Characterization of subclass A1 heat stress transcription factors in tomato

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1. Zusammenfassung

Die Tomate (*Solanum lycopersicum*) ist ein bedeutendes Nahrungsmittel und ihr kommt eine enorme wirtschaftliche Bedeutung zu. Sie zählt zudem zu den wichtigen Modellorganismen der modernen Biologie und wurde umfassend in Studien über die physiologischen und molekularen Wirkungen von Hitzestress eingesetzt. Wie in allen eukaryotischen Organismen werden die grundlegenden Prozesse der Hitzestressantwort in der Tomate von Hitzestresstranskriptionsfaktoren (Hsfs) gesteuert, welche die Transkription der meisten Stress-induzierten Gene, wie die der der Hitzeschockproteine (Hsps), regeln. Hsfs der Pflanze sind auf Basis ihrer strukturellen Besonderheiten in drei Klassen unterteilt, Klasse A, B und C, wobei Klasse A als transkriptionelle Aktivatoren agieren (Scharf et al., 2012). Die Unterklasse HsfA1 wurde in *Arabidopsis thaliana* und in der Tomate als Hauptregulator der Hitzestressantwort beschrieben (Mishra et a., 2002; Liu et al., 2011). Während diese Funktion in *A. thaliana* zwischen drei HsfA1 Proteinen aufgeteilt ist, wurde sie in Tomate nur für ein einziges Gen beschrieben, HsfA1a (Mishra et al., 2002; Liu et al., 2011). Dies wurde aus hoher Thermosensivität von transgenen HsfA1a co-suppressiven Pflanzen (A1CS) aufgrund ihrer Unfähigkeit eine Hitzestressantwort einzuleiten geschlussfolgert.

Die vollständige Sequenzierung des Tomatengenoms erlaubte die Identifikation von zusätzlichen Hsfs, was drei zusätzliche HsfA1-codierenden Gene einschließt. Unter Zuhilfenahme dieser Erkenntnisse wurde das Modell von HsfA1a als Hauptregulator der Hitzestressantwort überdacht. Klonierung und Expression von HsfA1a, HsfA1b, HsfA1c und HsfA1e in A1CS Protoplasten zeigten, dass die kurzen Interferenz-RNAs (siRNAs), welche in A1CS Pflanzen produziert werden, alle HsfA1 Gene unterdrücken können. In Konsequenz wurde eine ausführliche Analyse der HsfA1 Eigenschaften und Funktionen durchgeführt, um Einblicke in ihren Beitrag zur Hitzestressantwort zu erlangen und um spezifische Eigenschaften zu identifizieren, die zur Funktion der Hauptregulatorfunktion beitragen.

HsfA1a ist ein konstitutiv-exprimiertes Gen, dessen Protein in Zellen unter normalen physiologischen Bedingungen vorliegt, jedoch durch direkte Interaktion mit hochmolekulargewichtigen Chaperonen Hsp70 und Hsp90 inaktiv gehalten wird (Hahn et al., 2011). Eine Analyse der Transkript-Mengen von HsfA1 Genen in verschiedenen Geweben und Entwicklungsstadien mit Hilfe von qRT-PCR oder von verfügbaren RNAseq Daten, zeigte, dass HsfA1b während der Entwicklung reguliert und mit hoher Präferenz in sich entwickelnden Samen und reifenden Früchten exprimiert wird. Zusätzlich dazu werden HsfA1c und HsfA1e nur minimal exprimiert, sind jedoch in reifen Früchten hochreguliert. Zusätzlich wird HsfA1b als Reaktion auf einen 15 minütigen Hitzestress stark exprimiert, während HsfA1c und HsfA1e nur schwach durch einen lang anhaltenden Hitzestress hochreguliert werden. Dahingegen zeigt HsfA1a konstante Level in verschiedenen Geweben und unter Hitzestress, während

andere HsfA1 Gene entweder nur sehr schwach exprimiert werden oder präferentielle Induktion in verschiedenen Geweben und Stressstadien zeigen. Zusätzlich zeigte eine Analyse der Proteinstabilität jedes HsfA1 nach der Expression in Mesophyll Protoplasten der Tomate mit anschließender Hinzugabe von Cycloheximid, dass HsfA1a und HsfA1c stabil sind, wohingegen HsfA1b und HsfA1e schnell degradiert werden.

Die subzelluläre Lokalisation von N-terminal mit grün fluoreszierendem Protein (GFP) gekoppelten HsfA1 Proteinen, wurde mit Hilfe eines Konfokalen Laser Scanning Mikroskops in Protoplasten untersucht. HsfA1a, HsfA1b und HsfA1c besitzen ein Lokalisations- und Exportsignal für den Kern, jedoch zeigten nur HsfA1a und HsfA1c eine Verteilung zwischen dem Kern und dem Zytoplasma, während HsfA1b und HsfA1e nur im Zellkern detektiert werden konnten. Diese Ergebnisse könnten den schnellen Proteinabbau von HsfA1b und HsfA1e erklären, da bereits für HsfA1 und HsfB1 der Abbau über das 26S-Proteasom innerhalb des Zellkerns nachgewiesen wurde (Röth et al., 2016; Mesihovic PhD, 2018).

Die Fähigkeit von HsfA1 Genen zur trans-Aktivierung wurde mit Hilfe der GUS-Reporter Methode an Promotoren von Stress-induzierten Hsfs and Hsps getestet. HsfA1a aktivierte alle getesteten Promotoren, wohingegen HsfA1c und HsfA1e selektive spezifische Promotoren binden, zum Beispiel Hsp70-1 und HsfA7. HsfA1b hingegen zeigte geringe Aktivität an allen getesteten Reporter Konstrukten. Diese Befunde wurden mit Hilfe von transienter Expression der HsfA1 Proteine in Protoplasten und das Messen von deren Wirkung auf die endogenen Promotoren auf eine größere Gruppe von Stress induzierten Genen erweitert. DNA Bindung, als notwendiger Schritt zur Initiation der transkriptionellen Aktivität, wurde mit Hilfe von speziell entwickelten GUS-Repressor Reportern (Hahn et al., 2011) untersucht. Dabei zeigte HsfA1a im Vergleich mit anderen HsfA1 die höchste DNA Bindekapazität, was eine Erklärung der starken transkriptionellen Aktivität von HsfA1a liefern könnte. Diese Ergebnisse deuten darauf hin, dass unter allen HsfA1 nur HsfA1a die benötigte Aktivitätsspanne über verschiedene Gene besitzt, die für die Einleitung der Hitzestressantwort und die damit einhergehenden frühen Schutzmechanismen der Proteinhomöostase, wie die Akkumulation von verschiedenen Hsps, benötigt wird.

Eine wichtige Eigenschaft von HsfA1a ist die Fähigkeit heterooligomerische Komplexe mit HsfA2, HsA7 und HsfB1 zu bilden, welche eine starke Aktivierungsfunktion besitzen. Wie HsfA1a besitzen auch andere HsfA1 Proteine diese synergistische Aktivität, wenn sie mit HsfA2 gemeinsam exprimiert werden. Dies gilt jedoch nicht bei gemeinsamer Expression mit HsfA7 und HsfB1. Interessanterweise zeigt HsfA1b den stärksten synergistischen Effekt zusammen mit HsfA3. Diese Ergebnisse deuten auf die Entstehung von spezifischen Co-Aktivator-Komplexen unter Hitzestress hin, welche mutmaßlich

Funktionen in unterschiedlichen Stadien der Hitzestressantwort oder in unterschiedlichen Genregulationsnetzwerken einnehmen.

Trotz gemeinsamen Eigenschaften unter den HsfA1, insbesondere zwischen HsfA1a und HsfA1c, wurden auch erheblichen Unterschiede in der transkriptionellen Aktivität und Spezifität beobachtet. Aus diesem Grund wurden die Effekte der verschiedenen Domänen genauer untersuchten. Die synergistische Aktivität von HsfA1a mit HsfA2 und HsfB1 wurden der Oligomerisation zwischen HsfA1a und HsfA2 zugesprochen (Chan-Schaminet et al., 2009). Die C-terminale Domäne von HsfA1a wird benötigt um mit HAC1 zu interagieren und dadurch ternäre Komplexe mit HsfB1 einzugehen (Bharti et al., 2004). Darauf basierend wurde zuerst die transkriptionelle Aktivität von Mutanten, bei welchen die C-terminalen Domäne (CTD) sowie der Oligomerisationsdomäne (OD) vertauscht wurden, mit Hilfe des GUS-Reporter Systems untersucht. Interessanterweise wurden keine Unterschiede in der transkriptionellen Aktivität des nativen Proteins und der entsprechenden Austauschmutante beobachtet. Daraus kann geschlussfolgert werden, dass diese Domänen nicht oder nur gering zur unterschiedlichen Funktionalität von HsfA1a und HsfA1c beiträgt. Im Gegensatz dazu führte der Austausch der DNA binde Domäne (DBD) von HsfA1a und HsfA1c zu einem Tausch der transkriptionellen Aktivitäten und Spezifitäten bezüglich der Zielgene, wobei HsfA1a Mutanten, die die HsfA1c DBD besaßen, eine höheren Aktivität an Hsp70-1 sowie sehr viel niedrigere Aktivität an sHSPs zeigten und sich dabei wie HsfA1c verhielten. Diese Beobachtung wurde auch in anderen, verwandten Aktivitäten, wie der DNA Bindeaktivität und der Induktion von endogenen HS-Genen gemacht, was die entscheidende Rolle der DBD von HsfA1a bezügliche der DNA Binde- und Transaktivatorkapazität zeigt.

Ein Sequenzvergleich unter den DBDs von HsfA1 Proteinen führte zur Identifizierung von Aminosäuren, die zu der einzigartigen Aktivität von HsfA1a beitragen könnten. Es konnte eine Position innerhalb der β3-Loop-β4 Region der DBD identifiziert werden, an welcher HsfA1a und HsfA1b ein hoch konserviertes Argenine besitzen, wogegen in HsfA1c ein Leucin, und in HsfA1e ein Cystein zu finden ist. Interessanter Weise ist der Loop in pflanzlichen Hsfs kürzer als in den Hsfs der Metazoa und Hefe (Schultheiss et al., 1996). Dieser Loop ist wichtig für die Interaktion mit benachbarten Hsfs, wobei diese Hsfs zur Verstärkung der DNA Bindung und zur Definition der Spezifität für Zielgenen beitragen können (Ahn et al., 2001). Die Mutation von Arginin zu Leucin in HsfA1a resultierte in einem Verlust der DNA Bindekapazität und Aktivität an Promotoren der kleinen Hsps, wobei die Aktivität an dem Promotor vom Hsf70-1 kodierenden Gen nicht beeinträchtigt wurde. Verschiedene Mutationen in benachbarten Aminosäuren hatten keinen signifikanten Effekt auf die Aktivität oder Spezifität von HsfA1a. Dies zeigt, dass dieses Arginin eine wichtige Funktion einnimmt, indem es entweder zur direkten Bindung der DNA oder anderer Hsfs beiträgt oder die DBD Konformation stabilisiert, um effektiv an Promotoren mit spezifischen Hitzestresselementarchitektur binden zu können.

Die Analyse von insgesamt 60 HsfA1 Genen verschiedener Pflanzenspezies dokumentierte eine starke Konservierung des Arginins an dieser Position. Interessanter Weise existieren die beobachteten Unterschiede der Aminosäure an dieser Position innerhalb der Tomaten Hsfs auch in *Solanum tuberosum*, was vermuten lässt, dass eine Divergenz innerhalb der HsfA1 Familie über die Zeit der Solanacea Evolution aufgetreten ist.

Zusammenfassend unterstützen die hier gezeigten Ergebnisse das bisherige Model eines einzelnen Hauptregulators innerhalb des Hitzestressantwortsystems der Tomate. Jedoch erlaubt die Abwesenheit des Arginins in HsfA1c und HsfA1e die funktionelle Diversifikation, was eventuell Vorteile in der Regulation spezifischer Gen-Netzwerke unter Hitzestress oder anderer abiotischen Stresse bieten könnte. Genetische Ansätze könnten in Zukunft zu einem besseren Verständnis der spezifischen Rolle der individuellen HsfA1 Genen beitragen.

2. Abstract

Heat stress transcription factors (Hsfs) have an essential role in heat stress response (HSR) and thermotolerance by controlling the expression of hundreds of genes including heat shock proteins (Hsps) with molecular chaperone functions. Hsf family in plants shows a striking multiplicity, with more than 20 members in many species. In tomato (*Solanum lycopersicum*), HsfA1a was reported to act as the master regulator of the onset of HSR and therefore is essential for basal thermotolerance. Evidence for this was provided by the analysis of HsfA1a co-suppression (A1CS) transgenic plants, which exhibited hypersensitivity upon exposure to heat stress (HS) due to the inability of the plants to induce the expression of many HS-genes including HsfA2, HsfB1 and several Hsps. Completion of tomato genome sequencing allowed the completion of the Hsf inventory, which is consisted of 27 members, including another three HsfA1 genes, namely HsfA1b, HsfA1c and HsfA1e.

Consequently, the suppression effect of the short interference RNA in A1CS line was re-evaluated for all HsfA1 genes. We found that expression of all HsfA1 proteins was suppressed in A1CS protoplasts. This result suggested that the model of single master regulator needs to be re-examined.

Expression analysis revealed that HsfA1a is constitutively expressed in different tissues under control and stress conditions, while HsfA1c and HsfA1e are minimally expressed in general, and show an induction during fruit ripening and a weak upregulation in late HSR. Instead HsfA1b shows preferential expression in specific tissues and is strongly and rapidly induced in response to HS. At the protein level HsfA1b and HsfA1e are rapidly degraded while HsfA1a and HsfA1c show a higher stability. In addition, HsfA1a and HsfA1c show a nucleocytosolic distribution, while HsfA1b and HsfA1e a strong nuclear retention.

A major property of a master regulator in HSR is thought to be its ability to cause a strong transactivation of a wide range of genes required for the initial activation of protective mechanisms. GUS reporter assays as well as analysis of transcript levels of several endogenous transcripts in protoplasts transiently expressing HsfA1 proteins revealed that HsfA1a can stimulate the transcription of many genes, while the other Hsfs have weaker activity and only on limited set of target genes. The low transcriptional activity of HsfA1c and HsfA1e can be attributed to the lower DNA capacity of the two factors as judged by a GUS reporter repressor assay.

HsfA1a has been shown to have synergistic activity with the stress induced HsfA2 and HsfB1. The formation of such complexes is considered as important for stimulation of transcription and long term stress adaptation. All HsfA1 members show synergistic activity with HsfA2, while only HsfA1a act as co-activator of HsfB1 and HsfA7. Interestingly, HsfA1b shows an exceptional synergistic activity with HsfA3, suggesting that different Hsf complexes might regulate different HS-related gene networks.

Altogether these results suggest that HsfA1a has unique characteristics within HsfA1 subfamily. This result is interesting considering the very high sequencing similarity among HsfA1 members, and particularly among HsfA1a and HsfA1c.

To understand the molecular basis of this discrepancy, a series of domain swapping mutants between HsfA1a and HsfA1c were generated. Oligomerization domain and C-terminal swaps did not affect the basal activity or co-activity of the proteins. Remarkably, an HsfA1a mutant harbouring the N-terminus of HsfA1c shows reduced activity and co-activity, while the reciprocal HsfA1c with the N-terminus of HsfA1a cause a gain of activity and enhanced DNA binding capacity.

Sequence analysis of the DBD of HsfA1 proteins revealed a divergence in the highly conserved C-terminus of the turn of β3-β4 sheet. As the vast majority of HsfA1 proteins, HsfA1a at this position comprises an Arg residue (R107), while HsfA1c a Leu and HsfA1e a Cys. An HsfA1a-R107L mutant has reduced DNA binding capacity and consequently activity. Therefore, the results presented here point to the essential function of this amino acid residue for DNA binding function. Interestingly, the mutation did not affect the activity of the protein on Hsp70-1, suggesting that the functionality of the DBD and consequently the transcription factor on different promoters with variable heat stress element number and architecture is dependent on structural peculiarities of the DBD.

In conclusion, the unique properties including expression pattern, transcriptional activities, stability, DBD-peculiarities are likely responsible for the dominant function of HsfA1a as a master regulator of HSR in tomato. Instead, other HsfA1-members are only participating in HSR or developmental regulations by regulating a specific set of genes. Furthermore, HsfA1b and HsfA1e are likely function as stress primers in specific tissues while HsfA1c as a co-regulator in mild HSR. Thereby, tomato subclass A1 presents another example of function diversity not only within the Hsf family but also within the Hsf-subfamily of closely related members. The diversification based on DBD peculiarities is likely to occur in potato as well. Therefore this might have eliminated the functional redundancy observed in other species such as *Arabidopsis thaliana* but has probably allowed the more refined regulation of Hsf networks possibly under different stress regimes, tissues and cell types.

3. Abbreviations

aa	Amino acid
AHA	Aromatic, hydrophobic, acidic
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
A1CS	HsfA1 co-suppression
BF	Bright field
CaMV	Cauliflower mosaic virus
CHX	Cycloheximide
CLSM	Confocal laser scanning microscopy
CTAD	Carboxyl-terminal activation domain
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
EF1a	Elongation Factor 1 a
GFP	Green fluorescent protein
GUS	β-glucuronidase
HAC1	Histone acetyltransferase of CBP family 1
HA-tag	Hemagglutinin-tag
HS	Heat stress
HSE	Heat stress element
Hsf	Heat stress transcription factor
HSG	Heat stress granules
Hsp	Heat shock protein
sHsp	Small heat shock protein
HSR	Heat stress response
LRbc	Large subunit of RuBisCO
Mc	Monoclonal
MU	4-methylumbelliferone
MUG	4-methylumbelliferone β-glucuronide
NES	Nuclear export signal
NLS	Nuclear localization signal
OD	Optical density
OD Hsf	Oligomerization domain
OE	Overexpression
OL	Overlay
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
Pc	Polyclonal
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
PIC	Pre-initiation complex
RD	Repressor domain
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
RNAi	RNA interference
RNAP	RNA polymerase II
mRNA	Messenger RNA
RT-PCR	Reverse transcription-PCR
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
TBP	TATA-box binding protein
TF	Transcription factor
SDS	Sodium dodecyl sulfate
SE	Standard error
UTR	Untranslated region
Wt	Wild-type

4. Introduction

Global warming is considered as a major threat for most of the agriculturally important crops (Lobell and Field, 2007). The predicted increase of average annual temperature as well as the more frequent occurrence of extreme phenomena like heat waves is expected to have a significant negative impact on nearly all major plant developmental processes (Bokszczanin, 2013). Deciphering the molecular and physiological principles of heat stress response (HSR) and thermotolerance is the basis for the development of plants that will be able to perform well under unfavorable conditions (Fragkostefanakis et al., 2015a).

