA3509) carrying a single chromosome 8 segment which includes HsfA2. IL8-1 and the wild parent (*S. pennellii* LA716) showed similar splicing efficiency and which is different in the other parent *S. lycopersicum* cv M82. In addition, IL8-1 and the wild parent showed increased accumulation of Hsp17-CI protein in response to a 37.5°C heat stress. IL8-1 seedlings also showed higher thermotolerance than M82 ($P<0.001$) but is similar to *S. pennellii* confirming that HsfA2 plays an important role in thermotolerance. However, this is also highlighting its potential use in tomato breeding.

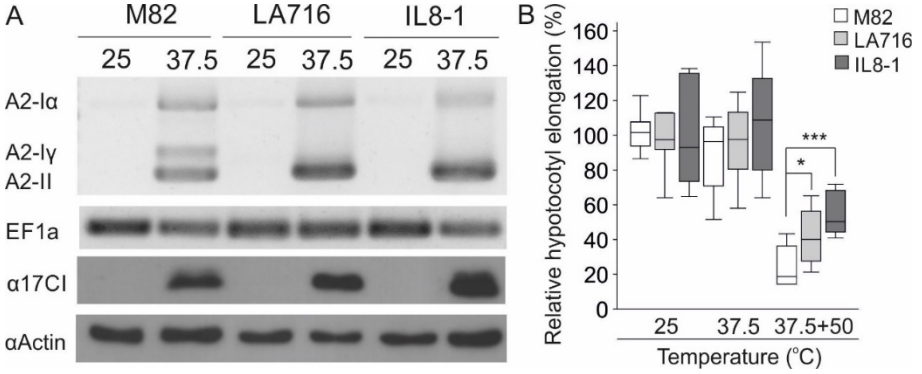

Figure 18. Introgression of wild HsfA2 into cultivated tomato enhances thermotolerance.
(A) Splicing patterns of HsfA2 and protein levels of Hsp17-CI in M82 (*S. lycopersicum*), LA716 (*S. pennellii*) and IL8-1 (Introgression line) with or without HS. The detached young leaves were kept at 25°C or 37.5°C for 1 hour. After HS treatment, samples were immediately collected and put in liquid nitrogen for further experiments. The splicing efficiency of HsfA2 was analyzed by RT-PCR using specific primers. The protein levels of Hsp17-CI was investigated

by western blot using antibody against Hsp17-Cl. (B) 4-day-old etiolated tomato seedlings of M82, LA716 and IL8-1 were exposed to 37.5°C for 90 min and treated a 90 min HS at 37.5°C and then directly applied a severe HS at 50°C for another 90 min. Seedlings with one asterisk or three asterisks had significantly higher values than M82 ($P< 0.05$ or $P<0.001$, respectively). Each data point represents the mean ± se of at least 10 seedlings for each line and treatment.

6.2.6 Alternative splicing is a common feature among HS-induced class A Hsfs

To determine whether other Hsfs undergo HS-dependent AS similar to HsfA2, RT-PCR splicing analysis was performed using primers pairs corresponding to the first and the last exon of each Hsf tested in the same cDNA samples as shown in Fig. 7A**.** Several important Hsfs, including seven members of class A Hsfs (HsfA1a, HsfA1b, HsfA1c, HsfA3, HsfA4c, HsfA6b and HsfA7) were selected for splicing analysis. Using the same procedure as for HsfA2 to examine alternative splicing, transcript variants of these genes were identified by RT-PCR in seedlings exposed to HS at different temperatures. Among these we found that HsfA1b, HsfA3, HsfA6b, and HsfA7 undergo AS in a temperature-dependent manner (Figure. 19A). In contrast, no AS was observed for HsfA1a (Fig. 19A), HsfA1c and HsfA4c (data not shown).

According to the current tomato genome annotation (SL2.5) of Sol Genomics Network (solgenomics.net) database, there is only one single intron in HsfA1a, HsfA1b, HsfA1c, HsfA3 and HsfA4c. Surprisingly, we detected five splice variants of HsfA1b and three for HsfA3. Based on the sequence analysis, AS in HsfA1b intron leads to the generation of four splice variants harboring a premature stop codon (dark arrowheads) and a fully spliced HsfA1b (asterisk). HsfA1b shows a significant induction at the transcription level after a 35°C treatment, which peaks at 42.5°C (Fig. 19A). Further temperature increases (from 42.5°C) cause suppression of expression which is almost abolished at 47.5°C. More interestingly, the splicing pattern of HsfA1b varies dramatically as temperature increases (Fig. 19A), suggesting that AS of HsfA1b, similar to HsfA2, is also sensitive to temperature changes as small as 2.5°C. For example, the isoform V of HsfA1b was mainly detected in seedlings exposed to 35°C to 45°C. Isoform II and III are generated under temperatures ranging from 40-45°C (Fig. 19A). Interestingly, HsfA3 already generates 3 splice variants under non-stress conditions (25°C, Fig. 19A). Sequencing analysis showed that retention or partial splicing of Intron 1 in HsfA3 results in generation of HsfA3-III and HsfA3-II, respectively (Fig. 19B). HsfA3-II and HsfA3-III contain a premature stop codon within the retained intron 1 portion that possibly leads to the NMD pathway or is translated into a truncated proteins.

There are two and four introns in HsfA6b and HsfA7, respectively. We detected two HsfA6b splice variants which have almost equal abundance under all temperatures ranging from 25-50°C. HsfA6b has two introns similar to HsfA2, however the second intron is in the region between the DBD and the oligomerization domain. Alternative splicing occurs under the

examined conditions only for intron 2, which results in generation of a putative protein isoform with harboring only the DBD but no other functional domains.

HsfA7 is strongly induced by moderate temperature ranging from (27.5°C to 40°C) but suppressed by severe HS (Fig. 19A). In addition, moderate HS induces the generation of four HsfA7 splice variants in total (Fig. 19A and B). HsfA7 shows complexity in intron-exon architecture, as it contains 4 introns, with three located in the 3'-terminus of the gene. Intron retention of introns 2-4 leads to generation of HsfA7-IV splice variant which encodes the full-length HsfA7-I protein, having an NES (Fig. 19B). This transcript variant is the most abundant in all samples. Interestingly, splicing of intron 2, accompanied by excision of intron 3 and 4, leads to the generation of 3 transcript variants which can potentially encode for a second protein isoform, lacking the NES as shown before for HsfA2. This suggests that HsfA7 might undergo a similar functional diversification by AS. Collectively, these results reveal that approximately 62.5% (5 out of 8) of Hsf genes tested here undergo HS-dependent AS, suggesting that upon heat stress conditions the complexity of HSR is far more complicated than previously thought.

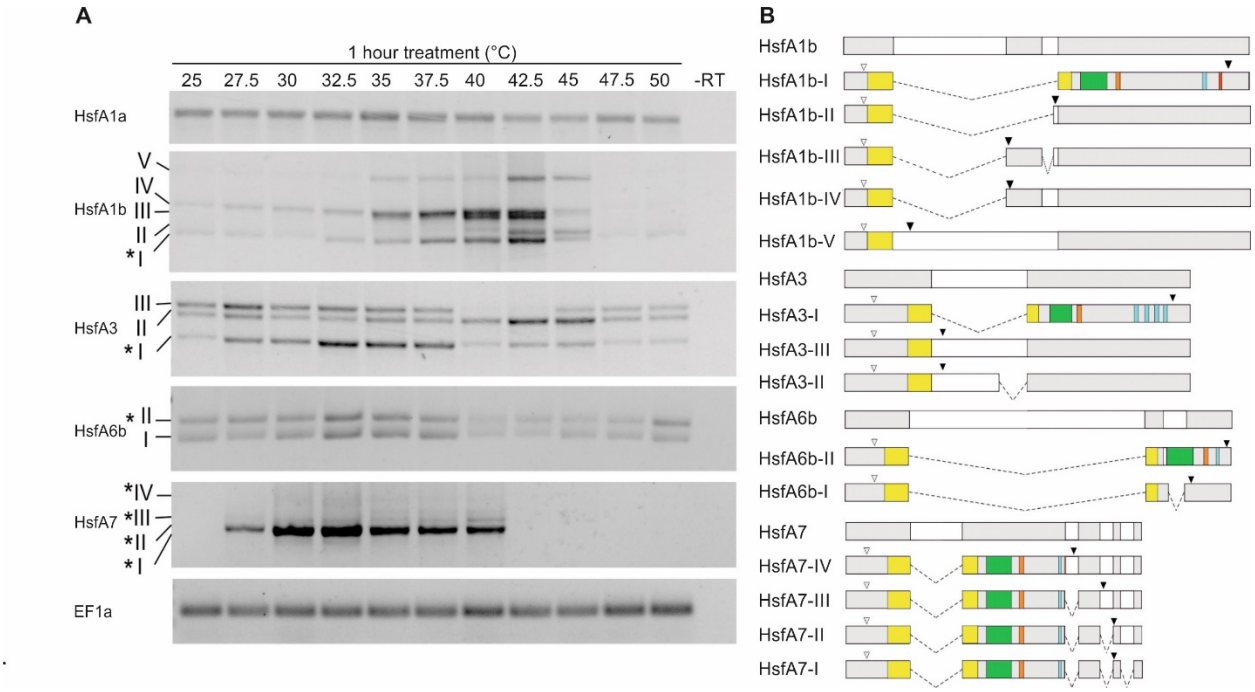

Figure 19. Splicing analysis of selected tomato Hsfs under different heat stress conditions.
(A) RT-PCR splicing analysis of the indicated tomato Hsfs in one-week-old tomato seedlings treated at elevated temperature (from 25-50°C) for 1 h. DNAse treated RNA (1µg) from these samples was used to prepare first-strand cDNA. An equal amount of first-strand cDNA (10ng) was used as a template in PCR with primers specific to the indicated tomato Hsfs (HsfA1a, HsfA1b, HsfA3, HsfA6b and HsfA7). The set of primers used anneal to the first exon and the 3'-UTR of each Hsf. Sequences of forward and reverse primers used in PCR are listed in Table 2. Elongation factor 1a housekeeping gene (EF1a) amplification was used to indicate an equal quantity of template in each PCR reaction. The names of Hsfs and a housekeeping gene are given on the left of each panel. Isoforms are numbered

in ascending order according to the size (Isoform I represents the smallest transcript). Asterisks denote transcripts that encode for a protein consisting all indicated functional domains. (B) The schematic diagram for each gene shows the gene structure and its alternatively spliced mRNA isoforms. The white and black arrowheads show the start and stop codons, respectively. Gray and white boxes denote exons and introns, respectively. Dashed lines represent the parts that are spliced out. Domains are presented as described in Fig. 7B.

6.3 Regulation of HsfA2 pre-mRNA splicing

6.3.1 SR protein family in tomato

Our results indicate that HsfA2 splicing is mediated by a splicing factor that acts as splicing silencer in case of SolycHsfA2 or splicing enhancer in case of SoperHsfA2. SR proteins have significant role in constitutive pre-mRNA splicing and are also important regulators of alternative splicing (Palusa et al., 2007; Reddy & Shad Ali, 2011). Consequently, we asked whether an SR protein is involved in splicing of HsfA2. SR proteins have a characteristic domain structure consisting of one or two RNA-binding motifs and a RS domain rich in serine/arginine dipeptides (Long & Caceres, 2009b). Based on the modular structure and the presence of specific domains (i.e. Zinc finger), plant SR proteins are classified into subfamilies. By orthology search using the annotated *A. thaliana* SR protein family and domain analysis, 17 genes encoding SR proteins were identified in tomato genome (Fig. 20). Previous report has shown that AS of *Arabidopsis thaliana* SR pre-mRNAs is dramatically altered in under heat stress conditions (Palusa et al., 2007). At first the global effect of HS on transcript level of tomato SR proteins was to be assessed. Transcriptome analysis for this study was performed on seedlings of tomato (cv. Moneymaker) for control and different HSR conditions. The gene expression values are presented as transcripts per million (TPM). The expression of genes encoding for SCL29, RSZ21, SC30a, SR32, SC30b, RS2Z36, SC24 and SR30 is higher either at 39°C or 45°C or at both temperatures compared to non-stress conditions (Fig. 20).

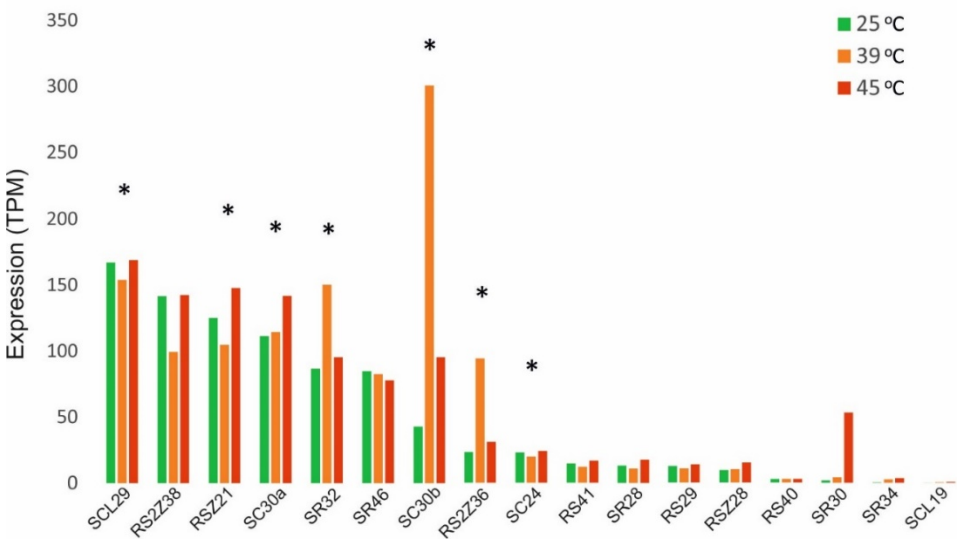

Figure 20. Transcript levels of SR protein-encoding genes in tomato seedlings exposed to heat stress.
Expression profiles of different SR protein-encoding genes in tomato seedlings under indicated temperatures (MACE, see material and methods). One-week-old tomato (*Solanum lycopersicum* cv. Moneymaker) seedlings were treated for 1 h at 39°C (orange) or 45°C (red) in water bath and another 90 mins at 25°C for recovery phase.

To analyze the effect of HS on the splicing pattern of SR genes we treated 4-day-old tomato wild type seedlings (cv Moneymaker) with either 1 h 37.5°C or 42.5°C heat stress and performed RT-PCR. Thirteen out of 17 genes exhibited AS, whereas only four genes (SR46, RSZ21, RS2Z38 and SC24) generated a single transcript under these conditions. Interestingly, in Arabidopsis, 15 out of 19 SR genes undergo AS and there are also four genes only produce a single transcript (Saiprasad Goud Palusa et al., 2007). This striking similarity on splicing pattern of SR genes indicates that SR proteins are highly conserved in plants. Furthermore, AS of tomato SR pre-mRNA is dramatically altered in response to HS (Fig. 21). For example, two additional splice forms of RS40 only appear under moderate HS condition (37.5°C), whereas some isoforms were reduced (Fig. 21). AS of RS26 produces more transcripts when the temperature is increasing (Fig. 20). Heat stress treatment reduces reduce the levels of all isoforms of SCL19 (Fig. 20 and 21). All these observations indicate that altered ratios of splice variants in response to heat stress may play a role in adaptation of plants to heat stress.