4.1. Heat stress response in plants

During their lifetime plants are continuously exposed to environmental changes which when exceed specific thresholds they can cause stress, such as nutrient deficiency, high temperatures, drought, salinity, but also many others (Mittler, 2006). Among these stresses, high temperatures causing heat stress (HS) is one of the major and most severe forms of stress that plants can experience in nature. It typically occurs when plants are exposed to temperatures 5-15˚C above their optimum for growth and development for a period of time (Fragkostefanakis et al., 2015a). HS disturbs cellular homeostasis mainly due to denaturation and aggregation of proteins, increased membrane fluidity and inactivation of chloroplast or mitochondrial enzymes, eventually leading to severe retardation in growth and development, or even death (reviewed by Hasanuzzaman et al., 2013; Fragkostefanakis et al., 2015). Tight regulation of cellular response under HS is therefore extremely important for the survival, adaptation and recovery of the plants from stress. Plants have evolved a complex and versatile network that enables them to respond quickly and effectively to temperature changes (Kotak et al., 2007a; Scharf et al., 2012; Qu et al., 2013; Röth et al., 2016b). The primary response is regulated at the transcriptional level, by induction of genes with important protective functions (Kornberg, 2007), however, several levels of regulation exist to orchestrate the response mechanisms, including among others pre-mRNA splicing, translation control and post translational modifications (Merrick and Hershey, 1996; Day and Tuite, 1998).

Sensing of high temperatures is thought to occur via different mechanisms, including reactive oxygen species (ROS) accumulation (Mittler et al., 2004), activation of Ca^{+2} signaling (Saidi et al., 2009; Saidi et al., 2010), changes in membrane fluidity (Murata and Los, 1997), histone occupancy (Kumar and Wigge, 2010) and alteration in protein homeostasis (Sugio et al., 2009; Walter and Ron, 2011). High temperatures can lead to an increase of membrane fluidity, which in turn leads to the opening of

calcium channels and influx of calcium ions into the cell that can regulate different signaling pathways (Murata and Los, 1997; Saidi et al., 2010). For example, there are indications that Ca^{2+}-dependent activation of a mitogen-activated protein kinase (MAPK) is a component of the heat stress signaling pathway, likely involved in the activation of heat stress transcription factors, Hsfs (Saidi et al., 2009). Imbalances in metabolic activities caused by HS lead to the accumulation of ROS, which can also mediate the stress signal by activating downstream pathways via certain Hsfs or MAPKs and by opening additional calcium channels at the plasma membrane (Mittler et al., 2004). Some studies suggest that a decrease in histone occupancy induced by HS might also function as a mean of temperature sensing through DNA-nucleosome fluctuations which in turn regulate the gene expression (Kumar and Wigge, 2010).

The deviation in physiological homeostasis caused by HS results in the activation of a highly conserved stress response pathway called HSR (Kotak et al., 2007a; Kim et al., 2013). This signaling pathway transiently upregulates the so-called heat shock proteins (Hsps) many of which act as molecular chaperones, with primary aim to maintain and protect protein homeostasis (Tissières et al., 1974). Molecular chaperones not only contribute to the initial folding of proteins, but are involved in maintaining proteins in a native state and protecting them from aggregation, while they are also involved in protein quality systems regulating protein fate (Hartl et al., 2011; Kim et al., 2013; Balchin et al., 2016).

In eukaryotes, the genes encoding components of the cytosolic HSR are under the control of heat stress transcription factors (Hsfs), which recognize and bind to promoters of genes containing characteristic conserved heat stress elements (HSE), consisting of consecutive palindromic nGAAn sequence motifs (Pelham, 1982; Parker et al., 1984; Wu, 1984; Pirkkala et al., 2001; Baniwal et al., 2004; Scharf et al., 1990).

Interestingly, some Hsfs are constitutively expressed even under non-stress conditions, during which they remain cytosolic in an inactive monomeric form due to association with high molecular weight chaperones such as Hsp70 and Hsp90 (Zou et al., 1998; Hahn et al., 2011). According to the chaperone titration model, HS results in a higher load of denatured proteins, which competitively bind to Hsp70 and Hsp90 due to their higher affinity for denaturated proteins (Hahn et al., 2011) . This leads to the release of the Hsfs from the chaperone complex, which then form trimers and translocate into the nucleus where they regulate the expression of a wide range of genes involved in numerous signaling and metabolic pathways (von Koskull-Döring et al., 2007). During HSR, additional Hsfs are expressed which can further stimulate the transcription of HS-induced genes, by in many cases forming hyper-active complexes (Scharf et al., 1998). Therefore, the reprogramming the expression of genes as a response to HS requires coordination of different factors and regulatory mechanisms.

4.2. The role of the transcription factors in stress response initiation

Eukaryotic gene transcription is a conserved process that involves a multitude of proteins, ranging from general transcription factors to chromatin regulators and sequence-specific DNA binding transcription factors (Sawadogo and Roeder, 1985; Sainsbury et al., 2015). RNA polymerase II, which is responsible for the transcription of nuclear protein-coding genes into RNA, is a twelve-subunit enzyme complex that functions in association with six general transcription factors (GTFs), named TFIIA, -B, -D, -E, -F and –H (Sawadogo and Roeder, 1985; Gasch et al., 1990; Baldwin and Gurley, 1996; Sainsbury et al., 2015). In general, gene promoter consists of a core promoter region, which contains the recognition elements required for transcription initiation, proximal and distal regions that primarily contain regulatory elements. The core promoter elements are specific DNA elements such as the TATA box, which is recognized by the TATA-binding proteins (TBP) that are enclosed in the TFIID complex (Sainsbury et al., 2015).

Initiation of transcription requires the recruitment and assembly of RNA polymerase II in association with other GTFs, to the promoter core elements in what called pre-initiation complex, PIC (Stargell and Struhl, 1996; Hahn and Young, 2011; Ma, 2011; Sainsbury et al., 2015). However, the activation of the transcription is initiated when sequence-specific transcription factors bind to the promoter of the corresponding gene as a response to cellular signals (Sainsbury et al., 2015). These transcriptional activators, in turn, recruit chromatin remodeling factors that modify the local chromatin environment, thereby enabling the assembly of PIC onto the promoter (Roberts, 2000; Maston et al., 2006). Specific transcription factors have been shown to directly recruit TFIID and other general transcription or elongation factors (Sainsbury et al., 2015).

These sequence-specific transcription factors will either increase (activators) or decrease (repressors) the rate of gene transcription by stabilizing or destabilizing the formation of the PIC (Sainsbury et al., 2015). They affect the stability of PIC, either by direct interaction with one or more components of the basal transcriptional machinery, or indirectly by interacting with co-regulators that affect the stability of the interaction between the transcription factor and the transcriptional machinery (Semenza, 1994). Co-regulators add yet another layer of regulation to the function of sequence-specific transcription factors. In particular, they increase the diversity of responses exhibited by a transcription factor. A transcription factor may act as an activator or a repressor, depending on the co-regulator it is associated with (Thomas and Chiang, 2006). Co-regulators are broadly defined as the factors that are required for the function of transcription factors, but which do not generally possess DNA-binding properties themselves (Malik and Roeder, 2000). Instead, they are recruited through protein-protein interactions. The chromatin regulators mentioned earlier are an example of co-regulators that work

by altering chromatin structure, thereby enhancing or restricting the access of the basal transcription machinery to DNA (Keung et al., 2014).

4.2.1. Heat stress transcription factors in plants

Hsfs are sequence-specific DNA binding transcription factors that regulate the expression of heat stress responsive genes (Scharf et al., 1990; Nover et al., 1996; Bharti et al., 2000a; Nover et al., 2001; von Koskull-Döring et al., 2007; Kotak et al., 2007a; Scharf et al., 2012; Qu et al., 2013; Röth et al., 2016b). Besides, they are involved in the regulation of cell growth and organismic development under physiological conditions. Due to their sessile nature and continuous exposure to environmental changes throughout their life cycle, plants have developed a more complex cellular response network than that of other eukaryotes (Scharf et al., 1990; Scharf et al., 1998). This complexity is exemplified by the presence of a high number of Hsfs (Nover et al., 1996; von Koskull-Döring et al., 2007; Kotak et al., 2007a; Scharf et al., 2012). Compared to one Hsf in yeast, invertebrates, nematodes and fruit flies or four Hsfs in vertebrates (Åkerfelt et al., 2010), the number of identified plants Hsfs ranges from 21 in *Arabidopsis thaliana*, 24 in *Solanum lycopersicum* to 52 in *Glycine max* (Scharf et al., 2012). The large number of Hsfs in angiosperms has been proposed to be the result of gene or whole genome duplications during evolution as well as gene loss (Proost et al., 2011).

4.3. Domain structure and classification of plant Hsfs

Hsfs have a modular structure, organized in such a way that different functions are divided into separate domains. Typical Hsfs are composed of an N-terminal DNA-binding domain (DBD), an oligomerization domain (OD) and a C-terminal domain (CTD) (Fig. 1; Scharf et al., 2012). Common to all Hsfs are the DBD (Wiederrecht et al., 1988; Scharf et al., 1990) and the OD (Perisic et al., 1989). The CTD is least conserved in terms of sequence and size representing the signature sequence for each Hsf (Scharf et al., 2012). This domain is characterized by the presence of nuclear localization signal (NLS), nuclear export signal (NES) and activator (AHA) or repressor (RD) motifs (Fig.1). According to their OD peculiarities, plant Hsfs are assigned to three classes, namely A, B and C (see section 4.3.2).

Further sub-classification due to the presence of sequence motifs with not necessarily known function found adjacent to functional domains has allowed additional classification. For example HsfA1 subfamily is consisted of four members in both *A. thaliana* and tomato, with both plants comprising HsfA1a, HsfA1b and HsfA1e, but *A. thaliana* having HsfA1d and tomato HsfA1c (Scharf et al., 2012). Such signature motifs might provide insights for the evolution of plant Hsf families (Scharf et al., 2012).

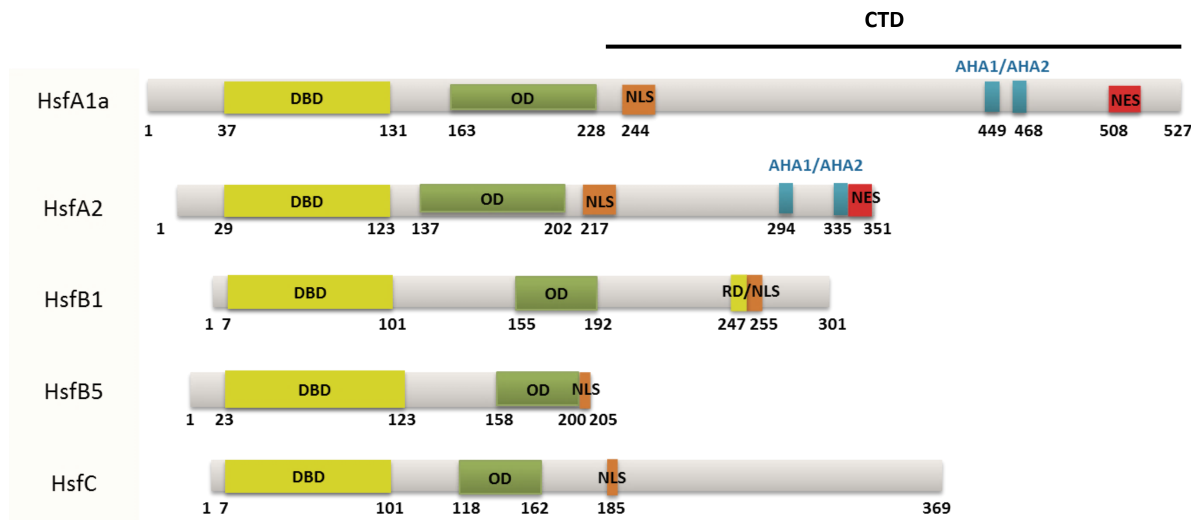

Figure 1. Domain structure of plant Hsfs.
The domain structure is exemplified for five representative tomato Hsfs from different classes A, B and C. Shown are the DNA binding domain (DBD); OD, oligomerization domain; NLS, nuclear localization signal; NES, nuclear export signal; AHA, activator motifs; RD, repressor domain (adapted from Scharf et al., 2012).

4.3.1. DNA binding domain and heat stress elements

DNA recognition and binding by Hsfs is accomplished via the N-terminal DBD. The DBD belongs to the family of helix-turn-helix (HTH) domains comprised of three major α-helices and a short, four-stranded β-sheet (Harrison et al., 1994; Schultheiss et al., 1996). The two helices represent the main feature of these HTH domains. They are orientated perpendicular to each other and connected with a short turn, whereby the first α-helix is inserted into the major groove of DNA for a base specific read out (recognition helix), and the second exhibits generic contacts to the DNA stabilizing the orientation of the first helix (Brennan and Matthews, 1989). Although this structure is conserved in all eukaryote, the DBD of non-plant Hsfs contains an additional long loop termed wing between strands three and four of the β-sheet which is absent in plant Hsfs (Schultheiss et al., 1996) . This wing has been shown to be involved in the DBD interaction of adjacent Hsfs (Littlefield and Nelson, 1999).

The DBD is highly selective in its function. This domain recognizes *cis*-regulatory elements called heat stress elements (HSE) upstream of TATA box in the promoter of target genes (Wray et al., 2003). HSEs consist of a highly conserved consensus repeats of 5'-nGAAn-3' sequence, where n is any nucleotide (Pelham, 1982; Nover, 1987; Amin et al., 1988). The G and C residues positioned in the major groove on opposite sides of the DNA helix are essential for HSE function, while one of other nucleotides (A or T) could be substituted without loss of function (Littlefield and Nelson, 1999). Usually more than two HSE motifs (one motif = nGAAn or nTTCn) are required due to the homo or hetero cooperative nature of Hsfs (Pelham, 1982; Topol et al., 1985; Xiao et al., 1991; Bonner et al., 1994; Fernandes et al., 1994;

Santoro et al., 1998). The fine structure/architecture of HSE motifs concerning sequence and number is crucial for efficient binding of Hsf oligomers. Interestingly, HSEs are abundant and can be found even in genes that are not induced by HS (Fragkostefanakis et al., 2015b). In addition, the number, sequence and organization including positioning of HSEs in genes show high variability which could be presumably allow the activity of different Hsf complexes.

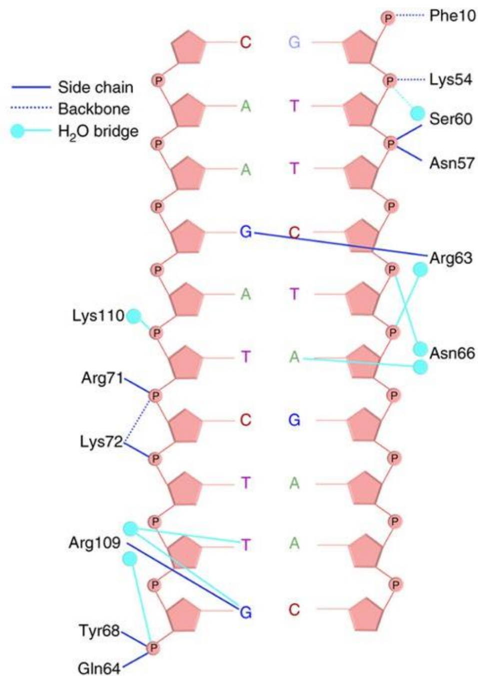

Figure 2. Ladder diagram of the direct and indirect possible contacts between the Hsf DBD and HSE DNA as exemplified for HSF2.
The sequence-specific interaction is largely mediated through a hydrogen-bonding interaction between Arg63 and the guanine of the nGAAn HSE motif. This Arg, which is conserved and is critical for DNA binding, is located within a recognition helix and is inserted into the major groove of DNA. In addition, numerous direct and indirect (water-mediated) contacts are made between the DBD and the DNA phosphate backbone (Jaeger et al., 2016).

The interaction of DBD with DNA is mainly mediated by the hydrogen bonds between arginine residue (Arg) in the highly conserved helix three (H3, recognition helix) of the DBD and the invariant G nucleotide of the HSE (Littlefield and Nelson, 1999). However, the surrounding conserved residues like serine (Ser), glutamine (Gln), asparagine (Asn), and tyrosine (Tyr) can foster the exact positioning of the DBD and strengthening of the interaction by hydrogen bonds and van der Waals interactions with the phosphate backbone of the DNA (Fig. 2 ; Littlefield and Nelson, 1999; Neudegger et al., 2016). Additionally, the methyl groups of the two T bases in nTTCn motif from the reverse-complementary DNA strand can also be engaged in van der Waals interactions with the side chains of Ser, Arg and Gln (Neudegger et al., 2016). The cluster of basic residues (Arg and Lys) in the C-terminus of the DBD can provide additional contacts across the major groove (Neudegger et al., 2016). However, Lys residues are accessible for modification by acetylation giving rise to a platform of DNA-binding regulation (Westerheide et al., 2009; Raychaudhuri et al., 2014). Acetylation removes the positive charge from Lys residues, which is required for ionic interactions with the DNA backbone, and thus lowers the overall affinity of the DBD to DNA (Zelin et al., 2012).

4.3.2. Oligomerization domain

Like DBDs, the OD is highly conserved among different organisms comprised of bipartite patterns of hydrophobic heptad repeats (HR-A and HR-B) with a potential linker in between forming a coiled coil domain (Sorger and Nelson, 1989; Peteranderl et al., 1999). The periodic repetition of Leu residues at every seventh position is responsible for the leucine zipper-type of interaction (Peteranderl et al., 1999). Based on the linker between HR-A and HR-B, plant Hsfs are classified into three classes: A, B and C (Scharf et al., 2012). Class B Hsfs are similar to all non-plant Hsfs which have a compact OD, meaning that no linker between the two subdomains exists. Classes A and C have an extended OD with a linker of 21 and 7 amino

acid residues, respectively (Nover et al., 2001; Kotak et al., 2007a; Scharf et al., 2012). Generally Hsfs are described to be active in homo-oligomeric trimers or hetero-oligomeric hexamer which mediated by HR-A and HR-B (Peteranderl et al., 1999; Chan-Schaminet et al., 2009). Hetero-oligomerization between different class A Hsfs can stimulate transcriptional synergy in which the regulatory effect of transcription factors working together is greater than the sum of the individual transcription factors giving rise to what is called a combinational control (Chan-Schaminet et al., 2009). As for example, the OD-mediated interaction of HsfA2 and HsfA1a in tomato yields a complex which possess a higher transactivation activity compared to the individual factors (Chan-Schaminet et al., 2009).

Additionally, the OD-mediated interaction between different members of the same family, as for example HsfA1a and HsfA1b in *A. thaliana* suggests a functional complementation that may have an effect on the expression of HsfA1a/HsfA1b target genes (Li et al., 2010). As mentioned above, the OD structure is variable among different classes of Hsfs. Accordingly, Hsfs of different classes, as for example HsfA1a and HsfB1 cannot interact but instead, they cooperate by forming C-terminal motif-mediated complexes (Bharti et al., 2000b).

4.3.3. Nuclear localization and export signals

Nuclear translocation and retention are required for the activation of the transactivation activity of Hsfs. Therefore, the nuclear localization signal (NLS), formed of a cluster of basic amino acid residues commonly following the OD, is essential and present in all Hsfs (Lyck et al., 1997). In contrast, nuclear export signal (NES) is not present in all Hsfs, which gives rise to an additional level of regulation by allowing the dynamic nucleocytoplasmic shuttling of only a subset of Hsfs (Scharf et al., 1998). In general, the NES is comprised of hydrophobic amino acid residues (typically rich in leucine) and located in the C-terminus of an Hsf and in some cases as in tomato HsfA2, it is adjacent to the activation motif

(Scharf et al., 1998; Heerklotz et al., 2001). The balance between NLS and NES is thereby controlling Hsf function and fate (Heerklotz et al., 2001).