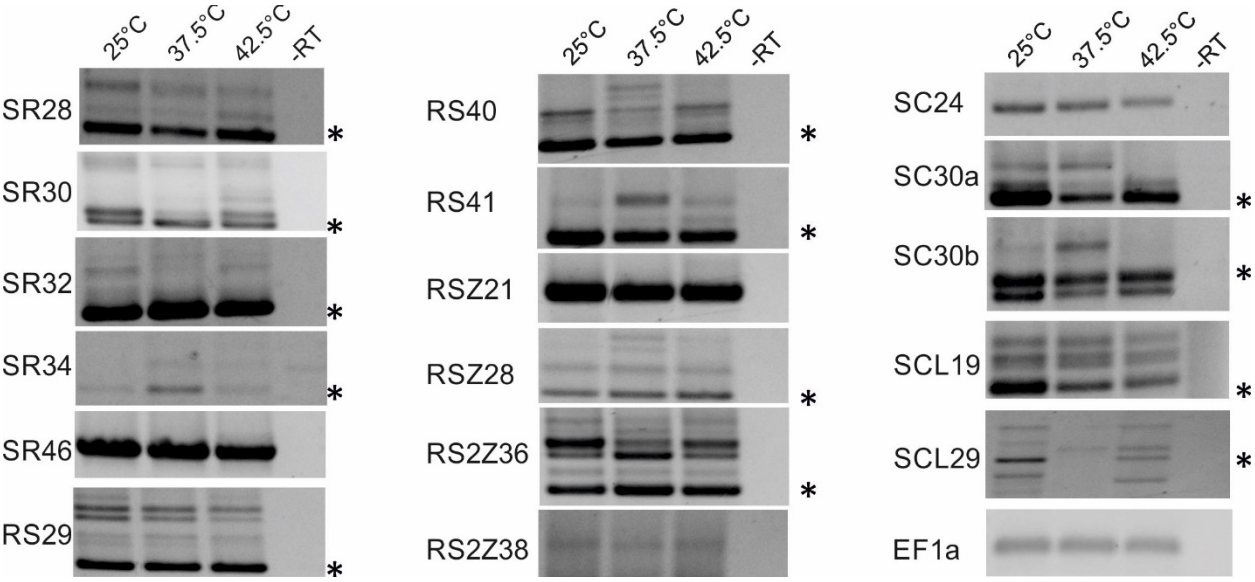

Figure 21. Effect of HS on the expression and AS of SR-protein encoding genes.
Agarose gel pictures of SR splicing products. One-week-old tomato (Solanum lycopersicum cv. Moneymaker) seedlings were kept at 25°C, 37.5°C or 42.5°C for 1 h. RNA from control (25°C) and treated samples was used for RT-PCR. The names of SR genes are given on the left of each panel. Asterisks indicate the transcripts that encode full-length proteins. EF1a was used as housekeeping gene.

6.3.2 Identification of the SR proteins involved in HsfA2 alternative splicing

To examine whether a member of the SR protein family regulates HsfA2 splicing under HS, the coding sequences of those SR proteins showing high expression levels in absence of heat stress (Fig. 20, genes marked with asterisks) were cloned into an expression vector under the control of the constitutively active CaMV-35S promoter and a 3×HA epitope encoding sequence fused N-terminally. Protoplasts were transformed with a constant amount of A2 minigene plasmid DNA and increasing amounts of SR proteins plasmid. Screening was performed by checking for changes in splicing based on proteins in relative ratio between GFP-A2-I and GFP-A2-II, using a GFP antibody. We identified two SR proteins, namely SCL29 and RS2Z36 that cause a significant increase of GFP-A2-I over GFP-A2-II specifically in the *S. lycopersicum* minigene, but had no effect on *S. peruvianum* (Fig. 22A and B).

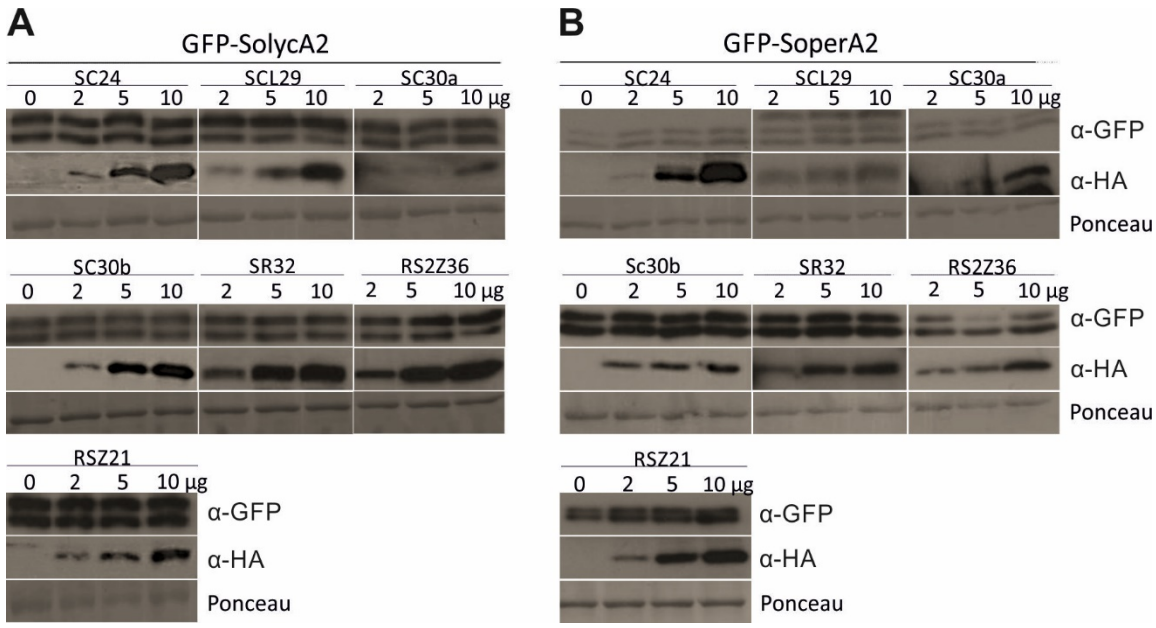

Figure 22. Screening for SR proteins involved in modulating HsfA2 splicing.
10^5 tomato mesophyll protoplasts were transformed with 4 µg GFP-SolycA2 (A) or GFP-SoperA2 (B) minigene plasmid DNA and indicated amounts of the indicated HA-SR protein construct or 10 µg Neo (control). After 24 hours of expression samples were harvested. Proteins were extracted and subjected to SDS-PAGE and western blot analysis with anti-GFP and anti-HA antibodies.

To further investigate the effects of SCL29 and RS2Z36 on the efficiency of HsfA2 pre-mRNA splicing at transcriptional level, we used the transient expression assay in tomato protoplasts isolated from young leaves. Four micrograms of A2-minigene were co-transformed with 10 µg of SCL29 or RS2Z36 in protoplasts. RNAs from these protoplasts were analyzed by RT-PCR. In protoplasts transformed with GFP-SolycA2 alone, the ratio of A2-I to A2-II transcripts was approximately 1. When co-expressed with RS2Z36, this ratio dramatically increased to approximately 9 to 1, indicating that RS2Z36 significantly inhibited the splicing of SolycA2. However, RS2Z36 had almost no effect on splicing efficiency of SoperA2 (Fig. 23B and C). These results indicate that RS2Z36 can bind or is associated with intron 2 of SolycA2 pre-mRNA but not SoperA2 or the binding affinity between the binding site of SoperA2 and RS2Z36 is very low. Furthermore, in protoplasts transformed with different amounts of SCL29 plasmid DNA, no significant altered ratio was observed for both GFP-SolycA2 and GFP-SoperA2 minigenes (Fig. 23B and C), indicating that SCL29 acts on the post-splicing level.

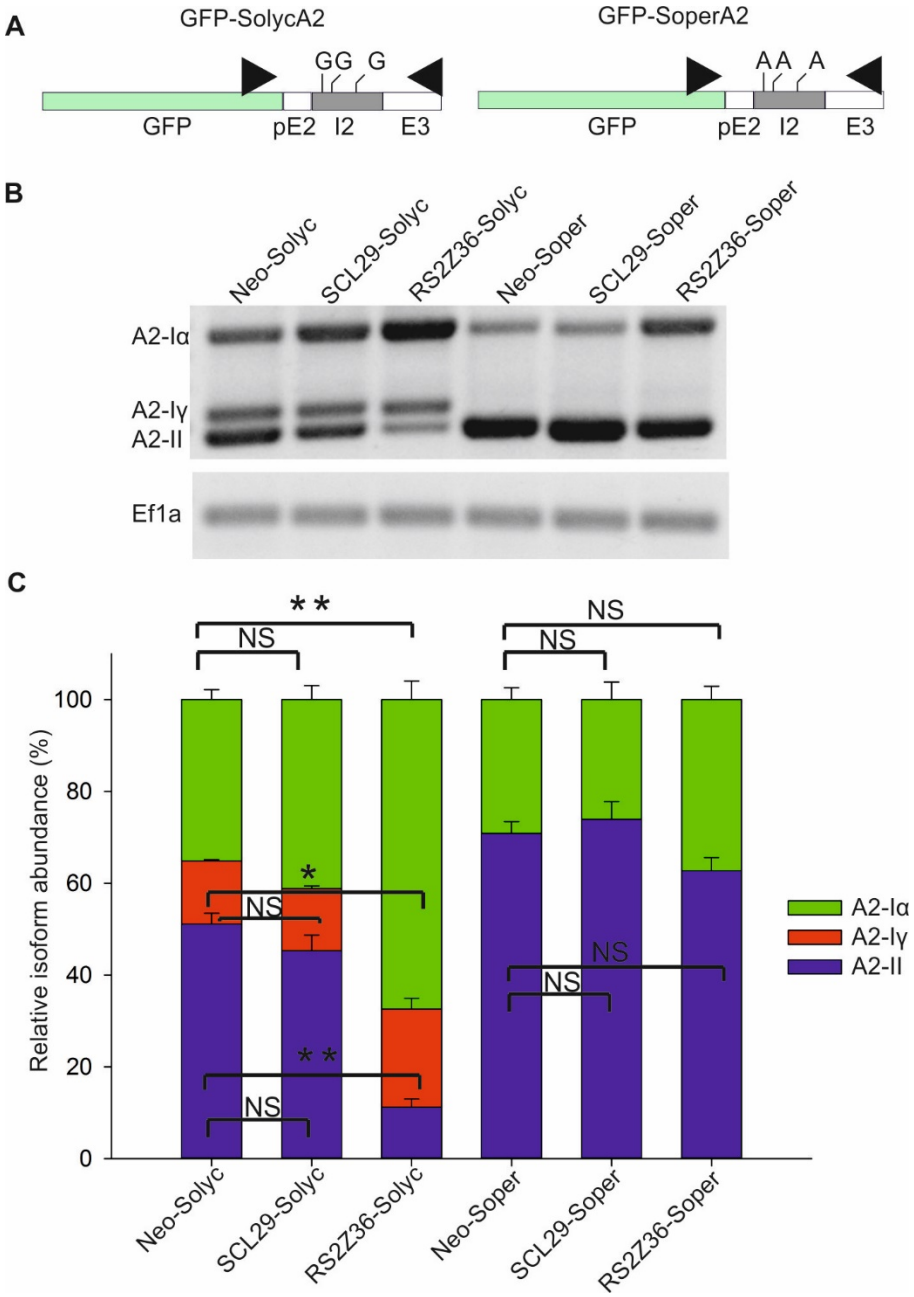

Figure 23. RS2Z36 inhibits Solyc-HsfA2 but not Soper-HsfA2 intron 2 splicing.

(A) Schematic representation of *S. lycopersicum* and *S. peruvianum* HsfA2-minigene splicing constructs fused to green fluorescent protein (GFP; green box). Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis are shown as black arrows. pE2, partial exon 2; I2, intron 2; E3, exon 3. (B) Reverse transcription polymerase chain reaction (RT-PCR) analysis of intron 2 splicing of 4 µg HsfA2 minigene construct (GFP-SolycA2 or GFP-SoperA2) co-expressed with *SI*SCL29 (10 µg) and *SI*RS2Z36 (10 µg) in tomato protoplasts. (C) The relative level of each HsfA2 isoform in a sample was calculated by dividing the level of each isoform by the total transcripts level of HsfA2. The relative isoform abundance is presented as the percentage of all isoforms in a sample. Quantification of transcripts was done by using the ImageJ program. Standard errors are calculated from three independent experiments. Two-tailed t-test is shown as: *, $P \leq 0.1$; **, $P \leq 0.05$; compared with experiments co-expressing Neo. NS, no significant difference.

7 Discussion

7.1 HsfA2 is involved in acquired thermotolerance

Plants have evolved different mechanisms to cope with various heat stress regimes occurring in nature. In tomato, HsfA1a has been shown to be the master regulator as it is required for the induction among others of several Hsps, but also Hsfs (Mishra et al., 2002). The accumulation of additional Hsfs, is thought to contribute to stimulation of HSR and transcriptional activity of HS-related genes during the different phases of the response.

HsfA2 is a HS-induced gene and considered along with HsfA1a and HsfB1 to make a triad of factors with essential functions for stress response and recovery (Scharf et al., 2012). Using HsfA2 knock-down seedlings we were able to show that HsfA2 is not involved in basal thermotolerance and induction of HS-induced genes like Hsp101 and Hsp17.7C-CI (Fig. 4). This is in agreement with the activity cycle of tomato HsfA2, which includes the recruitment of the protein in HSGs during an acute stress (Port et al., 2004; Scharf et al., 1998). In turn, HsfA2 is essential for enhancing the levels of acquired thermotolerance in seedlings, as in response to a second challenging HS after a pre-acclimation treatment seedlings of the A2AS lines showed reduced accumulation of Hsp101 and Hsp17-CI (Fig. 5). The reduced levels of HsfA2 and Hsps resulted in reduced hypocotyl elongation rate in the days following the stress, indicating reduced capacity to recover and increased sensitivity (Fig. 5). *Arabidopsis thaliana* HsfA2 has similar function in acquired thermotolerance (Charng et al., 2007) suggesting conserved function of this factor in different plant species. However, *A. thaliana* is required for the extension of the long but not short term acquired thermotolerance while in tomato we found the opposite result. This indicates a functional difference between the two plant species and it might be related to the spatial and temporal implication of other Hsfs which might compensate for HsfA2 suppression. For example, tomato HsfA7 which shares high sequence homology with HsfA2 (Scharf et al., 2012) might functional complement HsfA2 in the long term acquired thermotolerance. Interestingly, HsfA7 is induced in an HsfA2-independent manner in tomato leaves exposed to HS, while in anthers the induction is even stronger in the *A2AS* background (Fragkostefanakis et al., 2016). In the same study, it was shown that pre-formed HsfA2 during microsporogenesis enhances the capacity of pollen to withstand a direct HS suggesting an important developmental regulatory program for HsfA2 to prime pollen in case of an upcoming stress incident (Fragkostefanakis et al., 2016).

7.2 Functional diversification of tomato HsfA2 by alternative splicing

HsfA2 along with other Hsfs are core regulators involved in the induction of the majority of HS-induced genes (Bharti et al., 2004; Chan-Schaminet et al., 2009). In addition to transcriptional regulation, a multitude of regulatory mechanisms orchestrate the molecular responses under

stress conditions, including among others epigenetic modifications, pre-mRNA and mRNA processing, translation control, and post-translational modifications (Guerra et al., 2015). During the last years, mainly due to increasing use of Next Generation Sequencing (NGS) technologies which allow monitoring genome wide transcriptome changes it has become evident that alternative pre-mRNA splicing plays a very important role in plant adaptation to environmental stresses, including high or low temperature, changes in light quality and intensity, salt, and wounding (Boveet al., 2008; Filichkinet al., 2015; Hartmann et al., 2016; Marquez et al., 2012; Marshall et al., 2016; Palusa et al., 2007; Robinson & Parkin, 2008).

Many transcription factor-encoding (TF) genes are regulated by alternative splicing that result in loss or gain of protein domains that produce inactive, non-functional or hyperactive proteins. In most of the cases, the new isoforms lack DNA binding domain (DBD) or have truncated DBD (Liu et al., 2013; Penfield, Josse, & Halliday, 2010; Posé et al., 2013; Seo et al., 2012, 2011) while loss of the activation domain or nuclear export/localization signal is less frequent (Gagete et al., 2009). Interestingly, some AS of TF-genes leads to the generation of protein isoforms with opposite functions under specific conditions. For example, a cold-inducible alternatively spliced INDERMINATE DOMAIN 14 form (IDD14β) lacks functional DNA binding domain but forms heterodimers with the functional IDD14 form (IDD14α). This heterodimers are able to decrease the DNA binding activity of IDD14α to the promoter of *Qua*-Quine Starch (QQS) gene that plays an important role in starch accumulation (Seo et al., 2011). AS of potato BRANCHED1a (BRC1a) encoding a TCP transcription factor generates two proteins with antagonistic functions (Nicolas, Rodríguez-Buey, Franco-Zorrilla, & Cubas, 2015). In *Arabidopsis thaliana*, FLOWERING LOCUS M (FLM) is regulated by AS in a temperature-dependent manner. The two main FLM protein isoforms compete for interaction with the floral repressor SVP to regulate flowering in opposition (Posé et al., 2013). For Hsf, the mammalian HSF4 generates two isoforms (HSF4a and HSF4b) with different transcriptional activity by alternative splicing (M. Tanabe et al., 1999). Furthermore, Hsf1 and Hsf2 are subjected to AS and exhibit two AS isoforms, called α and β, and relative amount of Hsf2 isoforms determines Hsf1 transcriptional activity (Lecomte et al., 2013). Particularly in response to changes in ambient temperature, AS has been proposed to act as a ‘molecular thermometer’, allowing plants to quickly adjust the abundance of functional transcripts or activate RNA surveillance (Capovilla et al., 2015; Filichkin et al., 2015).