4.3.4. Activator and repressor motifs

As transcriptional activators, most of the class A Hsfs are characterized by the presence of activation motifs (AHA motifs) in their CTDs. These motifs are essential for transcriptional activation activatiy (Döring et al., 2000). AHA motifs are characterized by aromatic (W, F, Y), large hydrophobic (L, I, V) and acidic (E, D) amino acid residues (Hahn, 1993; Tjian and Maniatis, 1994; Triezenberg, 1995; Nover and Scharf, 1997). Exceptionally, in HsfA3, the CTD does not contain such distinct AHA motives but rather a characteristic pattern of tryptophan residues, which give additive contributions to the activator function (Bharti et al., 2000).

The number of activation motifs is ranging from two as in tomato HsfA1a and HsfA2 to four motifs as in tomato HsfA3 (Bharti et al., 2000; Döring et al., 2000). Most likely, these motifs with its acidic context present the putative contact sites for the interaction with the components of the basal transcriptional machinery (Döring et al., 2000). Among class A Hsfs, HsfA8-types form a marked exception since their CTDs lack an AHA motif (Kotak et al., 2007a).

In contrast to class A Hsfs, all class B Hsfs have no AHA motifs but instead have a neutral or positively charged motif (Döring et al., 2000). However, the C-terminal domain of tomato HsfB1 has an indispensable central Lys residue (GRGKMMK) which allows the formation of ternary complexes with the CREB binding protein (CBP) orthologue HAC1 and HsfA1a as shown in tomato (Bharti, 2004). These ternary complexes have a marked efficiency of transcription activation activity on specific genes such as small Hsps (Bharti, 2004).

The transcriptional repressors of class B Hsfs, except HsfB5, are characterized by the tetrapeptide -LFGV- in their CTDs, which is assumed to function as repressor motif by interaction with the corepressors in the transcriptional machinery (Czarnecka-Verner et al., 2004; Ikeda and Ohme-Takagi, 2009). Their function is important for the suppression of genes under non-stress conditions, or the reduction of transcription during the attenuation of the response (Ikeda et al., 2011). Class A1 Hsfs are characterized by the TDR domain, temperature dependent repression domain, involved in the interaction between HsfA1 and Hsp70 chaperon under non stress conditions (Ohama et al., 2015). This domain is conserved among HsfA1s and responsible for their feedback regulation.

4.4. Functional diversity of plant Hsfs

Despite the high level of structure conservation among members of the plant Hsf class, in several cases functional diversification has been reported (von Koskull-Döring et al., 2007; Kotak et al., 2007a; Scharf et al., 2012).

4.4.1. Class A Hsfs are the activators of heat stress response

Based on studies of *Arabidopsis* and tomato Hsf genes, members of subclass A1 serve as master regulators of HSR (von Koskull-Döring et al., 2007; Kotak et al., 2007a; Scharf et al., 2012). However, this function is also divergent among the two plants. In tomato, the constitutively expressed HsfA1a was assigned as a master regulator of HSR when transgenic tomato plant with suppressed HsfA1a levels showed hypersensitivity to high temperatures and eliminated transcriptional activation of major HS-induced Hsps and other Hsfs (Mishra et al., 2002). In contrast, no comparable exclusive master regulator activity could be identified for any of the four *Arabidopsis thaliana* HsfA1 genes (a, b, d and e), as single and even double knock-out mutants do not show a significant alteration in thermotolerance when compared to the wild-type (Liu et al., 2011; Yoshida et al., 2011). Instead, at least three AtHsfA1 genes need to be suppressed to have a significant reduction in thermotolerance showing that the role of master regulator is shared among the four paralogs (Liu et al., 2011). The knock-out of the four master regulators in *A. thaliana* result in a clear phenotype where alterations in morphology and retardation in growth were observed in the quadruple but not in triple KO mutants (Liu et al., 2011). Unlikely, there is no obvious phenotype for the single master regulator co-suppression (*A1CS*) in tomato under control conditions (Mishra et al., 2002).

The activation of HsfA1 in response to HS, leads to the induction of hundreds of genes including several Hsps and Hsfs (Mishra et al., 2002; Yoshida et al., 2011). Among them, HsfA2 becomes very abundant in tomato and *A. thaliana* heat stressed cells. In tomato, HsfA2 and HsfA1a form super active complexes, which possess very high transactivation activity compared to the activity of the individual factors (Chan-Schaminet et al., 2009). Consequently, suppression of tomato HsfA2 results in weaker induction of several HS responsive genes (Fragkostefanakis et al., 2016). In contrast, HsfA2 in *A. thaliana* can be active and functional in the absence of HsfA1s as over-expression of *At*HsfA2 in the HsfA1 quadruple knock-out mutant led to improved thermotolerance (Liu and Charng, 2013). Enhanced thermotolerance has also been obtained by ectopic expression of rice HsfA2e and lily HsfA2 (Yokotani et al., 2008; Xin et al., 2010).

Several other HsfA genes also function in the plant thermotolerance, as for example, ectopic expression of tomato HsfA3 and wheat HsfA3 in *A. thaliana* enhances thermotolerance (Li et al., 2013). Indeed, AtHsfA3 is exclusively expressed in response to heat stress and drought stress depending on the activity of the transcription factor called Dehydration-Responsive Element Binding Protein 2A (DREB2A) (Sakuma et al., 2006). Tomato and *A. thaliana* HsfA4 have been shown also to act as potent activators of HS gene expression and can be regulated by the specific repressor effect of HsfA5 (Baniwal et al., 2007). It was shown also that *At*HsfA6b and *At*HsfA7 is required for thermotolerance acquisition and plant viability under heat-stress, respectively (Larkindale and Vierling, 2007; Huang et al., 2016). Unlikely, AtHsfA9 plays a unique HS-independent role in embryogenesis and seed maturation (Kotak et al., 2007b). This role is controlled by the seed-specific transcription factor Abscisic acid–Insensitive 3 (ABI3) (Kotak et al., 2007b).

4.4.2. Class B Hsfs mainly act as repressors

In contrast to class A Hsfs, class B Hsfs have no transcriptional activator function on their own due to the lack of AHA-activator motifs (Döring et al., 2000). In *A. thaliana*, HsfB1 and HsfB2b act as repressors of HSR genes in non-stressed cells and during recovery from stress (Ikeda et al., 2011). However, hsfb1-hsfb2b mutants exhibit reduced acquired thermotolerance due to the weaker induction of HS-related genes (Ikeda et al., 2011). The mechanism by which HsfB members can have a stimulatory effect on transcription of HS-responsive genes remains unknown as only a direct repressor function has been reported yet in *A. thaliana*.

In contrast, the HS-induced tomato HsfB1 has been shown to act as a coactivator of HsfA1a by assembling into an enhanceosome-like complex resulting in a strong synergistic activation of expression of HS-genes (Bharti, 2004). The coactivator function of HsfB1 depends on the recruitment of the plant CREB binding protein (CBP) orthologue called histone acetyl transferase HAC1 (Bharti, 2004). Moreover, HsfB1 can cooperates with other transcriptional activators controlling housekeeping gene expression (Bharti, 2004). Thereby, HsfB1 acts as co-activator of HsfA1a, but as a transcriptional repressor on other Hsfs. This dual function of tomato HsfB1 has been shown to be responsible for the balance between growth and stress response under stress conditions (Fragkostefanakis et al., 2018). Together these findings point to the species-specific functional diversity of some members of the Hsf family and subsequent refined regulations.

4.5. Heat shock proteins acting as chaperones

The molecular chaperone concept was generally proposed in 1987 (Ellis et al., 1989). Ever since, chaperones have been defined as "proteins that interact with or aid the folding or assembly of other proteins without being part of the target protein final structure". Molecular chaperones cover a variety of functions and therefore interact with proteins through their entire life time – from synthesis at the ribosome until their degradation at the proteasome (Hartl et al., 2011). Chaperones are becoming very abundant under challenging situations such as heat stress, therefore they have been referred to as Hsps (Tissières et al., 1974). Molecular chaperones have been classified according to their monomeric molecular weight and functions in distinct families, including small Hsps (sHsp or Hsp20), Hsp60 (chaperonin), Hsp70, Hsp90 and Hsp100 (Wang et al., 2004).

4.5.1. Small heat shock proteins

sHsps are proposed to be the first line of defense during heat stress, interacting with denaturing proteins in an ATP-independent manner and providing access for other ATP-dependent Hsps like Hsp100 and Hsp70 chaperones that function in refolding and disaggregation of denatured proteins (Haslbeck and Vierling, 2015). Accordingly, sHsps by their own are not able to dissolve pre-existing aggregates but only prevent aggregation (Haslbeck and Vierling, 2015). This family of Hsps includes proteins which range in size from 12 to 42 kDa and comprised of a fixed α-crystalline domain flanked with divergent N-terminal arm of variable-length and short C-terminal tail (reviewed by Basha et al. 2012). Plant sHsps are distinguished by their intracellular localization; as they are found in all membrane-bound plant cell compartments: chloroplast, mitochondrion, endoplasmic reticulum, peroxisome and nucleus, as well as in the cytosol (Scharf et al., 2001; Sun et al., 2002). Among different subfamilies of cytosolic sHsps, the three subfamilies CI, CII and CIII are described in detail (Scharf et al., 2001; Siddique et al., 2003).

4.5.2. Hsp70 and Hsp40-proteins

Hsp70s (DnaK in prokaryotes) are an ubiquitous class of proteins that represent the central part of cytosolic chaperone network (Calloni et al., 2012). They are involved in a wide range of cellular processes including *de novo* protein folding, refolding of stress-denatured proteins, protein transport and protein degradation (Kim et al., 2013). Hsp70 chaperones interact with the co-chaperones of the Hsp40 family (also known as DnaJ proteins or J proteins) and nucleotide exchange factors (NEFs) (Laufen et al., 1999). Hsp70 proteins consist of two domains: an N-terminal nucleotide binding domain

(NBD) and a C-terminal substrate binding domain (SBD) which are connected via a conserved linker (Kim et al., 2013). The SBD binds substrate peptides in the open state, which is maintained by ATP binding to the NBD. ATP hydrolysis (accelerated by Hsp40s) enforces the allosteric rearrangement of the SBD into its closed state (Kampinga and Craig, 2010). Subsequently, nucleotide exchange factors (NEF) stimulate the release of ADP, resetting the SBD to the open state and releasing the bound substrate (Mayer and Bukau, 2005). Depending on the refolding state of the substrate protein it might be directly bound again to prevent aggregation and undergo further folding (Szabo et al., 1994) and then transferred to downstream chaperone machineries like Hsp90 or the chaperonin (Langer et al., 1992; Hartl et al., 2011). Due to their essential function, cells encode for constitutively expressed Hsp70 genes which are called cognates (Mayer and Bukau, 2005) . Under HS the increased demand for Hsp70 due to the accumulation of denatured proteins is supported by stress-induced Hsp70 genes.

4.5.3. The Hsp90 family

Hsp90 chaperones play an important role in the maturation and conformational maintenance of many signaling proteins in the cell, e.g. transcription factors, which are delivered to Hsp90 by Hsp70 chaperones and other cofactors (Kim et al., 2013). Hsp90 is a flexible dimer whereby each subunit is comprised of an N- terminal ATPase domain, a substrate-binding middle domain and C-terminal dimerization domain. Like Hsp70, Hsp90 undergoes an ATP dependent cycle which is characterized by an open, nucleotide-free state and a closed, ATP-bound conformation (Rehn& Buchner 2015). Hsp90 and its reaction cycle are tightly regulated by various co-chaperones. For example, human HOP and Cdc37 stabilize the open, substrate binding conformation of Hsp90, whereas human Aha1 stimulates ATP hydrolysis and formation of the closed state (Li et al., 2012). The closed state is stabilized by the human co-chaperone p23 (Li et al., 2012). Substrate folding likely occurs in the closed state of Hsp90, however the detailed mechanism of substrate binding and folding is not clear yet (Kim et al., 2013). Similar to Hsp70, cognate and stress-induced Hsp90 genes exist to satisfy the cellular demand for chaperones (Krishna and Gloor, 2001).

4.5.4. The Hsp100 family

In contrast to other chaperone families, members of the Hsp100 family can actively unfold protein aggregates and then deliver them to Hsp70-assisted refolding (Bosl et al., 2006). Hsp100 chaperones are characterized by two ATPase domains per monomer (Schirmer et al., 1996). The molecular mechanics of protein unfolding by Hsp100 are still unclear, however Hsp100 relies on the Hsp70 system

for proper functioning (Mogk et al., 2015). Furthermore, the cytosolic member of this family, Hsp101 can interact with sHsp chaperone system to resolubilize protein aggregates during heat stress and is essential for providing thermotolerance (Hong and Vierling, 2000; McLoughlin et al., 2016).

4.6. Crosstalk between Hsfs and Hsps

It has been described that Hsp70 in human cell culture binds Hsf1 under control conditions, only releasing it when levels of denatured proteins increase due to stress conditions (Abravaya et al., 1992). This release in turn leads to the activation of Hsf1 and subsequent higher expression of chaperones, until enough Hsp70 is produced to sequester Hsf1 from the DNA (Abravaya et al., 1992). In a similar manner, Hsp90 also keeps Hsf1 in an inactive state under normal conditions (Ali et al., 1998; Zou et al., 1998). This general system of regulation has been confirmed in plants, e.g. tomato HsfA1a and *A. thaliana* HsfA1d is regulated in a similar fashion by Hsp70 (Hahn et al., 2011; Ohama et al., 2015). It is proposed that this interaction depends on the presence of a specific temperature dependent repression domain (TDR) that represent the binding site for Hsp70 (Ohama et al., 2015).

Furthermore, regulation of tomato HsfA2 depends on the expression of cytosolic sHsps (Port et al., 2004). As stated above, during early HS, HsfA2 is retained in the nucleus by HsfA1a and together they form a super-activator complex (Scharf et al., 1998; Chan-Schaminet et al., 2009). During prolonged heat stress Hsp17.4-CII is expressed in sufficient amounts to recruit HsfA2 in cytoplasmic heat stress granules (HSGs). Thereby, the generally high expression in the presence of active HsfA2 is reduced but HsfA2 is also retained for later stages of HS or the recovery phase (Scharf et al., 1998). While the interaction and granule formation of HsfA2 is dependent on Hsp17.4-CII specifically, the dissolution of the granules is regulated by Hsp17-CI (Port et al., 2004). However, due to its function in dissolving protein aggregates Hsp101 might be involved in this process as well, albeit this has only been proposed (Tripp et al., 2009; Scharf et al., 2012).

Additionally, the DNA binding activity of tomato HsfB1 is modulated by Hsp90 which is also involved in HsfB1 degradation (Hahn et al., 2011; Röth et al., 2016a). It has been proposed that this represents a fine-tuning step for the attenuation of the recovery phase of the heat stress response, due to the repressor function of the class B Hsfs. The enhanced DNA binding of HsfB1 would result in a decreased expression of target genes and subsequently the removal attenuates the levels of HsfB1 (Hahn et al., 2011). Similarly, *A. thaliana* HsfA2 activity is regulated by Hsp90 and its specific co-chaperones ROF1 and ROF2 (Aviezer-Hagai et al., 2007). Upon HS the ROF1-Hsp90-HsfA2 complex is imported into the nucleus. In this context ROF1 and Hsp90 import is strictly dependent on HsfA2. Once imported, Hsp90 and ROF1 promote HsfA2 DNA binding and strongly enhance the transcriptional activity of HsfA2 (Meiri

and Breiman, 2009). One of the target genes is the co-chaperone ROF2 which in the recovery phase binds to ROF1 in the Hsp90-HsfA2 complex. This binding inhibits HsfA2 activity and target gene expression is reduced leading to the attenuation of the heat stress response (Meiri et al., 2010). Summarizing, the regulation of gene expression in response to HS is dependent on the interplay of Hsfs, Hsps and co-chaperones as exemplified for the main Hsfs.

4.7. Objectives of the study

In tomato, HsfA1a was characterised as the master regulator of heat stress response (Mishra et al., 2002). However, this function is shared between the four members of subclass A1 (HsfA1a, HsfA1b, HsfA1c and HsfA1d) in *A. thaliana* (Liu et al., 2011). This functional diversity of HsfA1 in different plants rise the question about the complexity of HsfA1 mediated-network in each plant. Up to now, nothing is known about other members of subclass A1 (HsfA1b, HsfA1c and HsfA1e) in tomato. Consequently, the aim of my study is to characterise these members in terms of activity and properties.

For this purpose, expression analysis as well as functional analysis is to be carried out *in vivo* and *in vitro*, respectively. Furthermore, to determine the specific features responsible for this dominant function of HsfA1a as a master regulator, the domain structure is to be swapped and examined.

During study, I got insights into the crucial role of DNA-binding domain (DBD) in the activity and coactivity of the HsfA1a. Accordingly, the next aim of this study is to analyze the structure of HsfA1a-DBD in terms of sequence to find out the impact of few amino acid variations. All in all, this study will fulfill the picture about function specificity of tomato HsfA1a as a master regulator of HSR giving some perspectives for structure-function relationship.

5. Materials and methods

5.1. Plasmid generation

Plasmids used in this study were generated according to the standard cloning procedures using T4 ligase (Thermo Fisher Scientific) and summarized in Table 1. Primers and cloning strategies were designed using Cloner Manager 9 (Black Mesa Laboratories). PCR products were cut of the agarose gel and purified using an E.Z.N.A Gel extraction Kit (OMEGA Bio-Tek Inc., Doraville, GA, USA). All restriction enzymes used were from Thermo Fisher Scientific. In some cases TA cloning was used (Ins TA clone™ PCR Cloning Kit, Thermo Fisher Scientific). Other constructs that used in this study but previously generated are listed in Table 2.