By RT-PCR on seedlings exposed to different temperatures we identified that HsfA2 undergoes AS which results into the generation of 7 splice variants (Fig. 7). Similar to *A. thaliana*, tomato HsfA2 exhibits AS in intron 1, resulting in generation of 3 transcripts that possess a PTC and long 3’-UTR which qualifies them as targets for NMD (Kalyna et al., 2012). The latter is supported by the higher accumulation of these transcripts in cells treated with cyclohexamide, as translation inhibition suppresses NMD (Fig. 8).

Interestingly, in *A. thaliana*, one of the two transcripts was shown to encode for a truncated isoform containing part of the DBD (Liu et al., 2013). Ectopic expression of this variant in *A. thaliana* plants stimulated HsfA2 accumulation and enhanced thermotolerance, suggesting that this isoform might regulate HSR and thermotolerance under severe stress conditions, by forming an autoregulatory loop (Liu et al., 2013). The truncated HsfA2 isoform was characterized by a hydrophobic leucine- and phenylalanine-rich carboxyl-tail (Liu et al., 2013). Despite the fact that A2-III putative proteins have the essential helix 2 and 3 required for DNA binding, A2-IIIγ has a very short C-terminal extension of 11 aa residues, while the other two that have longer carboxyl-tails they have lower leucine-content compared to *A. thaliana* variant (Fig. 8). In addition, the levels of HsfA2-III transcripts are very low and appear only at temperatures that are in general inhibitory for translation and transcription, thus it is most likely that AS in intron 1 is related to the attenuation of HSR by suppressing the levels of HsfA2 functional transcripts.

In contrast to *A. thaliana*, tomato HsfA2 possesses a second intron (Fig. 7B). AS in this intron, either by full or partial retention or full excision results in the generation of four transcripts, three of which encode for the already characterized HsfA2-I isoform (Scharf et al., 1998). Differences in the 3'-UTR of the three transcripts encoding for HsfA2-I might be related to mRNA stability but this needs to be further investigated. However, several preliminary lines of evidence point to this direction, as prolongation of stress favored the synthesis of specific HsfA2-I transcript variants over the other ones (Fig. 9). Most characteristically, the generally low abundant HsfA2-Iβ under short period of stress, becomes highly abundant after 2 hours of HS at 42.5^{o}C, while at the same time HsfA2-Iγ is reduced (Fig. 8). In addition, during recovery from 1 hour stress at 42.5^{o}C, HsfA2-Iα shows remarkable stability in comparison to HsfA2-Iγ. Interestingly, HsfA2-I transcripts are present at different temperatures ranging from 30 to 45^{o}C, highlighting the importance of this isoform for the pre-acclimation leading to acquired thermotolerance.

Splicing of intron 2 on the other hand results in a frameshift that generates a truncated HsfA2 protein (HsfA2-II). The presence of this protein isoform was confirmed by an antibody generated against a recombinant full length HsfA2 (Fig. 7D). The majorities of the studies conducted on tomato HsfA2 have utilized an antibody (PEP6) targeting the C-terminal NES peptide motif of the protein which is lacking in HsfA2-II, and this might be one reason why this important information was missed for such a well-studied protein. The two protein isoforms have significant differences in their activity cycle and therefore contributing by different means to the regulation of HSR.

HsfA2-II due to the absence of the NES, shows enhanced nuclear retention, increased activity but reduced stability compared to HsfA2-I (Fig. 10-13). The latter can explain the generally lower

levels of HsfA2-II in stressed seedlings (Fig. 7A) and might be related to the higher activity of this isoform. In a similar manner, the turnover of tomato HsfB1 is increased upon binding to HSEs (Röth et al., 2016), suggesting that this might be a more general mechanism to regulate the transcriptional activation activity of Hsfs.

Although HsfA2-II lacks five of the nine amino acids comprising AHA2, it does not loose its activator potential, as in the presence of HsfA1a the activity of the complex is similar to that of HsfA2-I/HsfA1a. Therefore both isoforms have the potential to stimulate stress response at the same level. GUS activity reporter assay showed that the transcriptional activity of HsfA2-II is 3-fold higher compared to HsfA2-I (Fig. 12). The stronger activity of A2-II can be attributed to its nuclear localization, since both isoforms exhibited similar activity in the presence of the nuclear export inhibitor leptomycin B (Fig. 12). Although HsfA2 is not expressed in vegetative tissues under non-stress conditions, it is present in early stages of pollen development (Fragkostefanakis et al., 2016; Giorno et al., 2010). However, at these stages only HsfA2-Iα transcript is present (data not shown) which might be related to the necessity of strict lower activity control, as increased levels of Hsps might interfere with pollen development (Fragkostefenakis et al., 2016).

Importantly, in contrast to HsfA2-I, HsfA2-II is by large able to escape the interaction with Hsp17-CII which prevents its accumulation in HSGs (Fig. 13). As the C-terminus of HsfA2-I is important for interaction with Hsp17-CII, either the absence of NES or the truncated AHA2 might contribute to Hsp17-CII avoidance. Interestingly, transgenic seedlings overexpressing the *S. peruvianum* HsfA2 showed enhanced accumulation of Hsp101 and Hsp17.7C-CI and subsequently increased basal thermotolerance compared to wild type (Fig. 6). This result suggests that ectopic levels of HsfA2 can overcome the repression effect caused by the interaction with Hsp17-CII.

Altogether these results suggest that HsfA2-I is synthesized under a wide range of temperatures to enhance the capacity of the cells to extend their ATT limits. On the other hand, under mild HS conditions (30-37.5^{o}C) HsfA2-II is produced to enhance the responsiveness of the cell thereby contributing to stress adaptation. We were able to confirm this notion in tomato wild accessions that exhibit higher splicing efficiency of intron 2 thereby accumulating higher levels of HsfA2-II. These accessions showed increased thermotolerance in response to a direct severe HS incident, in which seedling after a pre-acclimation to 37.5^{o}C, were exposed to the otherwise lethal 50^{o}C treatment (Fig. 17). So far, this novel post-transcriptional regulation mechanism for controlling tomato HsfA2 expression has not been described in any other plant species. This post-transcriptional regulation provides a versatile regulatory scheme for regulation of gene expression under heat stress conditions. Subtle changes in the ratio of full length Hsfs and

alternatively spliced variants has a profound effect on expression of their target genes, including HS-induced genes. It seems there is communication and coordination between alternative splicing and gene transcriptional regulation, maintaining the appropriate level of transcription factor activity in response to environmental fluctuations.

7.3 Natural variation in splicing efficiency of HsfA2

In light of the above results, the regulation of tomato HsfA2 AS is of great importance for understanding the regulation system of plant HSR. Apart from tomato, other Solanaceae species possess a second intron in the HsfA2 gene, which has not been reported up to now for any other plant family (Fig. 15). In all cases alternative splicing results in the generation of two isoforms, one with NES and one without, suggesting that this is a highly conserved mechanism in Solanaceae species. Worth mentioning, that in many species that encode for more than two HsfA2 paralogues (Scharf et al., 2012), at least one paralogue has NES and another not, suggesting that the functional diversification gained by AS in Solanaceae has been substituted by gene duplication in other families including several dicots.

From all tomato species (*Solanum* section *Lycopersicon*), *Solanum lycopersicum* is the only one which was domesticated (Peralta et al., 2006). Initially tomato domestication resulted in the generation of the small red fruited var. cerasiforme, which then through breeding was improved to generate the modern big fruit germplasm (Bai & Lindhout, 2007). Actually, several lines of evidence support the hypothesis that “cerasiforme” plants are not the direct ancestors of the cultivated tomato but modern culticars are rather a genetic mixture of wild and cultivated tomatoes (Nesbitt and Tanksley, 2002). The examination of splicing pattern of HsfA2 in accessions of different tomato species revealed the presence of 3 intronic SNPs that are related to pre-mRNA splicing (Fig. 17). *S. lycopersicum* accessions and the closely related *S. galapangense* possess in these position G nucleotide while the more phylogenetically distant wild accessions have an A on these positions (Fig. 17). Based on a minigene assay we were able to show that indeed the 3 SNPs are important cis-elements for intron splicing (Fig. 16). The third SNP is important for the generation of HsfA2-Iγ, as it is belongs to the dinucleotide GT serving as 5’-donor splicing site. However, the other two nucleotides are also important as a G to A substitution increases splicing efficiency allowing higher synthesis of HsfA2-II (Fig. 17).

Among the tomato species, only *S. pimpinelifolium* showed an interspecies polymorphism as one accession has no polymorphism compared to *S. lycopersicum* while the other has a G to A substitution in the second SNP position (Fig. 17). As mentioned above, the accessions with the “wild” polymorphic HsfA2 showed increased accumulation of HS-induced genes and seedling thermotolerance compared to the *S. lycopersicum* and non-polymorphic HsfA2 accessions (Fig. 17). Even the polymorphic HsfA2 *S. pimpinelifolium* accession showed increased

thermotolerance compared to the non-polymorphic which showed similar sensitivity to the domesticated accessions.

As in many crop plants, cultivated tomatoes contain only a very small fraction of the genetic variation that is available in related wild species and landraces (Tanksley & McCouch, 1997). The wild species are considered as a genetic resource of valuable traits and understanding HSR and thermotolerance in these species will stimulate breeding efforts. The exclusion of HsfA2 polymorphism during domestication cannot be explained by this data. Tomato breeding has been designed mainly for fruit size, uniformity of ripening and resistance to biotic and abiotic stress factors (Bai and Lindhout, 2007). On one side, the absence of HsfA2 polymorphism might be intentional as thermotolerance in many cases is related to lower yield due to the interference of stress and developmental networks (Fragkostefanakis et al., 2016). In the same direction, it is possible that the cultivation of tomato cultivars in more favourable environments alleviated the requirement of such stress tolerance related wild loci. On the other side, the locus of HsfA2 might include genes related to undesired traits. Nevertheless, wild species can provide wild genetic resources for thermotolerance traits that could be introduced into domesticated cultivars to generate heat stress resistance tomatoes.

To understand better these phenomenon, thermotolerance assays have to be performed under different climate scenarios and the outcome has to be directly related to fruit yield and quality, which is difficult to assess in wild species. Instead such efforts can utilize existing introgression lines, in which cultivated tomatoes harbour single chromosomal segments from wild relatives. In such an approach, we identified IL8-1 originating from crossing *S. lycopersicum* M82 x *S. pennellii* with HsfA2 carrying locus (Fig. 18). Seedlings of IL8-1 showed increased thermotolerance compared to M82 and similar to *S. pennellii*, highlighting the potential of using the wild tomato HsfA2 locus for breeding.

SNPs related to alternative splicing have been previously reported, emphasizing the importance of such a regulatory mechanism. Alignment of *BRC1a* genomic sequences of several tomato wild species, potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), wild and domesticated peper (*Capsicum annuum*), Petunia hybrid and tobacco (*Nicotiana tabacum*) revealed that the GT-AG nucleotides corresponding to donor site and acceptor site of canonical splice sites in all the Solanum species change to the GT-GG sequence in pepper, petunia, and tobacco. This change in spice site choice results in two splice variants of *BRC1a* in potato while only a single *BRC1a* transcript in petunia and pepper (Nicolas et al., 2015). Moreover, the single G to A polymorphism in the floral repressor locus *FLOWERING LOCUS C* (FLC) influences COOLAIR class II splicing and increases *FLC* expression. This SNP is identified as a major contributor to the natural variation in flowering in *Arabidopsis thaliana* accessions (Li et al.,

2015). Therefore, it is important to evaluate the functional relevance of SNPs identified by genome wide analyses and their relation to transcriptome and proteome alterations as well as their potential use in breeding for different traits.

7.4 Regulation of HsfA2 intron 2 splicing by RS2Z36

HsfA2 pre-mRNA splicing is an important stress related event. Natural variation in splicing efficiency is dependent on intronic cis-elements and is independent of the genetic background as splicing efficiency was high for *S. peruvianum* minigene in stressed *S. lycopersicum* protoplasts, but also splicing was similar when the *S. lycopersicum* minigene was expressed in *A. thaliana* protoplasts (Fig. 16).

SR proteins have been identified as essential splicing factors in both animals and plants. They can bind pre-mRNAs and act as splicing enhancers or repressors of both constitutive and alternative splicing and therefore they were considered as primary candidates of splicing regulators of HsfA2 pre-mRNA. The number of SR proteins in flowering plants is highest among eukaryotes, with 24 in rice and 19 in Arabidopsis (Iida & Go, 2006; Isshiki, 2006; Reddy, 2004). This expansion might be attributed to the differences between flowering plants and animals in exon/intron architecture, as well as plant-specific *cis*-elements involved in pre-mRNA splicing. SR proteins are identified as essential regulators of gene expression as they play important roles in plant pre-mRNA splicing (Long & Caceres, 2009). In addition, it has shown that AtSF1, the *Arabidopsis* SF1 homolog, is required for alternative splicing of AtHsfA2 intron 1 (Jang et al., 2014).

The minigene assay revealed that in non-stressed protoplasts the level of HsfA2-I and II transcripts of both *S. lycopersicum* and *S. peruvianum* are similar (Fig. 16). Under 37.5^{o}C stress conditions, splicing is inhibited in SolycHsfA2 and enhanced in SoperHsfA2. We assumed that the splicing factor regulating HsfA2 splicing is induced or repressed by HS. Although the induction or repression can be at different levels of regulation from transcription to post-translational modification, we checked for the transcript levels of the 17 SR proteins in a transcriptome dataset from seedlings exposed to two temperature stresses. Interestingly, we realized significant differences in the abundance of genes encoding for SR proteins, while some of them were also HS-induced (Fig. 20). Most of the SR proteins are extensively alternatively spliced with HS affecting the ratio among the various transcript variants (Fig. 21). The levels of the functional transcripts from some genes were induced (e.g. RS2Z36, SR32) or even repressed under HS conditions (e.g. SCL29, SC30a). These results show the extensive effect that high temperatures have on transcriptional and posttranscriptional regulation of SR proteins. Similar

regulation has been shown for *A. thaliana* SR co-orthologues (Palusa et al., 2007). The majority of AS events in SR proteins are related with PTC and NMD clearance mechanism, providing a means to the cell to rapidly adjust these regulators to the required levels.

Using an immunodetection system which allowed an initial screening of changes in the ratio of GFP-HsfA2 isoforms, two factors namely SCL29 and RS2Z36 which had an inhibitory effect on splicing of SolycHsfA2 but not SoperHsfA2 were selected for further analysis. We confirmed the inhibitory effect of RS2Z36 on splicing on the RNA levels for SolycHsfA2 but not for SoperHsfA2, which suggests that this SR protein acts as a splicing repressor. It is not clear how SCL29 might contribute to the higher accumulation of GFP-HsfA2-I isoform at the protein but not mRNA level.

SR protein are involved in several cellular processes including mRNA export from the nucleus, NMD, stability and translation (Long and Caceras 2009; Reddy et al., 2013). SCL29 might be involved in *S. lycopersicum* HsfA2 pre-mRNA nuclear export and preferably accelerate the translation of *S. lycopersicum* HsfA2-I splice variant leading to altered ratio of *S. lycopersicum* HsfA2-I to A2-II at protein level, which could be the reason why SCL29 can alter the ratio of *S. lycopersicum* HsfA2 isoforms at protein level but does not influence the spicing pattern of HsfA2 at the transcript level (Fig. 22, 23). Further experiments are required to examine this hypothesis and to unravel the function of SCL29.

7.5 Splicing of Hsfs as a general regulatory mechanism

Similar to HsfA2 another 4 class A Hsfs undergo AS in a temperature-dependent manner, which highlights the importance of AS in the regulation of HSR and thermotolerance (Fig. 19). The complexity of splicing differs among Hsfs, with HsfA6b generating only two and HsfA1b five variants. Interestingly, the expression levels of these HS-induced Hsfs reach the peaks at different temperatures, indicating that transcription and AS are major regulators of temperature-specific functions of Hsfs.

AS can regulate transcript abundance or stability by subjecting it to the NMD. Introduction of a PTC, due to intron retention, can lead to either generation of a shorter protein or degradation of the mRNA through NMD. However, it has been shown that most of the IR events in Arabidopsis that harbor features of NMD escape this mechanism, which prompts us to think what are the fates of these transcripts and the function of gene expression regulation by IR events (Kalyna et al., 2012). In plants, several reports have revealed that stable NMD-insensitive PTC-containing mRNAs play important roles in transcriptome adaptation to environmental stresses (Mastrangelo et al., 2012; Reddy et al., 2013; Staiger & Brown, 2013). AS transcripts that are subjected to NMD are often barely detectable. In support of this, similar to the splicing events

reported for *Arabidopsis* HsfA2 (Liu et al., 2013), AS of intron 1 in tomato HsfA2 generates three splice variants (Fig. 7). These three transcripts possess an in-frame PTC and only appear, with very low abundance, at severe HS or during the recovery from such a strong stress (Fig. 7 and 8) and are probably degraded by NMD (Fig. 8). In contrast, PTC-containing transcripts of HsfA1b and HsfA3 are generated in the absence of HS and become abundant in a broad range of temperatures (Fig. 19). However, the fate of these transcripts is still not clear. They could be degraded by NMD or might escape from degradation by NMD and stored in the nucleus until further processed to generate truncated but functional proteins upon recovery from stress.