Table 1. DNA constructs generated during this study

Construct name	Gene ID	Primer sequence (5' to 3')	Orientation, stock no.	Restr. Site
pRT-35S-3HA-HsfA1a	Solyc08g005170	CTGGGTACCAATGGAGCCGAATTCTTATGG	Forward 9985	Acc65I
		GGGTCTAGATTAGATCATATGTTTTTGTTGTTTG	Reverse 9758	XbaI
pRT-35S-3HA-HsfA1b	Solyc03g097120	TGGGTCGACAGACGGAGTTCATGAG	Forward 9079	SalI
		GTCTCTAGAATCAAATTTGGGCAGCCG	Reverse 9014	XbaI
pRT-35S-3HA-HsfA1c	Solyc08g076590	CTAGTCGACGGACGCCGGAGGAGGTTC	Forward 8318	SalI
		CCATCTAGATTCATACTTTTTTAGTGTTTGATG	Reverse 9318	XbaI
pRT-35S-3HA-HsfA1e	Solyc06g072750	CTAGGTACCGTCGCCGGACGGCGAA	Forward 8320	Acc65I
		CTATCTAGACTACAGTTTATCACAATC	Reverse 8321	XbaI
pRT-d35S-GFP-HsfA1a	Solyc08g005170	CTAGGTACCATGGAGCCGAATTCTTATG	Forward 9757	Acc65I
		GGGTCTAGATTAGATCATATGTTTTTGTTGTTTG	Reverse 9758	XbaI
pRT-d35S-GFP-HsfA1b	Solyc03g097120	GGTACCGACGGAGTTCATGAGGCTACGG	Forward 9379	Acc65I
		GTCTCTAGAATCAAATTTGGGCAGCCG	Reverse 9014	XbaI
pRT-d35S-GFP-HsfA1c	Solyc08g076590	AGATCTGGACGCCGGAGGAGGTTCTCC	Forward 9380	BglII
		CCATCTAGATTCATACTTTTTTAGTGTTTGATG	Reverse 9318	XbaI
pRT-d35S-GFP-HsfA1e	Solyc06g072750	AGATCTGGCGTCGCCGGACGGCGAAAAAAC	Forward 9381	BglII
		CTATCTAGACTACAGTTTATCACAATC	Reverse 8321	XbaI

5.2. Quick change site-directed mutagenesis

Targeted changes in nucleotide sequence in a plasmid construct were performed by an adapted Quick change mutagenesis PCR from Agilent with a single oligonucleotide. First the oligonucleotide (Sigma-Aldrich) for mutagenesis was phosphorylated with T4-polynucleotide kinase (PNK) in buffer A according to the manufacturer's protocol (Thermo Fisher Scientific) for half an hour at 37°C followed by an incubation at 70°C for 15 min. Subsequently a standard PCR was done with the following alterations: 10 ng plasmid DNA as a template, 5 pmol of phosphorylated primer, 0.5 mM NAD^+, 5U Ampligase (Biozym),

Table 2. DNA constructs used in this study

Construct name	Reference/source
pRT103 (pRT-Neo)	Töpfer et al., 1987
pRT-HsfA2	Treuter et al., 1993
pRT-HsfA7	Kindly provided by Dr.Mesihovic
pRT-HsfA3	Bharti et al., 2000
PGmHsp17.3B-CI:GUS/PHsp17*:GUS	Treuter et al., 1993
pRT-PHsp21.5ER:GUS	Kindly provided by Dr.Mesihovic
pRT-PHsfA2:GUS	Kindly provided by Dr.Mesihovic
PHsfA7:GUS	Kindly provided by Dr.Mesihovic
PHsp70-1:GUS	Kindly provided by Dr. Fragkostefanakis
pRT-HSE3:GUS	Treuter et al., 1993
pRTds-AtEnp1-herry	Kindly provided by Dr. Sascha Röth

Pfu buffer and Pfu polymerase in a 25 µl reaction. The temperature used for annealing was chosen according to the primer specific melting temperature. Elongation time was chosen according to the plasmid size (1 min for 0.5 kb for Pfu polymerase) and 28 cycles were employed. DpnI (Thermo Fisher Scientific) was added directly to the product and kept over-night at 37°C for digestion of the template DNA. Subsequently the mixture was directly chemically transformed into competent *E. coli* DH5α cells. Sequencing of putative positive clones was done by GATC Biotech and analyzed for presence of the mutation with Clone Manager software. Oligonucleotides designed for site mutagenesis in this study are summarized in Table 3.

Table 3. Oligonucleotides used for site directed mutagenesis PCR

Template	Primer sequence (5' to 3')	Stock no.	Restr. site
pRT-35S-3HA-HsfA1a	CCACCTGGACACAGCGCTTCCGTTGGGGCTTG	10093	AfeI
	GGCAAAAGCTGTCAACAGCCCCGGGTTCTTGGCAC	10215	XmaI
pRT-35S-3HA-HsfA1c	GCTGCATAGTCAGAGCGCTTCAGTTGGGGCTTG	10094	AfeI
	GCTGTTAACAGCCCCGGGTTCTTTGCACAG	10097	XmaI
Template	**Primer sequence (5' to 3')**		**Mutation**
pRT-35S-3HA-HsfA1a	CGCCGTTTCTGATGAAGACGTATGATATGG	10787	V42M
pRT-35S-3HA-HsfA1a	AAAGGTTGATCCAGACCTCTGGGAATTTGC	10788	R107L
pRT-35S-3HA-HsfA1a	GAAAGGTTGGTCCAGACCGCTGGAAATTTTCTAATGAGGGATTC	10508	D104G/E109K/A111S

5.3. Plasmid DNA preparation

5.3.1. Transformation of competent *Escherichia coli*

Chemically competent *E. coli* DH5α cells (Dagert and Ehrlich, 1979) were mixed with equal volume of transformation buffer (100 mM $CaCl_2$; 50 mM $MgCl_2$) and ~10 ng plasmid DNA of interest or ligation reaction. The mixture was incubated on ice for 30 min after which a heat shock was performed at 42°C for 90 s in a water bath. Cells were immediately placed on ice for 5 min followed by addition of 800 µl LB medium (Luria-Bertani, 10 g l^{-1} tryptone; 5 g l^{-1} yeast extract; 10 g l^{-1} NaCl) and incubation at 37°C for 1 h with shaking at 750 rpm. Cells were pelleted at 5000 rpm and room temperature for 5 min and the pellet was resuspended in a small amount of medium, plated on LB plates (LB medium with 15 g l^{-1} agar) with the appropriate antibiotic and incubated overnight at 37°C.

5.3.2. Mini-preparation of plasmid DNA

In order to extract plasmid DNA and screen for positive clones, plasmid mini-prep was used based on the protocol of Sambrook et al., 1989 with minor modifications. Colonies were inoculated into 3 ml of LB medium with the appropriate antibiotic and cultures were incubated overnight at 37°C in a rotary shaker (120 rpm). On the next day cells were harvested at 8,000 rpm for 5 min and the pellet was air-dried and resuspended in 100 µl P1 (50 mMTris/HCl pH 8.0; 10 mM EDTA; 100 µg ml^{-1} RNAse A) by vigorous vortexing. Afterwards, 200 µl of P2 solution (0.2 M NaOH; 1% SDS) and 150 µl of P3 solution (3 M potassium acetate; 11.5% (v/v) glacial acetic acid) were added. Samples were mixed gently by inverting, kept on ice for 10 min and centrifuged for 15 min at 14,000 rpm at 4°C. The supernatant was transferred to a new tube, mixed with 240 µl isopropanol and precipitated at -20°C for at least 1 hour. Plasmid DNA was pelleted (14,000 rpm at 4°C for 15 min) and washed with 70% ethanol (14,000 rpm at 4°C for 5 min). In the end, the DNA pellet was dried at 42°C for 15 min and resuspended in 50µl sterile ddH_2O.

5.3.3. Midi-preparation of plasmid DNA

In order to prepare plasmid DNA of high-quality and yield required for efficient protoplast transformation, plasmid midi-preparation was employed (Sambrook et al., 1989) with minor modifications. In short, a bacterial culture (50-100 ml) was harvested by centrifugation and the bacterial pellet was resuspended in 3 ml of lysis buffer (50 mMTris/HCl pH8.0; 50mM EDTA pH8.0; 15% [w/v] sucrose). Seven milliliters of freshly prepared 0.2 M NaOH/1% SDS buffer was added, mixed

gently and kept at room temperature for 10 minutes. Next, 3.5 ml of P3 solution (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. After 1h at -20°C and subsequent centrifugation (20 min at 11,000 rpm) the pellet was resuspended in 500 µl of TE buffer (10 mMTris/HCl pH 7.6; 1 mM EDTA pH 8.0) and mixed with an equal volume of 5 M LiCl/50 mM MOPS buffer. After 30 minutes on ice the samples were centrifuged at 8,000 rpm for 10 minutes at 4°C and the supernatant was transferred to a new tube and precipitated with 2.5V EtOH (96%) and 0.1V 3M Na-acetate. After incubation at -20°C for at least 30 minutes the samples are centrifuged (11,000 rpm for 20 minutes at 4°C) and dry pellet was resuspended in 300 µl TE buffer and 10µl of each RNase A (500 U ml^{-1} in 5mMTris/HCl pH8.0; Roth) and RNase T1 (500 units/ml in 5mM Tris/HCl pH8.0; Thermo Fisher Scientific). The mix was then 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 min 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 then transferred to a new Eppendorf tube and precipitated with ethanol and sodium acetate. Finally, following centrifugation, the pellet was washed with 70% ethanol, dried at 42°C for 15 min and resuspended in TE buffer. Concentration was adjusted to 1 µg $µl^{-1}$.

5.4. DNA sequencing

Sequencing reactions were prepared using 80-100 ng of plasmid DNA and 5 µl of 10 µM primer in a final 10µl reaction, as recommended by GATC Biotech (Konstanz, Germany). Sequence verification was done using Clone Manager 9 software.

5.5. Protoplast isolation and transformation

Mesophyll protoplasts were isolated from young leaves of tomato plants and transformed with the plasmid of interest as described previously (Mishra et al., 2002). In short, leaves of 6-7 week old sterile grown tomato plants (*Solanum lycopersicum* cv. Moneymaker) or A1CS transgenic plants were detached and lightly cut using a sterile scalpel in a petri dish containing 10 ml enzyme solution (K3M-S solution (0.4M mannitol, 24.7mM KNO_3, 1.01mM $MgSO_4x7H_2O$, 1.09 mM $NaH_2PO_4x2H_2O$, 1.01 mM $(NH_4)2SO_4$, 6.12 mM $CaCl_2x2H_2O$, 0.56 mM m-inosid, 3mM NH_4NO_3, 5 ml L^{-1} of 2.3 g $250ml^{-1}$ $FeSO_4EDTA$

[Sigma-Aldrich], 1x Trace elements (500ml 100x stock: 37.5 mg KJ, 500 mg $MnSO_4xH_2O$, 100 mg $ZnSO_4xH_2O$, 150 mg H_3BO_3, 12.5 mg $Na_2MoO_4x2H_2O$, 1.25 mg $CoCl_2x6H_2O$, 1.25 mg $CuSO_4$), 5.57 mM NES, 0.89 mM BA, 29.65 mM vitamin B1, 4.86 mM vitamin B6, 8.12 mM nicotinamide, 5 mM MES; pH5.7-5.8) containing 0,25% cellulose and 0.1% macerozyme (Duchefa). Leaves were incubated overnight in dark at room temperature. On the next day, the protoplast containing solution was separated from leaf debris by gentle shaking and passing through a sieve. Remaining protoplasts were collected by washing the plate with K3-2S solution (K3M-Swith 6% [w/v] sucrose) and then separated by centrifugation at room temperature for 7 minutes at 470 rpm. The upper 2/3 of the supernatant 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 7 min at 670 rpm and finally re-suspended in K3Msolution (K3M-S with 3% [w/v] sucrose) to a final concentration of 10^6 protoplasts per ml. For each sample 100,000 protoplasts (in 100 µl) were mixed with 20 µg total plasmid DNA and equal volume of PEG solution (25% PEG6000, 0.45 M mannitol, 0.1 M Ca $(NO_3)2x4H_2O$, pH 6.0 (KOH)) and incubated at room temperature for 25 minutes. The transformation was then stopped by adding K3M (K3M-S with 3% [w/v] sucrose) to a final volume of 1 ml. To allow expression of transformed plasmid, samples were incubated for the individually assigned expression times at 25°C under light. Protoplasts were harvested by centrifugation at 14,000 rpm at 4°C for 5 min and supernatant is removed by aspiration. Samples were frozen in liquid nitrogen and kept at -80°C until further processing.

5.6. Temperature Treatments

Heat stress treatments were performed on leaves and protoplasts of wild type plants according to the experimental setup. Leaf tissue treatments were done by incubation of detached young leaves in petri dishes on wet towels in a water bath. Control samples were kept at 25°C in a climate chamber.

5.7. Protoplast sample preparation

For SDS-PAGE and subsequent western blot analysis pellets of 100,000 protoplasts were re-suspended in 60 µl of high salt buffer: 20 mM Tris/HCl pH7.8; 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-mercaptoethanol; proteinase inhibitor cocktail: Pefabloc (in 10mMHepes pH7.5) [10 µg ml^{-1}]; Pepstatin A (in ethanol or isopropanol) [1 µg ml^{-1}]; Leupeptin [2 µg ml^{-1}]; Aprotinin [2 µg ml^{-1}]; TLCK (in 0.1 N HCl) [50 µg ml^{-1}]; TPCK (in ethanol) [20 µg ml^{-1}]; Benzamidine [150 µg ml^{-1}]. Samples then were vigorously vortexed and subsequently centrifuged for 5 min at 14,000 rpm at 4°C. The supernatant was mixed with 4xSDS

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 at 95°C. 10-20µl were used for SDS-gel electrophoresis.

5.8. Immunoblot analysis

Protein samples were first separated based on molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Together with Unstained Protein Molecular Weight Marker (Thermo Fisher Scientific), samples were loaded on 10-12% SDS-polyacrylamide gel. G els were run for 1 hour at 0.5 mA cm^{-2} with 1x SDS buffer (10x stock solution: 0.5 M Tris, 3 M glycine and 1%(v/v) SDS) and then proteins were transferred to an Amersham Protran 0.45 µm nitrocellulose blotting membrane (GE Healthcare) using the semi-dry method (40 mM Tris; 39 mM glycine; 0.037% SDS; 20% methanol; 1 mA $cm^{-2}$of transferred area for 75 minutes). After protein transfer the membrane was stained with Ponceau S (0.4% (w/v) Ponceau S, 3% (v/v) trichloroacetic acid (TCA), 1% (v/v) acetic acid) and RuBisCO large subunit staining was taken as a loading control. Blots were blocked in 5% (w/v) non-fat dry milk solution in phosphate-buffered saline (1x PBS; 140mM NaCl, 2.6mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 1 hour at room temperature and incubated for another 1 hour with the appropriate primary antibodies. All primary antibodies used in this study are listed in Table 4. Membranes were washed three times with PBS solution for 5 min and then incubated with the secondary antibody. Secondary antibodies are conjugated to horseradish peroxidase (Sigma-Aldrich) and signals were detected with enhanced chemiluminescence (ECL kit, Perkin-Elmer Life Sciences).

Table 4: Antibodies of Hsfs and Hsps used in this study.

Primary antibody		Dilution	Secondary antibody
mc α-HA	(Covance)	1: 2000	mouse
pc HsfA2/pep6	(Lyck et al. 1997)	1: 5000	rabbit
pc HsfA3	(Bharti et al. 2000)	1:5000	mouse
pc HsfA7	(Anida Mesihovic)	1:7500	mouse
pc HsfB1	(Lyck et al. 1997)	1: 4000	rabbit
pc GFP	(Roche)	1:5000	mouse

Secondary antibodies were used in dilution of 1:10,000 (Sigma-Aldrich)

5.9. Plant material and heat stress treatment

For expression analysis samples from different tissues were taken from 8 week old tomato plants (*Solanum lycopersicum* cv. Moneymaker), frozen in liquid nitrogen and stored at -80°C until further

processing. Plants were grown in a greenhouse at a temperature of 25°C day/20°C night and 16h day/8h night cycle. For heat stress experiments, young leaves from respective 8 week old plants were detached and incubated in a petri dish with wet filter paper in a water bath with desired temperatures or kept at 25°C (control). All samples were harvested at desired time points, frozen in liquid nitrogen and stored at -80°C until further processing.

Plants for protoplast preparation were grown on gelrite-solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 20 gl^{-1} sucrose (25°C day/ 23°C night, 16h light/8h dark).

5.10. RNA extraction

Frozen tissue samples (50-100 mg) were homogenized using a Tissue Lyser MM300 (Qiagen/Retsch) 2 times for 30 sec at 30 strokes per second. Total RNA was extracted using the E.Z.N.A. Plant RNA kit (Omega Bio-Tek) from tissues and protoplasts according to the manufacturer's protocol. Genomic DNA was removed by DNase I treatment according to the manufacturer's instructions. For protoplasts, 1µg RNA was treated with DNase I (Applichem) for 30 min at 37°C to eliminate any remaining plasmid DNA. Total RNA was quantified at 260 nm with a NanoDrop™ 1000 (PEQLAB, Erlangen, Germany). The purity of obtained RNA was inspected by the A260/A280 ratio.

5.11. 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 (T24VN) and RNase-free water at a final volume of 11 µl. The sample was incubated at 70°C for 5 minutes to remove RNA secondary structures and kept on ice for another 5 minutes. A 9 µl aliquot of reaction mix for Reverse transcription (RT) containing 1µl Revert Aid reverse transcriptase (Thermo Fisher Scientific, 200 U), 4 µl of 5X RT-buffer, 2 µl dNTP mix (10 mM) and 2 µl RNase-free water was added and then incubated at 42°C for 1 hour for cDNA synthesis. Subsequently, the reactions were incubated at 70°C for 15 minutes to deactivate the enzyme and kept at -20°C till further use.

5.12. Reverse Transcriptase PCR

cDNA samples generated by reverse transcription of the RNA were used as template for further PCR analysis of various genes. Equal loading and normalization of samples were inspected by RT-PCR using primers for Elongation factor 1 alpha (EF1α, Solyc06g005060) as a housekeeping gene (Fragkostefanakis et al., 2016). PCR reactions were performed in a final volume of 20 µl using Taq polymerase, 2 µl of 10x Taq buffer (100 mM Tris-Hcl pH 8.3; 15 mM $MgCl_2$; 500 mM KCl), 0.2 mM dNTP mix and 1 µM of each forward and reverse primer. Annealing and elongation temperature were adjusted depending on the primer melting temperature and amplicon length. PCR reactions were analyzed on a 2% agarose gel. The gels were incubated in ethidium bromide (0.5 mg ml^{-1}) for 5-10 min and placed on an UV-transilluminator (TFX-20M, Vilber Lourmat) for detection.

Table 5. Oligonucleotides used in qRT-PCR

Amplified gene	Gene ID	Primer Sequence (5' to 3')	Orientation, stock no.
EF1α	Solyc06g005060	GGAACTTGAGAAGGAGCCTAAG	Forward 7038
		CAACACCAACAGCAACAGTCT	Reverse 7039
HsfA1a	Solyc08g005170	ACAAATGATGTCGTTCCTGGC	Forward 6570
		GAAAGCTCCCTCAACATTGCC	Reverse 6571
HsfA1b	Solyc03g097120	CCTGAAGCTGATGCATTGAA	Forward 6433
		GGCATACCATCCAGCAAAAC	Reverse 6434
HsfA1c	Solyc08g076590	GAGGGGCAATGATTCTTTCA	Forward 6572
		CCCAGGAGGGATCAGTAACA	Reverse 6573
HsfA1e	Solyc06g072750	GGCTTCAATACACCGGAAAC	Forward 6574
		ACCAGTGAGAGGGCTTGCTA	Reverse 6575
HsfA2	Solyc08g062960	GGCGACCATAACTCTATCCTTCCC	Forward 6576
		GCCTCCTCCACTATTCCAGTATCC	Reverse 6577
HsfA7	Solyc09g065660	GCGTGACAAGCAAGTTTTGA	Forward 6437
		CAAACTCGGGATTTTGCATT	Reverse 6438
Hsp101	Solyc03g115230	GTGGCAAGTGTACCATGGAGA	Forward 6233
		GACTTGCCTCAACTGCTCGT	Reverse 6234
Hsp70-1	Solyc06g076020	GCTCGAGGGCATCTGTAATC	Forward 6235
		GAAACCAACTAGTATCTTTATCAACC	Reverse 6236
Hsp70-5	Solyc04g011440	GGAAGTGGACTAAGCTCCACA	Forward 6243
		CGAAGGATATTTCTACATACACAAA	Reverse 6244
Hsp70-6	Solyc11g066100	GACAGCAACCAACTTGCAGA	Forward 6245
		CCTGCACCACCACCAACAGAAGG	Reverse 6246
Hsp90-3	Solyc06g036290	TTCTTGGTGACAAGGTCGAA	Forward 6249
		ATCAGGATTAATTTCCATCGTCT	Reverse 6250
Hsp90-4	Solyc03g007890	TGACCCGAATACATTTGCTG	Forward 6251
		ACCGCATATAGATGACGCAT	Reverse 6252
Hsp17.7A-CI	Solyc06g076520	ATGGAGAGAAGCAGCGGTAA	Forward 6263
		ATGTCAATGGCCTTCACCTC	Reverse 6264
Hsp15.2-CI	Solyc09g015000	TTGGAAAGAGACCCCACAAG	Forward 6265
		TTGAACTTACCGCTGCTCCT	Reverse 6266

5.13. Quantitative real-time PCR

To determine the relative transcript levels of selected genes qRT-PCR was employed using PerfeCTa SYBR® Green Fast Mix (Quanta Biosciencies, Gaithersburg, MD, USA) on a Strata gene Mx3000P cycler (Agilent Technologies, Palo Alto, CA, USA). The qRT-PCR reactions were done in a final volume of 10 µl contained the 2 µl of 1:10 diluted cDNA (corresponding to 5 ng of total RNA), 1µl of gene-specific forward and reverse oligonucleotides (0.3 µM each primer; summarized in Table 5, Sigma-Aldrich) and 5µl of SYBR Green Fast Mix – Low ROX (Quanta Biosciences).