AS of HsfA2 results in the generation of protein isoforms with distinct and significant functions in thermotolerance of young tomato seedlings. In a similar manner, AS of HsfA6b and HsfA7 results in putative protein isoforms with diverse functions. In case of HsfA7, temperature-dependent AS leads to generate a new protein isoform missing the C-terminal NES. In case of HsfA6b, the new HsfA6b isoform, HsfA6b-II, is generated from AS event in the second intron of HsfA6b pre-mRNA and predicted to encode for a putative truncated protein with a full DBD (Fig. 19), but no other functional domains including activation domains. To get insights into the properties and functions of these isoforms and their role in HSR a series of established assays to study Hsf activity, DNA binding and localization will be utilized. First, the subcellular localization of HsfA7 isoforms will be investigated as shown for HsfA2. This is of particular interesting since efficient Hsf nuclear retention is required for activation of target genes and this capacity can be influenced by interactions with chaperones, other Hsfs but also by the intrinsic properties of the protein due to the presence of NLS and NES motifs. Second, the transcriptional activation activity of specific isoforms with altered activation domains has to be examined using a GUS reporter assay. Third, the effect of altered DBD structure on the ability of and Hsfs to bind HSEs is needed to be evaluated by employing the repressor reporter assay. In this reporter construct, HSE motifs are inserted downstream of the TATA box in the 5’ UTR of the GUS reporter genes. Recognition of these elements by Hsf will affect the progression of RNA polymerase-II. GUS can be detected only when no Hsf is bound to HSEs, as in case of Hsfs affected in DNA binding.

Due to loss of activation domains (Fig. 19), HsfA6b-II might not act as a transcriptional activator. Recently, it has been shown that *A. thaliana* HsfA6b plays an important role in ABA-mediated salt and drought resistance and positively regulates thermotolerance (Huang et al., 2016). The role of HsfA6b-II in HSR and thermotolerance in plants worths further investigation.

8 Conclusion and outlook

The natural genetic and phenotypic variations that occur in crop plants are the main resources for contemporary breeding strategies. Till now, the extent of intra-species variations of alternative splicing in crops has been poorly evaluated. Genome-wide studies can be applied to crop species, including a large collection of related domesticated cultivars and wild accessions, exposed to different stress treatments. Hopefully, some stress-related alternative splicing events that are important for resistance would be identified, which might provide insights into molecular basis of stress resistance mechanisms. Furthermore, the investigation of genetic variability in cultivars and wild species might help to select genotypes for alternative splicing events with a clear effect on stress resistance. This might represent a good strategy for decreasing the drop in crop yields under stressful conditions.

It is becoming increasingly clear that alternative splicing can be influenced by changes in temperature and it is a mechanism that responses to cold or high temperatures (Bove et al., 2008; Capovilla et al., 2015; Palusa et al., 2007; Robinson & Parkin, 2008; Simpson et al., 2007; Yan et al., 2012). In here, we show that the heat stress transcription factor HsfA2, undergoes alternative splicing to generate two protein isoforms, HsfA2-I involved in acquired thermotolerance, and HsfA2-II in direct heat stress response. Variation in splicing efficiency among cultivated and wild accessions is due to three intronic nucleotide polymorphisms. Seedlings of many wild accessions carrying the polymorphic allele, exhibit stronger stress responses and enhanced tolerance against a severe stress incident, due to higher HsfA2-II levels. Seedling thermotolerance of cultivar M82 was increased by the introgression of HsfA2 allele from *S. pennellii*, highlighting the potential of this polymorphism for breeding. We propose that the HsfA2 polymorphic allele has stimulated the survival of many wild accessions in harsher environments, but was probably excluded during tomato improvement as breeding took place under more favorable environmental conditions. Future efforts to understand the broader effect of HsfA2 splicing in thermotolerance should concentrate on agronomically relevant traits, such as pollen quality, fruit set and fruit yield and quality. The investigation in this direction could involve different tomato introgression lines generated by crossing tomato cultivated and wild species.

As alternative splicing is a major step towards regulation of HSR and thermotolerance, the molecular mechanism underlying specific critical events, like Hsf splicing should be investigated into detail. This includes the identification of proteins involved in splicing, for example by RNA-protein pulldown approaches. Based on the results of this thesis, RS2Z36 seems to be a major candidate for regulation of HsfA2 intron 2 splicing, however further evidence to prove direct interaction or the nature of the association is required. In a similar manner, the investigation of the broader range of action of RS2Z36 is of major interest and should be approached.

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10 Supplemental information

Supplemental table S1- Oligonucleotides for RT-PCR

Amplified transcript	Gene ID	Primer numbers	Sequence
*Sl*HsfA2 (1st exon + last exon)	Solyc08g062960	3485 (F)	TTCCACCACATTGTTGCCTA
		6027 (R)	GGACATTCCTCCAGACTAAC
*Sl*HsfA2 (2nd exon + last exon)	Solyc08g062960	6026 (F)	AGGCCGGATTCTGTTGTGAC
		7332 (R)	GAGACCGCCTCAAAGCTTCCTG
(*SI&SP*)HsfA2-minigene	Solyc08g062960	6997 (F)	TCCAATTGGCGATGGCCCTGTCC
		6027 (R)	GGACATTCCTCCAGACTAAC
*St*HsfA2	PGSC0003DMG400008223	7896 (F)	AGGGCCTGAAGATATTTGGGA
		7897 (R)	ACTGCCTCAAAGCTTGCTTC
*Ca*HsfA2	CA08g05000	7898 (F)	TTGTGACAGCAAGTGGAACC
		7899 (R)	AAAAAGCTGTGAAGTGGCAGT
*Nb*HsfA2	Nb101Scf01777g02001.1	7900 (F)	CAAGGTCGGCTTCTGTTGTG
		7901 (R)	ACTGCTCCAACATAACTGCC
*Ph*HsfA2	Peaxi162Scf00327g00413	7904 (F)	CCGAGCTTGCAAATATGGGA
		7905 (R)	TGTTACCGCATCCCTCACTT
*St*EF1α	PGSC0003DMG400008117	7972 (F)	ATTGGAAACGGATATGCTCCA
		7973 (R)	TCCTTACCTGAACGCCTGTCA
*Ca*UBI3	AY486137.1	7968 (F)	TGTCCATCTGCTCTCTGTTG
		7969 (R)	CACCCCAAGCACAATAAGAC
*Nb*EF1α	NbS00021090g0020.1	8054 (F)	GGAAACTGGTGTCCTCAAGC
		8055 (R)	GGAGTTGGAAGCAACAAACC
*Ph*GAPDH	*SGNU209515*	7970 (F)	CCTGGTCAAATTGGAAACGG
		7971 (R)	CAGATCGCCTGTCAATCTTGG
*(SI&SP)*EF1α	Solyc06g005060	7038 (F)	GGAACTTGAGAAGGAGCCTAAG
		7039 (R)	CAACACCAACAGCAACAGTCT
*Sl*HsfA1a	Solyc08g005170	6570 (F)	ACAAATGATGTCGTTCCTGGC
		6571 (R)	GAAAGCTCCCTCAACATTGCC
*Sl*HsfA1b	Solyc03g097120	6376 (F)	AATGGACGGAGTTCATGAGG
		7343 (R)	GGTCCGATATGATAGATAGTG
*Sl*HsfA1c	Solyc08g076590	6378 (F)	CCTAATCCCCAAAACCCTGT
		7429 (R)	CCCGTGACCCAATACACTAAG
*Sl*HsfA3	Solyc09g009100	7341 (F)	CTCCACCGACTTTACAGAG
		7342 (R)	CTATCCCATAACTCGGCACCAG
*Sl*HsfA4c	Solyc02g072000	7431 (F)	GGAAGAAAGATATTCGGGAATG
		7432 (R)	CTCATATCATGCAATGCACAGC
*Sl*HsfA6b	Solyc06g053960/50	6087 (F)	TTACCTTTGCCAAGATCCTCT
		7340 (R)	GCTTCTTCAAGTTGTTTCC
*Sl*HsfA7	Solyc09g065660	6386 (F)	GCTTCTTTTATCCATGGTGTCC
		6473 (R)	CCATAAACTTGATCAGGATCTGC

*Sl*HsfB1	Solyc02g090820	6380 (F)	TGCCCTTTTGCTTTTCAGTT
		6381 (R)	CCGTGAACTGGGACAACTTT
*Sl*HsfB2a	Solyc03g026020	6382 (F)	CCCACACCCACTCACCTCTA
		7344 (R)	GACATAGTTGAATTACCCG
*Sl*SR30	Solyc01g099810	7043 (F)	TTTTTGCTTTCTACGCAACC
		7044 (R)	GATCTGGATCTTGATAGAGAT
*Sl*SR34	Solyc09g075090	7045 (F)	GAACATGCTGTTTGCTTCAC
		7046 (R)	AATCCACAAACCGCCGCA
*Sl*SR32	Solyc03g082380	7047 (F)	GAACTCTCTGGAAGAAGTGCAG
		7048 (R)	AAAACCACTGTGCAAAAGTCTAC
*Sl*RS29	Solyc01g096180	7049 (F)	TCGGTCCCGTAGAACTTTTC
		7050 (R)	CAAATTGAACTGCCTAACTCTCTA
*Sl*SC24	Solyc10g009330	7051 (F)	ATGAAATCTGGGTATGCTTTCGT
		7052 (R)	ACATTCATAGGAAATGAGAATACTAC
*Sl*RS40	Solyc03g026240	7053 (F)	CGCTTTGCTGTTGCAGTTAG
		7054 (R)	TCCAAGCAACTTCAAGGTGTAA
*Sl*RS41	Solyc11g072340	7055 (F)	CTTTTCAGAAGATATGGGAGAG
		7056 (R)	AGCGGGATCTTTCTCGAGTT
*Sl*RSZ28	Solyc11g072340	7057 (F)	GTTGAGCGTTGGGTGTGC
		7058 (R)	GGTTCTTAAAAAGGGTAACATTGA
*Sl*RS2Z38	Solyc05g054920	7059 (F)	ATGGAAAACAGAGAGACCAC
		7060 (R)	TTTGATGGAGGTGGTGATGA
*Sl*SCL29	Solyc01g005820	7061 (F)	TGAACCAACCATCAATTTGG
		7062 (R)	TTTCCCATGCATACACGAAG
*Sl*SC30a	Solyc04g074040	7063 (F)	ACCGGACATCAAAGACACCT
		7064 (R)	CCACATTGACTTTTCACGACC
*Sl*SC30b	Solyc01g105140	7065 (F)	ATTACAAACGGGGTGACGAA
		7066 (R)	ACCTCACCAACCTTCTCTCG
*Sl*SCL19	Solyc01g080660	7067 (F)	CGAGGGTTTTGGATTTTCTC
		7068 (R)	CTTTAAGTCTCTTTCTCAATTTAGC
*Sl*SR46	Solyc10g005590	7069 (F)	CAATGGCGAAACCAGGTC
		7070 (R)	TCCTAAGGGGCTCCTTTCTC
*Sl*RS2Z36	Solyc09g005980	7071 (F)	AATAGCACTCGTCTCTATGTG
		7072 (R)	TGAAGGACTGACGCTCCTCT
Putative SR protein	Solyc03g093350	7406 (F)	CAATCTCAATCTCCCGCAGT
		7407 (R)	CATATTCTGCATCCGCACCATAGG
*Sl*SR28	Solyc06g009060	9616 (F)	ATGAGTCGTTCAAGTAGGACG
		9617 (R)	CCACAATATAGCTCTCACCTG
M13 (Sequencing)		7316 (F)	GTAAAACGACGGCCAGT
		7317 (R)	CAGGAAACAGCTATGAC

Supplemental table S2. Oligonucleotides for qRT-PCR			
Amplified gene	Gene ID	Primer number	Sequence
*Sl*EF1α	Solyc06g005060	7038 (F)	GGAACTTGAGAAGGAGCCTAAG
		7039 (R)	CAACACCAACAGCAACAGTCT
*Sl*UBI	Solyc07g064130	5743 (F)	GGACGGACGTACTCTAGCTGAT
		5744 (R)	AGCTTTCGACCTCAAGGGTA
*Sl*HsfA2 (total)	Solyc08g062960	6347 (F)	AGGTGGATCCTGACAGATGG
		3486 (R)	GCAAGCACCAGATCCTTGTT
*Sl*HsfA2-IIIα	Solyc08g062960	7450 (F)	GGAATATGAGTTTTATTGAC
		3486 (R)	GCAAGCACCAGATCCTTGTT
*Sl*HsfA2-IIIβ	Solyc08g062960	7449 (F)	GAACACCGAGGTGATCACT
		6092 (R)	TCCACCTTTCTAAATCCCTAGCT
SlHsfA2-IIIγ	Solyc08g062960	10022 (F)	CATTCGGCAGCTTAACACATATATTG
		6092 (R)	TCCACCTTTCTAAATCCCTAGCT
*Sl*Hsp17.7CCI	Solyc09g015020	6023 (F)	ATGGAGAGAAGCAGCGGTAA
		6024 (R)	ATGTCAATGGCCTTCACCTC
*Sl*Hsp70-5	Solyc04g011440	6243 (F)	GGAAGTGGACTAAGCTCCACA
		6244 (R)	CGAAGGATATTTCTACATACACAAA
SlAPX3	Solyc01g104740	6259 (F)	CCGCCCTCTAGTCGAGAAAT
		6260 (R)	AGAACCAGACTGATCTCCAGAGA

Supplemental table S3. Antibodies used in this study			
Primary antibody	Antigen	Organism	Dilution
α-HA	3HA-Tag	Mouse	1:2000
α-GFP (monoclonal)	GFP-Tag	Mouse	1:5000
α-30HN	*Sl*HsfA2	Rabbit	1:10000
α-pep6	*Sl*HsfA2-I	Rabbit	1:5000
α-8HN	*Sl*HsfA1	Rabbit	1:5000
α-17CI	*Sl*Hsp17CCI	Rabbit	1:3000
α-101	*Sl*Hsp101	Rabbit	1.3000
Secondary antibody	Antigen	Organism	Dilution
α -mouse HRP	IgG heavy chain mouse	Goat	1:10000
α-rabbit HRP	IgG heavy chain rabbit	Goat	1:10000

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Thank you

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12 Curriculum Vitae

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Conference and workshop

02/2016	29th Conference of Plant Molecular Biology, Dabringhausen, Germany (Poster)
03/2015	Stress Biology and Crop Fertility, Sorrento, Italy (Poster)
03/2014	Physiological, biochemical and cell biological tools for the analysis of PTT, Frankfurt (workshop)
11/2013	SPOT-ITN conference, Arnhem, Netherlands (Poster)

Publications

Hu Y, Mesihovic A, Röth S, Scharf KD, Schleiff E, Frakostefanakis S. Alternative splicing of a heat stress transcription factor mediates natural variation in thermotolerance of tomato species (In preparation)

Hu Y, Lang M, Simm S, Fragkostefanakis S, Schleiff E. High temperature differentially modulates transcription in young seedlings of heat-tolerant and heat-sensitive tomato cultivars (In preparation)

Keller M, Hu Y, Mesohovic A, Paul P, Fragkostefanakis S, Schleiff E, Simm S (2016) Alternative splicing in tomato pollen in response to heat stress. *DNA Research*, doi: 10.1093/dnares/dsw051

Fragkostefanakis S, Simm S, Mesihovic A, Paupière MJ, Hu Y, Paul P, Mishra SK, Tschiersch B, Theres K, Bovy A, Schleiff E, Scharf KD (2016) HsfA2 controls the activity of developmentally and stress–regulated heat stress protection mechanisms in tomato male reproductive tissues. *Plant Physiology doi:10.1104/pp.15.01913*

Fragkostefanakis S, Mesihovic A, Hu Y, Schleiff E. (2016) Unfolded protein responses in pollen Special Issue on “Pollen development and Stress response” in the journal of Plant Reproduction) 29: 1, 81–91 *doi: 10.1007/s00497-016-0276-8*

Erklärung

Ich erkläre hiermit, dass ich mich bisher keener Doktorprüfung unterzogen habe.

Frankfurt am Main, den ……. ……

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation über

„Alternative splicing of HsfA2 mediates thermotolerance in tomato species"

Selbstständig angefertigt habe und keine anderen als die hier genannten Hilfsmittel verwendet habe. Alle Entlehnungen aus anderen Veröffentlichungen wurden entsprechend im Text gekennzeichnet.

Ich versichere, dass ich keine Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen habe.

Frankfurt am Main, den ……. ……..