Reactions were done in a white-standard 96-well plate (Thermo Fisher Scientific) and sealed with optically clear flat 8 cap strips (Thermo Fisher Scientific) and run in a Stratagene Mx3000P (Agilent Technologies). 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 and Schmittgen, 2001). EF1α (Solyc06g005060) gene was 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.

5.14. β - Glucuronidase reporter assay

The transcriptional transactivation activity of Hsfs on different promoters was determined *in vivo* by transient GUS reporter assays in tomato mesophyll protoplasts. When transcription factors with activator potential bind to the promoter harboring the reporter gene GUS, the β-glucuronidase is expressed 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 thereby can be analyzed photometrically (Gallagher, 1992). In triplicates, 50, 000 protoplasts were co-transformed with 0.5 µg Hsf expression construct and 1 µg GUS reporter plasmid DNA. Total DNA amount was adjusted to 10µgwith pRT-Neo construct. Neo is a plasmid encoding for the Neomycin resistance gene, which is used as mock plasmid DNA. After expression protoplasts were harvested by centrifugation. The supernatant was removed and protoplasts were resuspended in 50 µl of GUS buffer containing β-mercaptoethanol (50 mM $NaPO_4$, pH 7.0; 10 mM EDTA $(Na)_2$ pH 8.0; 0.1% N-Laurylsarcosine-Na-salt (v/v), 0.1% Triton X-100 (v/v); 14.3 mM β-mercaptoethanol). Samples were vigorously vortexed and frozen in liquid nitrogen. Lysis of the protoplasts was achieved by three cycles of freeze-thaw and vigorous vortexing. Following lysis, the samples were centrifuged at 20,000xg 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$mgml^{-1}$ in GUS buffer). The 96-well plate was incubated at 37°C in dark. MU-fluorescence was measured for several time points in the “Fluostar” fluorometer (BMG Lab

Technologies 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. The rest of the samples were pooled and used for Western blot analysis.

5.15. Protein turnover assay

In order to determine protein turnover in a protoplast chase experiment plasmid DNA coding for the protein of interest was transformed into protoplasts in as many replicates as time points. After 4 h of expression cycloheximide (CHX; translation inhibitor) was added to a final concentration of 20 mg ml^{-1}. Protoplasts were then harvested at different time intervals and protein extraction was performed as described for protoplast sample preparation. After SDS-PAGE and immunoblotting, protein signals were quantified using ImageJ. For every immunoblot at least 3 exposure times were used for quantification. The mean values for each time point were used for curve building.

5.16. Microscopic analysis

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

15.17. *In silico* analysis tools and statistical analysis

The domains in HsfA1 protein sequences were annotated with help of the Heatster database (*applbio.biologie.uni-frankfurt.de/hsf/heatster*) (Scharf et al., 2012). Genomic information was obtained by SOL genomics network (solgenomics.net). Information for expression of Hsf genes was obtained by TomExpress database (tomexpress.toulouse.inra.fr.) (Zouine et al., 2017). Protein sequence alignment was performed with Clustal Omega (ebi.ac.uk/Tools/msa/clustalo).

The statistical analysis in this thesis included ANOVA and Duncan's Multiple Range test and was performed using the Sigma Plot software.

6. Results

6.1. Expression of HsfA1 genes in A1CS protoplasts

In tomato, HsfA1a has been described as the master regulator of heat stress response (HSR) and thermotolerance based on hypersensitivity obtained in A1CS plants when exposed to HS (Mishra et al., 2002). The RNAi approach used in that investigation was originally designed to suppress HsfA1a as based on the limited information about the completeness of tomato Hsf family. Therefore, the possibility that other closely related recently identified members, HsfA1b, HsfA1c and HsfA1e are also co-suppressed in A1CS cells was examined.

For this purpose, the coding regions of all HsfA1-genes were amplified from tomato leaf cDNA and cloned into a pRT expression vector with an N-terminal 3xHA-tag for transient expression in tomato mesophyll protoplasts under the control of the constructively active and Hsf-independent CaMV 35S promoter. Protoplasts of wild-type (*cv*. Moneymaker) and A1CS plants were prepared and transformed with HA-HsfA1 expression plasmids. The accumulation of expressed proteins was detected using α-HA antibody for immunodetection after 6 hours of expression at 25°C. As expression control HsfA1 constructs were co-transformed with GFP encoding plasmid.

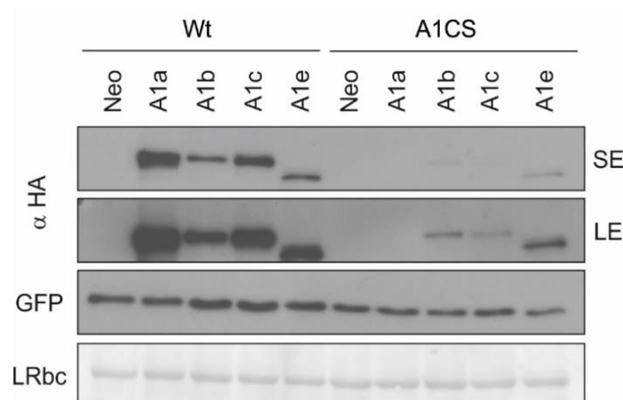

Figure 3. HsfA1-RNAi can suppress all tomato HsfA1-genes.
Protoplasts of wild type and A1CS plants were transformed with αHA-HsfA1s expression plasmids. After 6 hours protoplasts were harvested and subjected to SDS-extraction for SDS-PAGE and immunoblot analysis. The specific antibody (α-HA) was used for immunodetection. Two exposure times of the same blot are shown (SE=short exposure, LE = long exposure). Large subunit of RuBisCO (LRbc) serves as a loading control while GFP as a transformation control.

Although protoplasts were transformed with the same amount of Hsf encoding plasmids, HsfA1a and HsfA1c showed higher protein levels compared to HsfA1b and HsfA1e in wild-type protoplasts (Fig. 3). The protein levels of all Hsfs are remarkably reduced in A1CS protoplasts compared to wild-type, with HsfA1a and HsfA1c being affected the most (Fig. 3). Instead the levels of GFP are similar in all samples confirming that transfection and expression are not affected by HsfA1 expression or by HsfA1 suppression. This result suggests that all HsfA1-members are potentially co-suppressed in A1CS plants,

and therefore the master regulator function previously appointed only to HsfA1a needs to be revisited. In this direction, a detailed characterization of the recently identified HsfA1b, HsfA1c and HsfA1e is to be conducted.

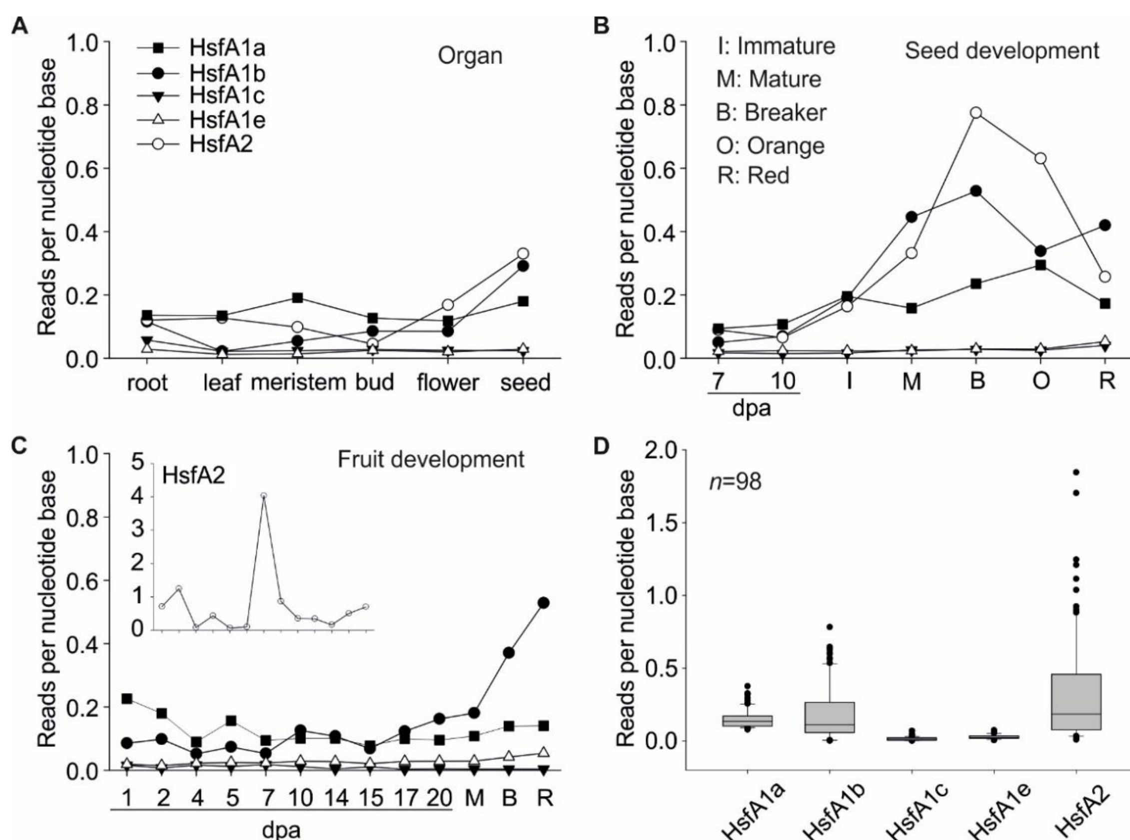

Figure 4. Transcript profile of HsfA1 genes in tomato RNAseq datasets.
(A) Expression of HsfA1 and HsfA2 genes in different tomato organs, (B) during seed development, and (C) fruit development and ripening. dpa: days post anthesis, M: mature green fruit, B: Fruit at breaker stage, R: red ripe fruit. (D) Box plots of transcript levels of HsfA1 and HsfA2 genes in tomato tissues, indicating the relative levels and variation in expression of each gene across 98 samples. Data is obtained by TOMEXPRESS (Zouine et al., 2017).

6.2. Transcript abundance of HsfA1-genes under control and aberrant temperatures

The absolute transcript levels of tomato HsfA1 genes were obtained from existing RNA-seq databases (TOMEXPRESS) (Fig. 4). The levels of the stress-inducible HsfA2 were obtained for comparison. HsfA1a and HsfA1b are the most abundant HsfA1 genes in roots and flowers, while HsfA1a shows higher levels compared to HsfA1b, HsfA1c and HsfA1e in leaf and meristem tissues (Fig. 4A). HsfA1b is the highest expressed HsfA1 in seeds, while in general HsfA1c and HsfA1e are only weakly expressed (Fig. 4A). Apart from leaves, HsfA2 and HsfA1b have similar pattern of expression. This was confirmed in seeds from different developmental stages, as the transcripts of HsfA1b and HsfA2 are upregulated in seeds

of immature green fruits and peak in seeds of breaker fruits (Fig. 4B). HsfA1a shows a steadier expression profile, while HsfA1c and HsfA1e only low levels throughout all developmental stages.

Similar expression patterns are present during fruit development, with HsfA1e showing a very weak induction in red ripe fruit, but HsfA1b after the onset of ripening at the breaker stage (Fig. 4C). HsfA2 showed two peaks, one strong during fruit growth at 14 dpa, and a much weaker during ripening. In general, HsfA1a shows steady state levels in most of the tissues examined and as such is in some cases the most abundant HsfA1. HsfA1c and HsfA1e are only weakly expressed, while similar to HsfA2 in several cases HsfA1b is developmentally-regulated and preferentially expressed in specific tissues (Fig. 4D).

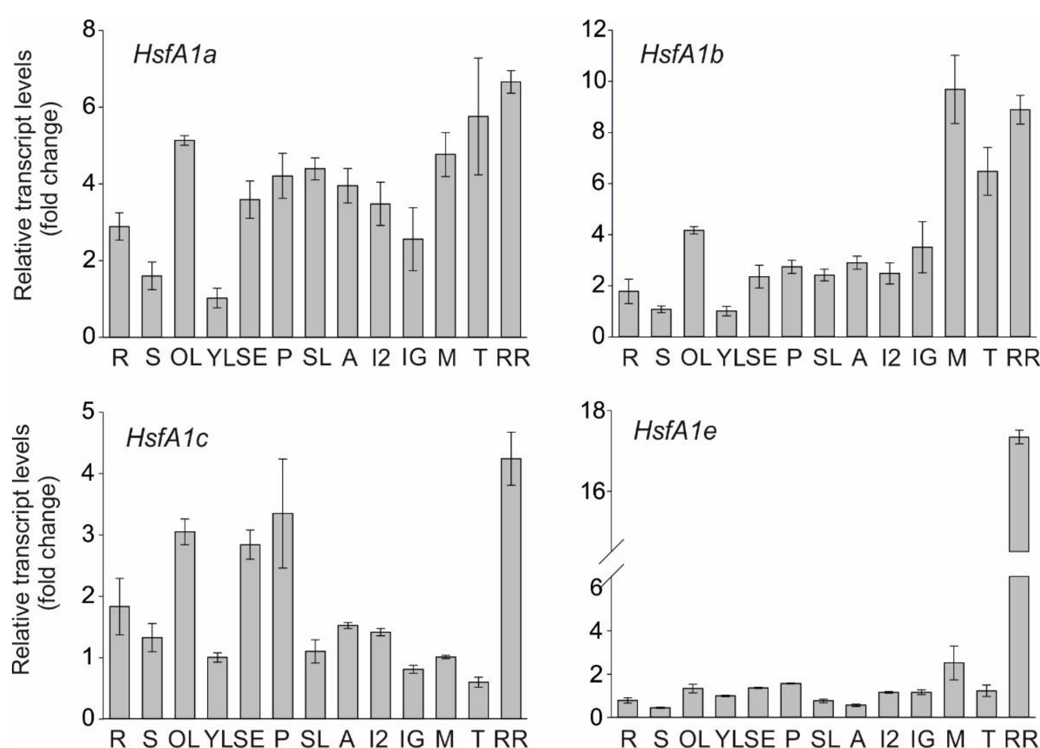

Figure 5. Relative transcript levels of four HsfA1- genes in different tomato tissues using qRT-PCR.
RNA of different tissues was isolated under control conditions including root(R), stem (S), old leaves (OL), young leaves (YL), sepals (SE), petals (P), style (SL), anther (A), immature green fruit 2 cm in diameter (I2), immature green fully grown (IG), mature green fruit (M), turning fruit (T) and red ripe fruit (RR). The transcript level of the individual HsfA1 was quantified in the corresponding cDNA using qRT-PCR. Ct values were normalized to the Ct value for EF1a used as housekeeping gene and calculated relative to the levels of the gene in young leaves (YL). Bars represent the mean value for three replicates ± SE.

The transcript levels of the four HsfA1 genes were examined using qRT-PCR as well in both vegetative and reproductive tissues in the cultivar Moneymaker (Fig. 5). HsfA1a shows steady state levels among different tissues and only slightly reduced levels in young leaf and stem tissues. As shown in transcriptome data, HsfA1b is upregulated in ripening fruit. HsfA1c shows an approximately 5-fold induction in red ripe fruit when compared to mature green fruit, but is also expressed at increased

levels in old leaves, sepals and petals when compared to the rest tissues. Last, HsfA1e shows steady state levels across all samples with the exception of the red ripe fruit, in which an approximately 17-fold induction was detected (Fig. 5). Altogether, these results point to possible tissue-specific functions for HsfA1 members.

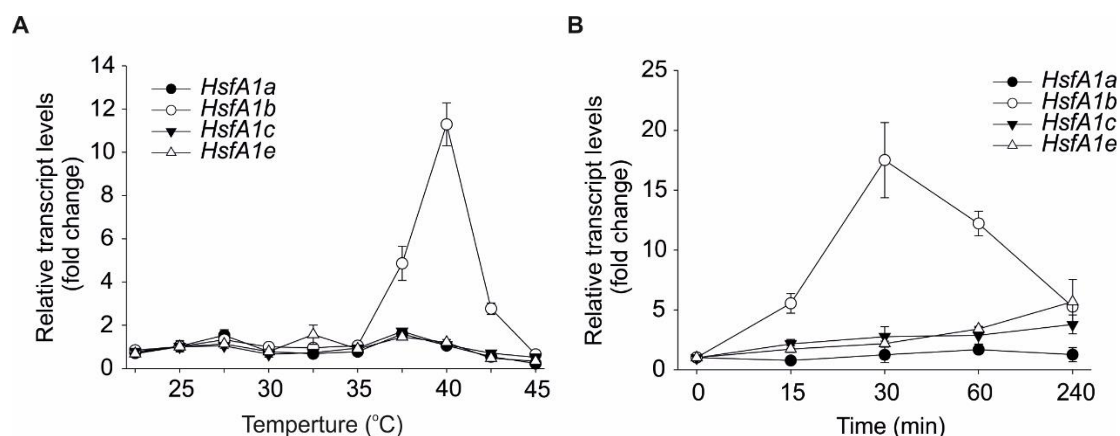

Figure 6. Transcript levels of HsfA1 genes in response to heat stress examined by qRT-PCR.
RNA of tomato leaf tissue was isolated after (A) different heat treatments for 1hour or (B) 40°C treatment for different time intervals. After cDNA preparation, transcript level was quantified using qRT-PCR. Ct values were normalized to the Ct value of EF1a used as housekeeping gene and calculated relative to 25°C or 0 time point. Bars represent the mean value for three replicates ± SE.

To investigate the expression pattern of HsfA1-encoding genes under HS, young leaves of tomato plants were collected and exposed for 1 hour at temperatures ranging from 22.5 to 45°C. All HsfA1s except HsfA1b show a remarkable stability in expression under all temperatures. Only HsfA1b is induced at temperatures above 35°C, having maximum levels at 40°C followed by a gradual decrease at higher temperatures (Fig. 6A). In addition, the levels of HsfA1 transcripts were examined in young leaves exposed to 40°C for different time points. Interestingly, HsfA1b transcripts upregulate already 15 minutes after the heat treatment, peak at 30 minutes and then gradually decrease, remaining at higher levels than the non-stressed sample (T0) even after 4 hours of treatment (Fig. 6B). HsfA1e transcripts show a 5-fold induction after 4 hours of stress, suggesting that in contrast to the early responsive HsfA1b, HsfA1e is a late responsive gene. Instead, HsfA1a and HsfA1c retain steady state levels at all time points.

6.3. Characterization of the properties of HsfA1 proteins

6.3.1. Transactivation activity

In contrast to the well-characterized HsfA1a, the other tomato HsfA1-proteins have not been analyzed so far. As the rapid and strong induction of several genes is essential for cell survival under HS, a master regulator is thought to have a wide range of activity on many genes (Mishra et al., 2002). The activity

of HA-tagged HsfA1-proteins was examined using a GUS reporter assay in tomato protoplasts. In which, GUS open reading frame is under the control of different HSE-containing promoters, including promoter from tomato Hsps and Hsfs, namely Hsp21.5-ER, Hsp70-1, HsfA2, and HsfA7 (Fig. 7). In addition, GmHsp17* a promoter fragment derived from the soybean (*Glycine max*) Hsp17.3B-CI gene containing two Hsf binding sites was used (Treuter et al., 1993). The promoters used have different number, and distribution of HSEs (Fig. 7A). Protein levels of all transformed HsfA1s were examined in the same samples by immunoblotting using an anti-HA antibody.

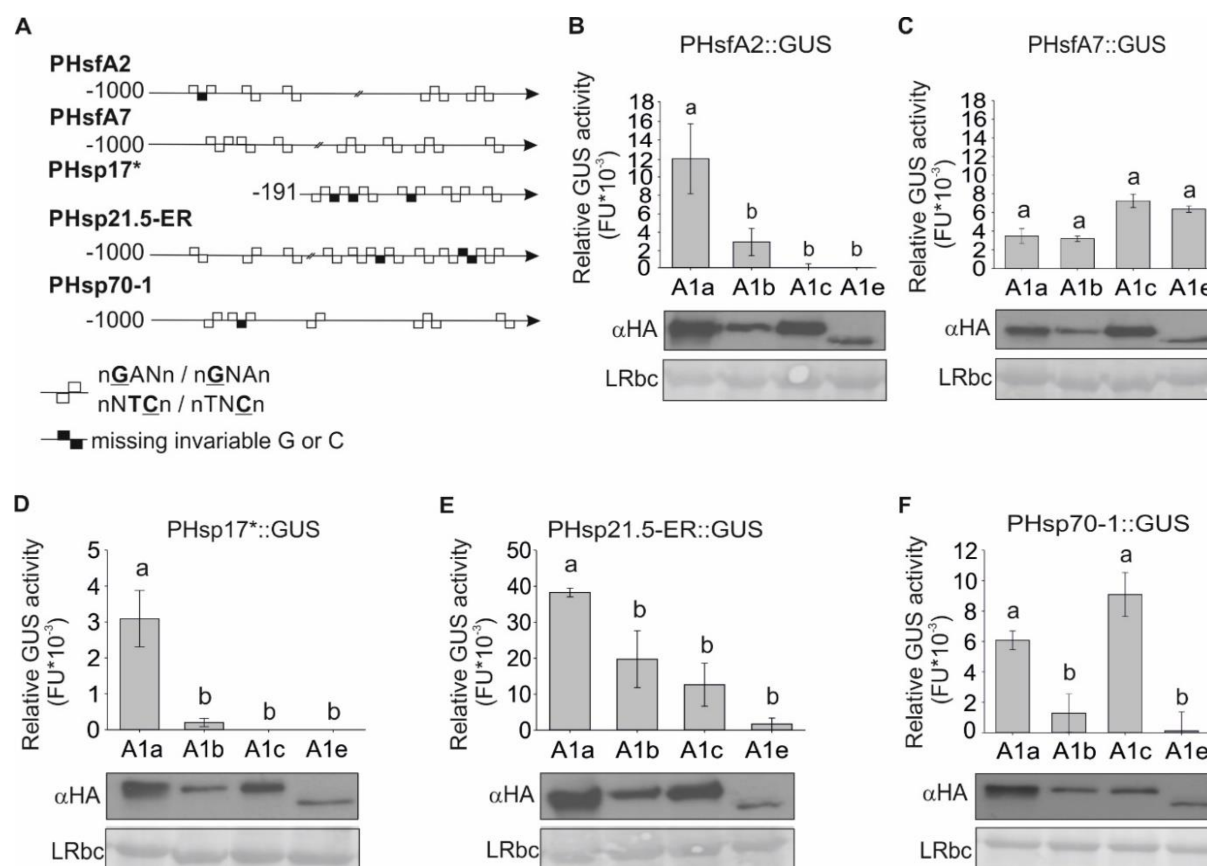

Figure 7. Activity of HsfA1 members on different reporter constructs.
(A) Schematic representation of the different *GUS*-driving promoters with different HSE-architectures. White squares represent the functional HSE motifs while black ones the non-functional HSE motifs. (B-F) HsfA1 proteins activity monitored by co-expression of HA-HsfA1 expression plasmids with (B) PHsfA2, (C) PHsfA7, (D) PHsp17*, (E) PHsp21.5, (F) PHsp70-1 GUS reporter constructs into tomato mesophyll protoplasts. Cells were harvested after 7 h of expression at 25°C and prepared for GUS assay. Bars represent the average GUS activity in fluorescence units (FU*10^{-3}) of the indicated HsfA1-proteins ±SE of three replicates from independent transformations. The average was normalized to the average of corresponding Neo (empty vector without Hsf). After GUS fluorescence measurements, samples were subjected to immunodetection of the respective Hsfs using the specific antibody α-HA. Large subunit of RuBisCO (LRbc) serves as a loading control. Statistically significant difference ($p < 0.05$) represented in different letters was calculated by ANOVA test and Duncan Mutliple Range test.

HsfA1a showed enhanced GUS activities, suggesting that HsfA1a is able to activate all tested promoters and with the activity being the highest among HsfA1 proteins on Hsp17*, Hsp21.5 and HsfA2

promoters (Fig. 7B-F). HsfA1b showed also enhanced GUS activity, which however is much weaker than that of HsfA1a, with the exception of HsfA7 promoter. Instead HsfA1c and HsfA1e were inactive on Hsp17*, HsfA2 and only HsfA1c activated Hsp21.5-ER. Interestingly, all HsfA1-proteins have the same activities on HsfA7 promoter (Fig. 7C), while only HsfA1a and HsfA1c share the stronger activity on Hsp70-1 promoter (Fig. 7F).

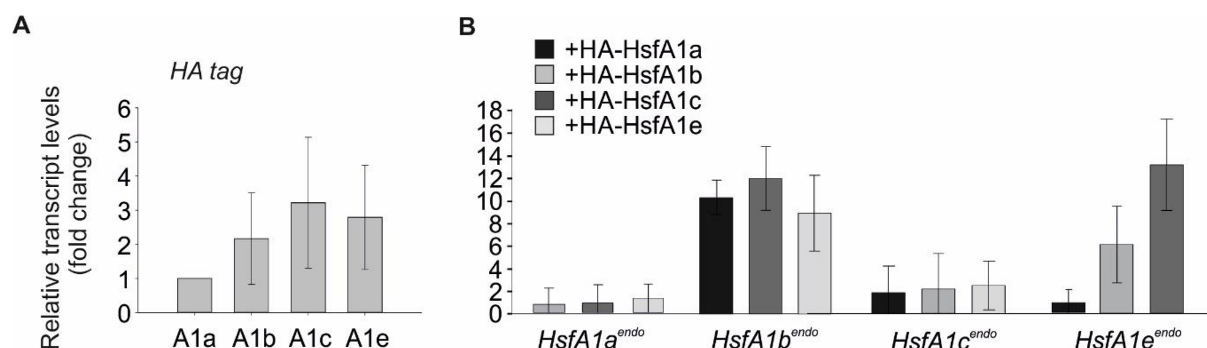

Figure 8: Transcript levels of the transgenic and endogenous HsfA1 genes in protoplasts using qRT-PCR. Tomato protoplasts were transformed with HA-HsfA1 expression plasmids and incubated for 8h at 25°C. After cell harvest and RNA-extraction, the total transcript levels of the transgenic HA-tagged genes (A) and the HsfA1 genes (B) were determined using HA-tag and specific primers for HsfA1 genes, respectively. Ct values were normalized to the Ct value of EF1a used as housekeeping gene and calculated relative to Neo sample (empty vector without Hsf). Shown are the mean values of 3 biological replicates with error bars representing ±SE.

Although protoplasts were transformed with the same amount of expression plasmids, the detected levels of the HsfA1b and HsfA1e proteins were lower than that of HsfA1a or HsfA1c in all cases (Fig.7). Therefore, the transcript level of transgenes was determined by qRT-PCR using primers annealing to the HA-coding region. The transcripts encoded by the transgene cassette of each HsfA1 had similar abundance supporting uniformity in expression of the HA-HsfA1 genes in different protoplasts (Fig. 8A).

In addition, the total levels (endogenous and transgene) of each HsfA1 gene were determined using gene specific primers (Fig. 8B). *HsfA1a* and *HsfA1c* maintained steady levels in protoplasts expressing other HsfA1s. Instead *HsfA1b* is upregulated in protoplasts expressing HsfA1a, HsfA1c and HsfA1e, while endogenous *HsfA1e* is induced in protoplasts expressing HsfA1c. Therefore, these results suggest a possible crosstalk in the regulation of HsfA1 subfamily.

The interesting observation of preferential activity of HsfA1-members for specific genes was further examined on the transcript level of endogenous genes of mesophyll protoplasts transformed with individual HsfA1 expression plasmid under control conditions (Fig.9). A set of known HS-induced genes encoding for transcription factors and chaperones including *HsfA2*, *HsfA7*, *Hsp17.7C-CI*, *Hsp15*.2CI, *Hsp70s*, *Hsp90s* and *Hsp101* were determined by qRT-PCR analysis (Fig. 9). All tested HS-genes, and particularly *sHsps*, were highly induced in response to HsfA1a overexpression compared to other HsfA1-members, with the exception of *Hsp70*-1 which was upregulated in similar level by HsfA1a and

HsfA1c (Fig. 9). HsfA1b on the other hand, did not show any preferential stimulatory effect. In general these results are in agreement with the GUS reporter assay and confirm the wide range of activity of HsfA1a but the preferential activity of HsfA1c and HsfA1e on specific genes.

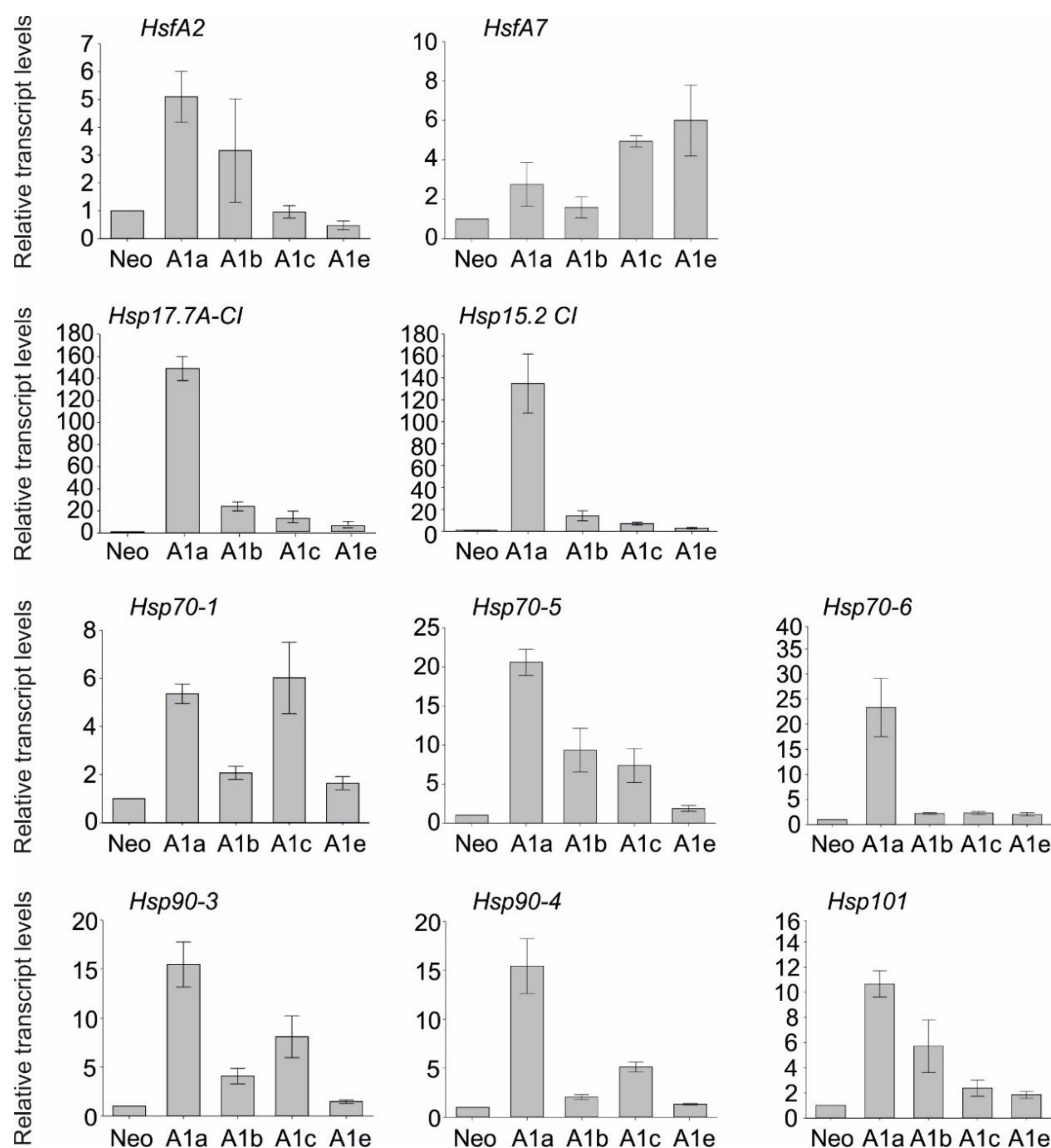

Figure 9. Effect of expression of HsfA1 protein on transcript levels of endogenous HS-induced genes.
Tomato protoplasts (cv. Moneymaker) were transformed with HA-HsfA1 expression plasmids and incubated for 8h at 25°C. After cell harvest and RNA-extraction, transcript levels of the indicated endogenous genes were determined by qRT-PCR. Ct values were normalized to the Ct value of EF1a used as housekeeping gene and calculated relative to Neo sample (without Hsf). Shown are the mean values of 3 biological replicates with error bars representing ±SE.

6.3.2. Co-activator capacity of HsfA1 proteins

HsfA1a can form co-activator complexes with HsfA2 by direct interaction and with HsfB1 by the formation of ternary complexes with HAC1/CBP (Bharti et al., 2004; Chan-Schaminet et al., 2009).

These co-activator complexes can stimulate transcriptional synergy in which the activity of Hsf complexes is greater than the sum the activity of the individual factors. Therefore, the capacity of HsfA1-members to form such complexes, already characterized for HsfA1a, was examined using a reporter assay on the PHsp17*-GUS reporter construct (Treuter et al., 1993; Bharti, 2004). Protoplasts were transformed with HA-HsfA1 encoding plasmids either alone or in combination with tomato HsfA2, HsfA3, HsfA7 and HsfB1 expression plasmids (Fig. 10).

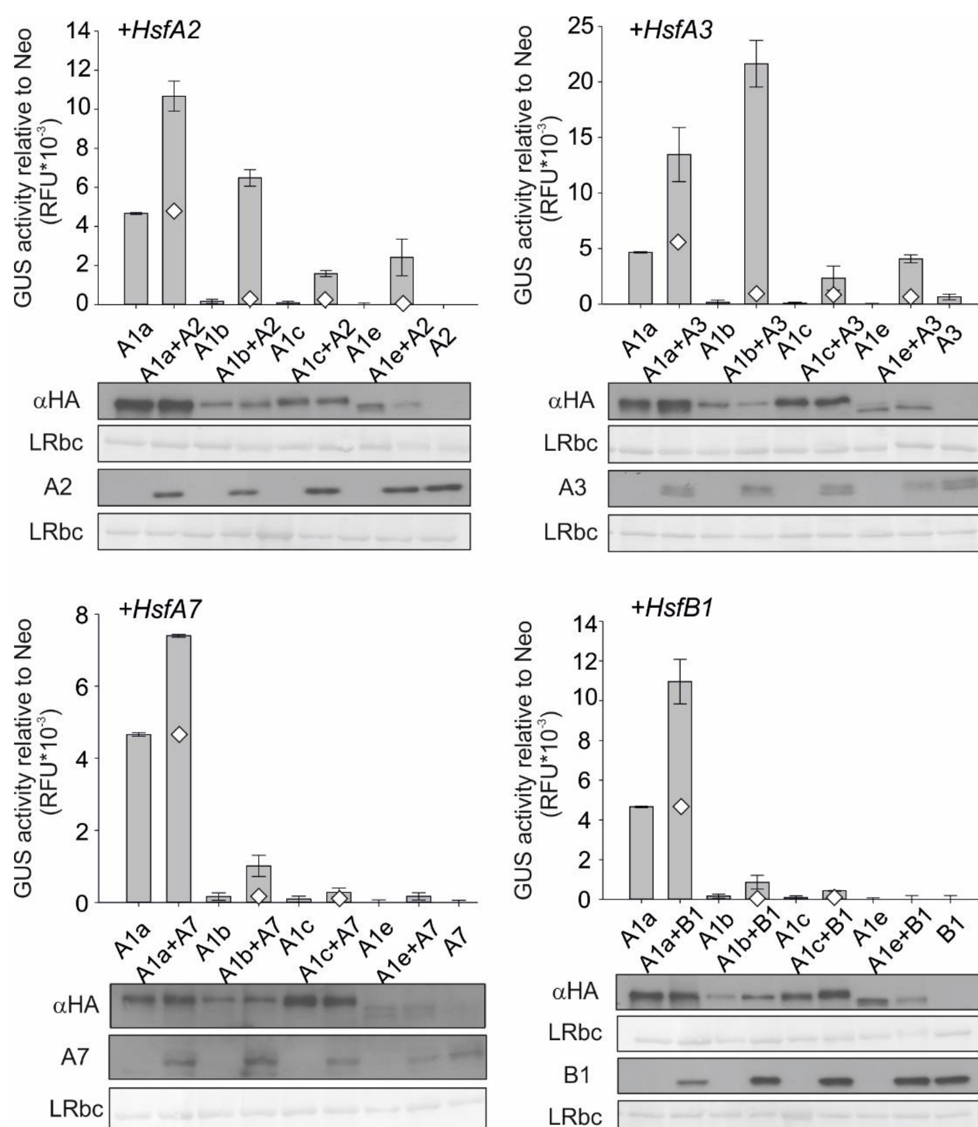

Figure 10. Co-activator capacity of HsfA1 proteins with other Hsfs.

HsfA1s co-activity was monitored by co-expression of HsfA1s constructs and HSE-containing GUS reporter construct PHsp17*-GUS (Bharti et al., 2004) with and without HsfA2, HsfA3, HsfA7 and HsfB1 in tomato mesophyll protoplasts. Samples were harvested after 7 h of expression and prepared for GUS assay. Bars represent the average GUS activity of the indicated Hsfs alone or Hsf combinations and error bars are ±SE of three replicates. The activity was normalized to Neo sample (without any Hsf) and is presented in relative fluoresence unit (RFU). Following GUS fluorescence measurements, samples were subjected to immunodetection using specific antibodies against HsfA2, HsfA3, HsfA7 and HsfB1 or αHA antibody against HsfA1 proteins. Large subunit of RuBisCO (LRbc) serves as a loading control.

HsfA1a shows a strong activity already when expressed alone in protoplasts, but its activity is further stimulated in the presence of the co-activators HsfA2, HsfA3, HsfA7 and HsfB1 (Fig. 10).

HsfA1b showed only strong synergistic activity in the presence of HsfA2 and HsfA3, with the latter being even stronger than that of HsfA1a-HsfA3 (Fig. 10). Instead, the inactive per se HsfA1c and HsfA1e on Hsp17* promoter, had an only weak co-activity with the tested coactivators except HsfB1. These findings suggest that HsfA1a can form co-activator complexes with several other Hsfs, while in the case of HsfA1b the co-activator capacity is preferential with specific Hsfs, like HsfA2 and HsfA3. Instead, HsfA1c and HsfA1e have only limited synergistic activity, the weakest among HsfA1- members.

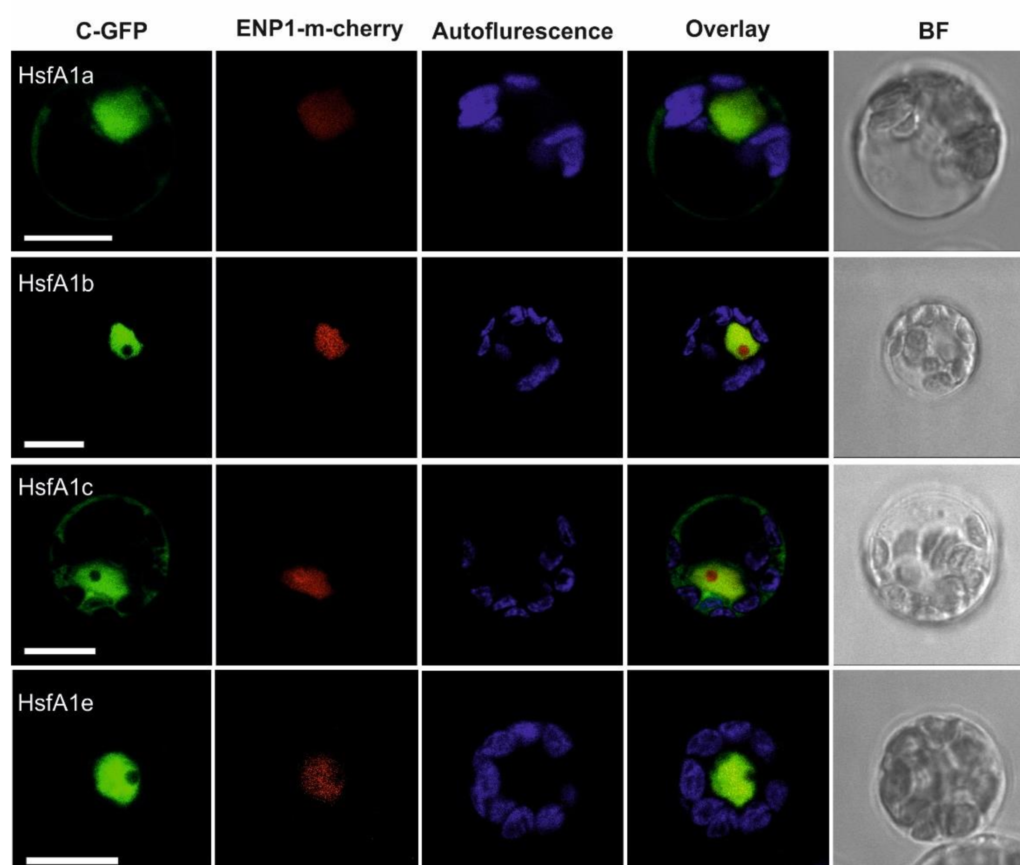

Figure 11. Intracellular distribution of HsfA1 proteins using CLSM.
Tomato mesophyll protoplasts (MM) were co-transformed with 10 µg of GFP-HsfA1 expression plasmids and 10µg of ENP1-mCherry expression plasmid as a nuclear marker. Images were recorded after 8 h of incubation at 25°C. OL, overlay; AF, autofluorescence; BF, bright field. Scale bar =10 µm. The same images were recorded in more than 15 protoplasts in individual experiments.

6.3.3. Intracellular localization and stability of HsfA1 proteins

Hsfs carrying NLS and NES are thought to shuttle between the nucleus and the cytoplasm, with the balance of the distribution determining the activity and fate of the factors(Scharf et al., 1998; Röth et al., 2016a). Sequence analysis predicts the same domain structure among HsfA1-members with the

exception for HsfA1e where NES is not identified yet (Supplemental Fig. 1). To examine the intracellular distribution of HsfA1 proteins, a plasmid carrying an expression cassette of each HsfA1 gene fused to the C-terminus of GFP was transformed into tomato mesophyll protoplasts. As a nuclear marker, ENP1-mCherry (Missbach et al., 2013) expressing plasmid was co-transformed with each GFP-HsfA1. Intracellular localization was then detected using CLSM (Fig. 11). As previously reported (Scharf et al., 1998), the GFP signal obtained for HsfA1a is distributed between the nucleus and the cytoplasm with a stronger signal in the nucleus (Fig. 11). HsfA1c shows a similar nucleocytoplasmic distribution, while HsfA1b and HsfA1e show a strong nuclear retention (Fig. 11).

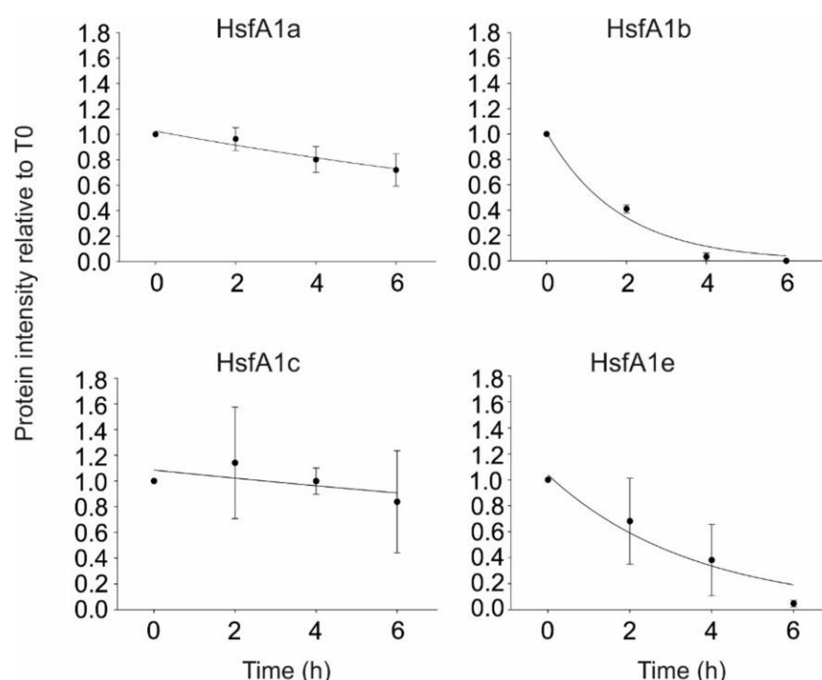

Figure 12. Protein stability of HsfA1 members in the presence of the translation inhibitor cycloheximide. Tomato mesophyll protoplasts were transformed with plasmids coding for HA-HsfA1 genes and allowed to express the proteins for 4 h after which the translation inhibitor cycloheximide was added at a final concentration of 20 µg ml^{-1}. Samples were further incubated at 25°C and harvested at different time points. Protein extracts were subjected to SDS-PAGE and immunoblotting, followed by detection with α-HA specific antibody and quantification of protein signals using ImageJ. Data points represent the immune signal intensity calculated relative to T0. Error bars are ±SE of three independent experiments.

These results do not directly correlate with the activity of HsfA1-members, as a stronger activity is expected for factors with strong nuclear retention, indicating the existence of certain regulatory mechanisms controlling the activity of Hsfs. One of these mechanisms could be the protein turnover, as in all activity assays the levels of HsfA1b and HsfA1e were lower when compared to HsfA1a and HsfA1c, while the transcript levels of the transgenes are similar (Fig. 8A).

To compare the rate of degradation of HsfA1 proteins, protoplasts were transformed with plasmids coding for HA-HsfA1 members under the control of the CaMV 35S promoter. Protoplasts were allowed

to express the proteins for 4 h and subsequently treated with the translation inhibitor cycloheximide (CHX). Protein levels were monitored in protoplasts for 6 hours following CHX treatment at 25°C (Fig. 12). Interestingly, both HsfA1a and HsfA1c remained quite stable during the whole experiment while HsfA1b and HsfA1e had a rapid turnover, with a half-life of approximately 2-4 hours. Thus, we can conclude that differences in protein abundance determined by immunoblotting are due to differences in protein stability.

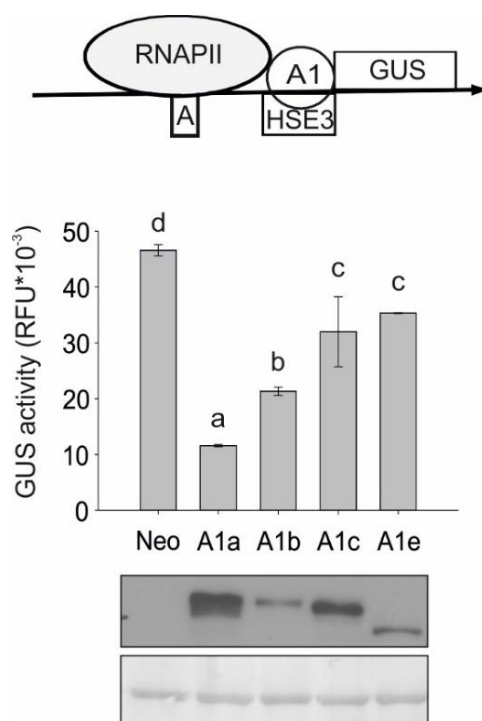

Figure 13. DNA binding of HsfA1-proteins on the HSE containing promoter based on a repressor assay.
Tomato protoplasts were transformed with GUS repressor construct alone or with αHA-HsfA1 expression plasmids. After 7h incubation at 25°C, cells were collected and prepared for GUS assay. Scheme on top represents the basis of the GUS repressor assay. Bars represent the average of 3 replicates derived from individual transformation events ±SE. The protein levels of the transformed HsfA1s were detected by α-HA antibody on immunoblots. Large subunit of RuBisCO (LRbc) serves as a protein loading control. Different letters indicate statistically significant difference ($p < 0.05$) calculated by ANOVA test and Duncan Mutliple Range test.

6.3.4. DNA binding capacity of HsfA1 proteins

DNA recognition by Hsfs is a critical step for transcriptional activity initiation. As obtained before in GUS assay, HsfA1a had a transcriptional activity on different promoters including HsfA2, HsfA7 and Hsps (Fig. 7). Similar activities were obtained in transcriptomic analysis of endogenous HS-genes in response to ectopic expression of HsfA1s with prominent activity of HsfA1a on sHsps (Fig. 9). Instead, HsfA1c and HsfA1e showed a preferential activity toward Hsp70-1 and HsfA7 which even stronger than that of HsfA1a (Fig. 9).

To describe the relation between DNA-binding capacity and transcriptional activity, the previously established GUS repressor assay was used (Treuter et al., 1993; Hahn et al., 2011). In which GUS reporter gene expression is regulated by the constitutive CaMV 35S promoter. A synthetic sequence containing three adjacent HSEs has been inserted in the region between the TATA box and the start codon of the GUS open reading frame. In the presence of Hsf and in case of Hsf binding to the HSE motifs in the promoter, GUS activity is reduced as following transcriptional initiation polymerase II

(RNAPII) is prevented from passing through to the open reading frame (Fig. 13; Treuter et al., 1993). Thus, the reduction in GUS activity is considered as an indicator of Hsf DNA-binding.

Protoplasts were co-transformed with GUS repressor construct and HsfA1s expression plasmids, allowed to express proteins for 7 hours and prepared for GUS repressor assay. In parallel, GUS repressor construct was transformed alone without any Hsfs as a control (Fig.13). Strong reduction of approximately 75% was observed in GUS activity in the presence of HsfA1a. HsfA1b expression led to an about 50% GUS repression while only weak reduction obtained with HsfA1c and HsfA1e (Fig. 13). Thus, we can conclude that HsfA1a, among different HsfA1-members, has the strongest binding capacity to such HSE motifs. This finding points to the strong relationship between DNA-binding and activity of HsfA1s which is likely to be responsible for that difference in activity and specificity toward different HS-genes.

6.4. Analysis of HsfA1a and HsfA1c domain-swapping mutants

The marked differences in properties among members of the HsfA1 subfamily prompted us to investigate the contribution of each domain in the activity and specificity. Here we focused on the comparison between HsfA1a as a master regulator and HsfA1c of the same phylogenetic origin that showed similar expression pattern and some properties like stability and nucleocytoplasmic distribution but differ significantly in transcriptional activity and specificity (Fig. 7 and 9). Each Hsf was divided into three regions: N-terminal region containing the DBD, middle-region containing the oligomerization domain, and C-terminal region containing the NLS, AHA motifs and NES (Fig. 14). These mutations did not have any effect on activity, stability or DNA binding capacity of Hsfs.

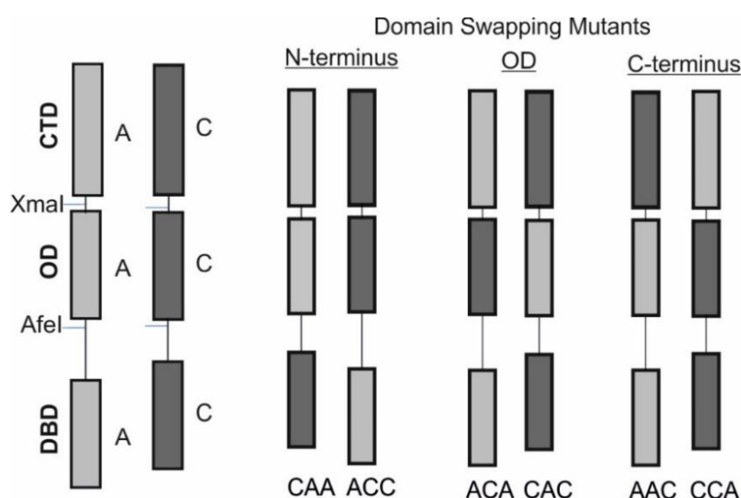

Figure 14. Schematic representation of different domain swaps.
Domain swapping mutants including DBD containing N-terminal swaps (N-terminus); Oligomerization domain swaps (OD) and C-terminal domain swaps (C-terminus). Each domain is represented with one capital letter, A for HsfA1a or C for HsfA1c with the domain order from left to right DBD, OD, CTD.

6.4.1. Transcriptional transactivation activity of oligomerization domain and C-terminal swapping mutants

HsfA1a has a unique synergistic activity among HsfA1-proteins with HsfA2 and HsfB1 (Fig.10). This has been previously attributed to the oligomerization of HsfA1a and HsfA2 (Chan-Schaminet et al., 2009) and the C-terminal domain of HsfA1a required for interaction with HAC1 for ternary complex formation with HsfB1 (Bharti et al., 2004). Based on this, first the transactivation activity of the oligomerization domain and C-terminal swapping mutants was examined in a GUS activity assay using the PHsp17*:: GUS reporter construct. Interestingly, *ACA* mutant has similar activity with HsfA1a (*AAA*), while *CAC* has similar activity to HsfA1c (*CCC*) when the proteins were expressed alone (Fig. 15B). Same results were yielded when the wild-type and swapping mutants were expressed with either HsfA2 or HsfB1

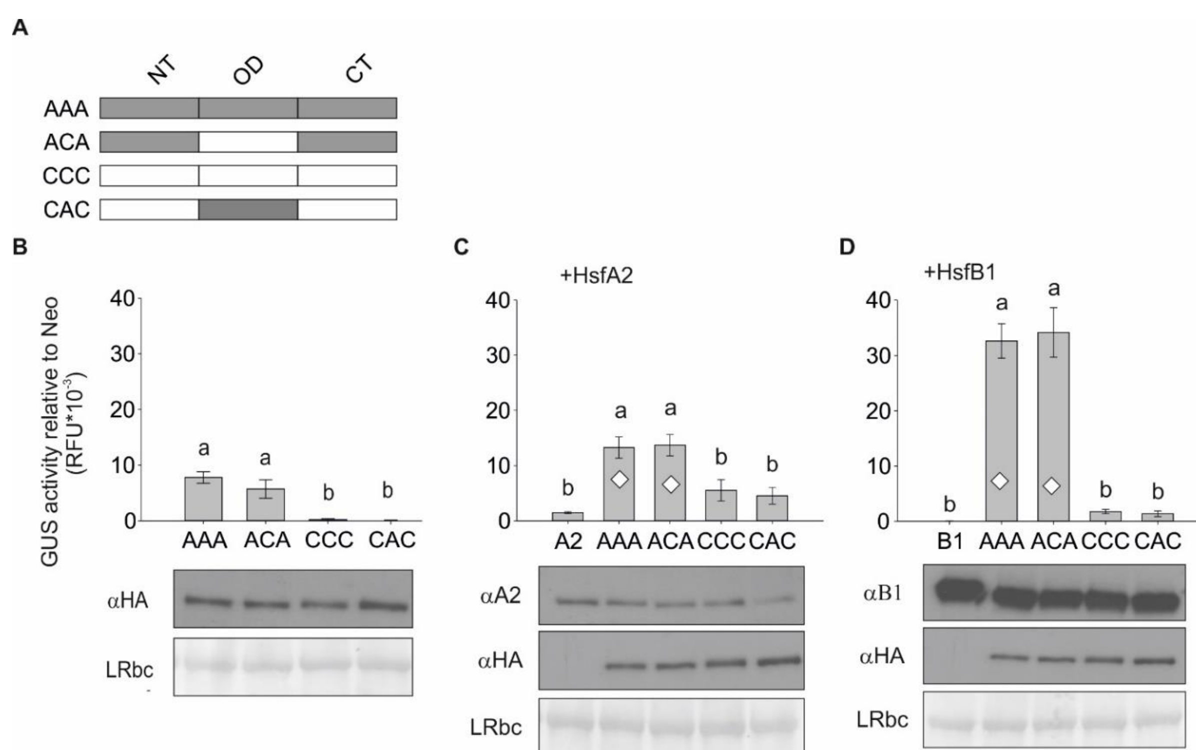

Figure 15. Contribution of the oligomerization domain of HsfA1a and HsfA1c in activity and cooperation with other Hsfs.

(A) Schematic representation of OD swapping mutants. NT, N-terminus; OD, Oligomerization domain; CT, C-terminus (B) The transcriptional activity of OD swapping mutants using HSE-containing GUS reporter construct PHsp17*::GUS (Bharti, 2004) in tomato mesophyll protoplast system. (C-D) The co-activity of OD swapping mutants with HsfA2 and HsfB1, respectively. The synergistic activity was monitored using the same GUS reporter construct PHsp17*::GUS. Bars represent the average GUS activity of the indicated HsfA1s or Hsf combinations and error bars are ±SE (n=3). Different letters indicate statistically significant difference ($p < 0.05$) calculated by ANOVA test and Duncan Mutliple Range test. After the GUS fluorescence measurements, samples were subjected to immunodetection of the respective Hsfs using α-HA against HA-HsfA1s while specific antibodies against HsfA2 and B1. Large subunit of RuBisCO (LRbc) serves as a loading control.

(Fig. 15 C-D), suggesting that differences in the activity of HsfA1a and HsfA1c are likely not due to discrepancies in the oligomerization domains of the two proteins.

Next we analysed the possibility that the functional differences are conferred by the C-terminal region of HsfA1s. The swapped region contains the activation motifs as well as the NLS and NES. The replacement of HsfA1a C-terminus with that of HsfA1c (*AAC*) did not result in any change in transactivation activity in comparison to HsfA1a (AAA) either when was expressed alone or in combination with HsfA2 or HsfB1 (Fig. 16). On the other hand, HsfA1c hybrid containing HsfA1a C-terminal region (*CCA*) did not show any gain of activity when compared to HsfA1c (CCC). Instead, the low synergistic activity of HsfA1c was completely abolished in *CCA* mutant (Fig. 16C-D).

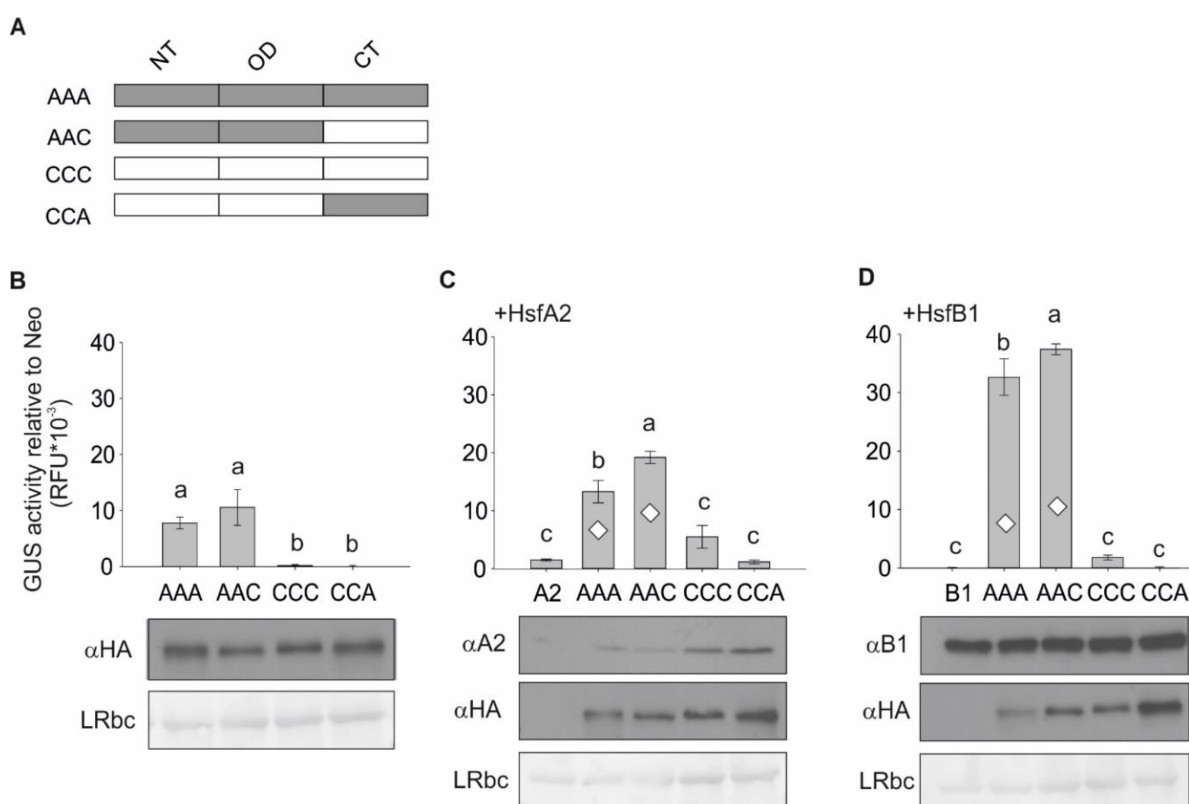

Figure 16. Contribution of the C-terminal domain of HsfA1a and HsfA1c on activity and cooperation with other Hsfs.

(A) Schematic representation of CTD swapping mutants. NT, N-terminus; OD, Oligomerization domain; CT, C-terminus. (B) The transcriptional activity of CTD swapping mutants using HSE-containing GUS reporter construct PHsp17*::GUS (Bharti, 2004) in tomato mesophyll protoplast system. (C-D) The coactivity of CTD swapping mutants with HsfA2 and HsfB1, respectively. The synergistic activity was monitored using the same GUS reporter construct PHsp17*::GUS. Bars represent the average GUS activity of the indicated HsfA1s or Hsf combinations and error bars are SE of three replicates. Different letters indicate statistically significant difference ($p < 0.05$) calculated by ANOVA test and Duncan Mutliple Range test. After the GUS fluorescence measurements, samples were subjected to immunodetection of the respective Hsfs using α-HA against HA-HsfA1s while specific antibodies against HsfA2 and B1. Large subunit of RuBisCO (LRbc) serves as a loading control.

Together these results suggest that the differences observed regarding transactivation activity and co-activity do not account for discrepancies in the oligomerization domains or C-termini of the two proteins.

6.4.2. Transcriptional activity and DNA-binding capacity of N-terminal swaps

The transcriptional activity of N-terminal swapping mutants was examined using GUS reporter assay, in which the transcriptional activity was measured using PHsp17*:: GUS reporter construct. Protoplasts were co-transformed with reporter construct and expression plasmids of intact HsfA1a, HsfA1c or N-terminal swaps (*CAA* or *ACC*). Interestingly, the *CAA* mutant has very low transactivation activity similar to HsfA1c (CCC) while the *ACC* enhanced activity similar to HsfA1a (AAA) (Fig. 17).

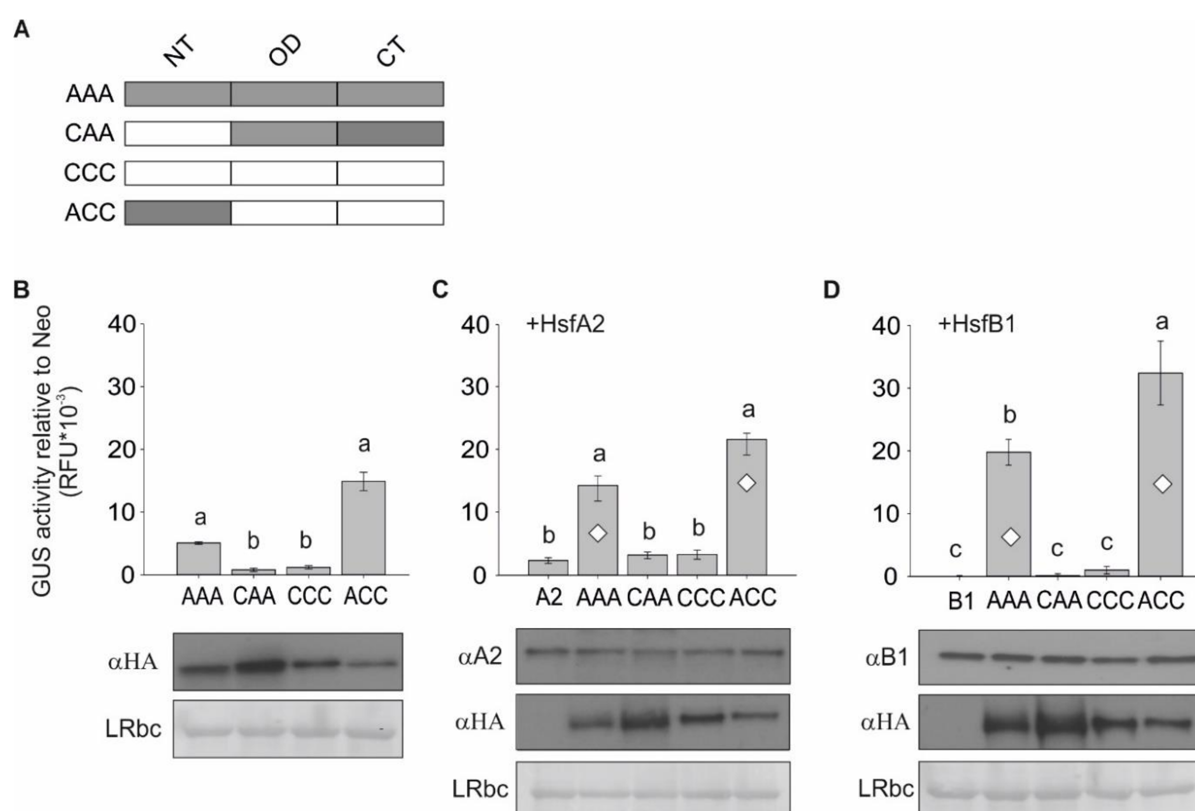

Figure 17. Activity and coactivity of N-terminal HsfA1a and HsfA1c swapping mutants using a GUS reporter assay.
(A) Schematic representation of DBD swapping mutants. NT, N-terminus; OD, Oligomerization domain; CT, C-terminus. (B) The transcriptional activity of DBD swapping mutants using HSE-containing GUS reporter construct PHsp17*::GUS (Bharti, 2004)in tomato mesophyll protoplast system. (C-D) The co-activity of N-terminal swapping mutants with HsfA2 and HsfB1, respectively. The synergistic activity was monitored using the same GUS reporter construct PHsp17*::GUS. Bars represent the average GUS activity of the indicated HsfA1s or Hsf combinations and error bars are SE of three replicates. Different letters indicate statistically significant difference (p < 0.05) calculated by ANOVA test and Duncan Mutliple Range test. After the GUS fluorescence measurements, samples were subjected to immunodetection of the respective Hsfs using anti-HA antibody against HA-HsfA1s while specific antibodies against HsfA2 and HsfB1. Large subunit of RuBisCO (LRbc) serves as a loading control.

Synergistic activity of the N-terminal swaps with HsfA2 and HsfB1 was also examined using the same PHsp17*:: GUS reporter construct(Fig. 17C-D). As previously obtained, the wild-type HsfA1a (AAA) has a strong synergistic activity with both HsfA2 and HsfB1 while HsfA1c has no further transcriptional activation with both Hsfs (Fig. 17C-D). Similarly, no co-activity could be detected for HsfA1a-hybrid *CAA* with neither HsfA2 nor HsfB1, while the hybrid of HsfA1c with DBD-containing N-terminus of HsfA1a (*ACC*) has an enhanced activity with both HsfA2 and HsfB1 similar to wild type HsfA1a (Fig. 17C-D). These differences observed in N-terminal swapping mutants might be due to the capacity of the mutants to bind to HSE-containing promotor, as shown earlier for HsfA1a and HsfA1c (Fig. 13). Therefore, the DNA-binding capacity was examined in a GUS repressor assay. Interestingly, *CAA* mutant shows reduced repressor activity compared to HsfA1a (AAA) while *ACC* mutant has enhanced repressor activity compared to HsfA1c and similar to HsfA1a (Fig. 18).

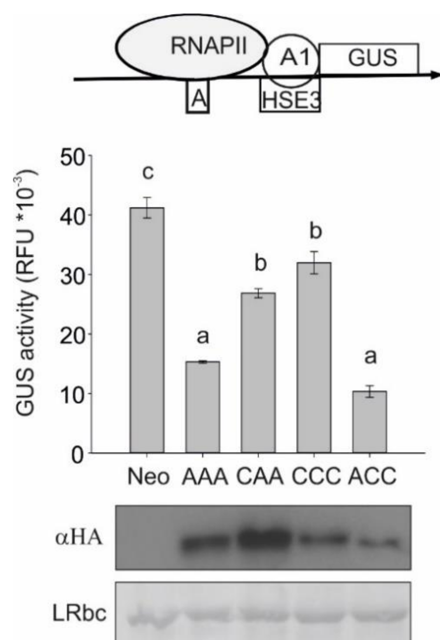

Figure 18. DNA binding capacity of N-terminal HsfA1a and HsfA1c swapping mutants based on a repressor assay.
Protoplasts of wild type tomato were transformed with GUS repressor construct alone (Neo) or with expression constructs of intact and DBD swaps of HsfA1a and HsfA1c. After 7h incubation at 25°C, cells were collected and prepared for GUS assay. The mean value of 3 technical replicates was calculated ±SE. Different letters indicate statistically significant difference ($p < 0.05$) calculated by ANOVA test and Duncan Mutliple Range test. The protein levels of the transformed HsfA1s were detected by α-HA antibody on immunoblots. Large subunit of RuBisCO (LRbc) serves as a loading control.

For further examination of N-terminal swapping mutants but on endogenous genes, the wild type and N-terminal swaps were expressed in tomato protoplasts while the transcript levels of several genes was monitored by qRT-PCR as shown earlier (Fig. 9). Interestingly, protoplasts expressing the *CAA* hybrid have reduced transcript levels of HsfA2, Hsp15.2-CI and Hsp17.7A-CI when compared to protoplasts expressing HsfA1a. In contrast, protoplasts expressing *ACC* hybrid had enhanced transcript abundance of Hsp15.2-CI, Hsp17.7A-CI and HsfA2 compared to HsfA1c (Fig. 19). Remarkably, the activity of proteins on HsfA7 and Hsp70-1 was similar for the mutants carrying either HsfA1a or HsfA1c DBD confirming the preferential activity of HsfA1c that seems to be mediated by DNA-binding affinity of its DBD (Fig. 19). All in all, these findings indicate that the DBD containing N-terminal region is responsible for the specific activity of the two HsfA1s on specific sets of genes which is likely related to DNA-binding affinity. On the other hand, the function of OD and CTD seems to be affected by the

N-terminus peculiarities pointing to the importance of the proper DNA-binding for activity and coactivity of HsfA1s.

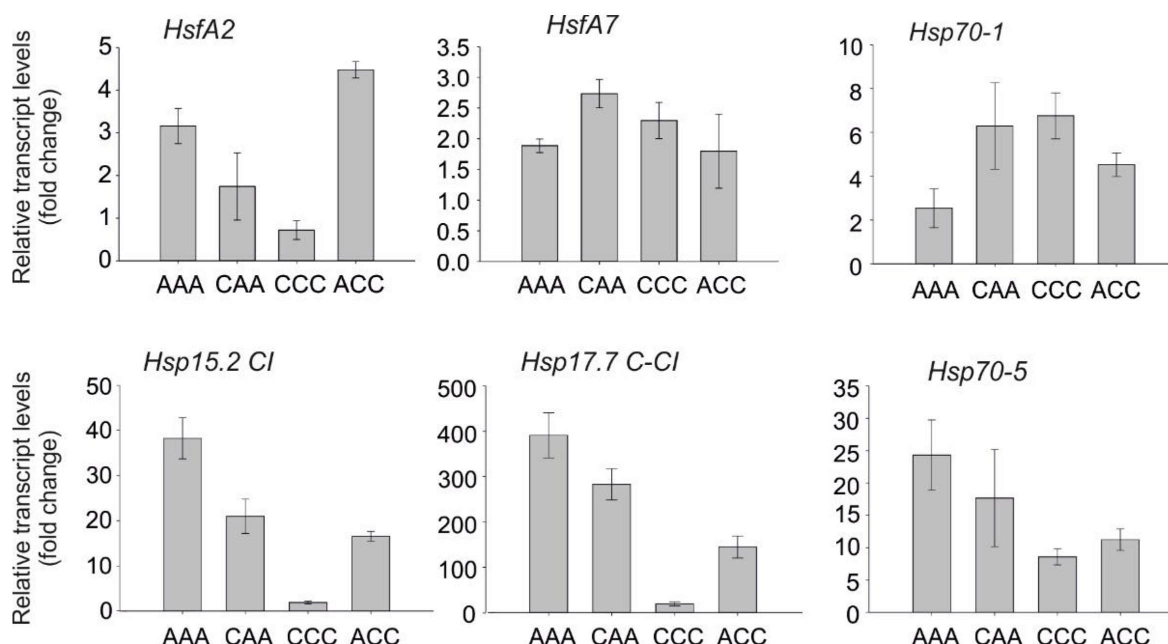

Figure 19. Effect of expression of HsfA1a, HsfA1c and their N-terminus swapping mutants on expression of heat stress induced genes in tomato protoplasts.
Tomato mesophyll protoplasts were transformed HsfA1a (AAA), HsfA1c (CCC), and the respective N-terminal swapping mutants (*ACC* and *CAA*). The transcript levels of the indicated heat stress induced genes were determined by qRT-PCR. Ct values were normalized to the Ct value of EF1a (housekeeping gene) and calculated relative to Neo sample (transformed with the mock pRT-Neo plasmid). Shown are the mean values of three technical replicates with error bars representing ±SE.

6.5. DNA-binding domain properties related to the functional specificity of HsfA1a

The functional discrepancies between HsfA1a and HsfA1c are most likely due to differences in the DBD of the two proteins. Sequence alignment of the DBD in different HsfA1-members shows differences in some amino acids (Fig. 20). To identify whether specific amino acids conferring to the wide range of activity and DNA-binding capacity, we checked for some differences with putatively significant effect on the structure of the DBD.

First a variation in helix 1 was examined, in which Val residue in HsfA1a is Met in HsfA1c, or Asn and Ser in HsfA1b and HsfA1e, respectively. Mutation of the Val to Met in HsfA1a (M1) did not result in any significant alteration in HsfA1a transactivation activity on neither PHsp17* nor PHsf70-1 containing reporter constructs. In support of this, DNA binding as judged by DNA reporter assay of the mutant was similar to the wild type HsfA1a (Fig. 21). The C-terminus of the DBD, containing the β3-linker-β4,

represents the least conserved region of the DBD. Although the relevance of this region not well understood as it is thought not to interact with the DNA, it might be important for the stabilization of the structure of the DBD. Interestingly, the sequence in the β3-turn-β4 sheet is very conserved among 160 plant Hsfs and shows exceptional diversity in the linker where the VDPDR in HsfA1a is VGSDL in both tomato and potato (*S. tuberosum*) HsfA1c (Supplemental fig. 2). In addition, the WEFAN sequence in β4 strand is WKFSN in HsfA1c in both tomato and potato HsfA1c. It should be noted that up to date an HsfA1a has not been identified in potato genome. With few exceptions the vast majority of the examined plant HsfA1 genes have the conserved sequence present in tomato HsfA1a.

```
                                                          α1                β1
HsfA1a    MEPNSYGSGKAAVGDGVGAPMLQTAPAPAPIPSANAPPPFLVKTYDMVDDPSTDKIVSWS
HsfA1c    ------------MDAGGGSPV--PEITPAPMPSSNAVPPFLMKTYDMVDDPSTDKIVSWS
HsfA1b    ----------------------MDGVHEATVAGNLTSPPFLNKTYDMVDDPSTDSVVSWS
HsfA1e    MSPDGE------------------KTKGGVHEIGNSVPPFLSKTYDMVDDRSTDAVVSWS
                                               : **** ******** *** :****

             β2      α2                       α3          β3       β4
HsfA1a    PTNNSFVVWDPPEFAKDLLPKYFKHNNFSSFVRQLNTYGFRKVDPDRWEFANEGFLRGQK
HsfA1c    PTDSSFVIWDPPEFAKQLLPKYFKHSNFSSFVRQLNTYGFRKVGSDLWKFSNDGFIRGQK
HsfA1b    KSNNSFVVLNVPEFARDILPKYFKHNNFSSFVRQLNTYGFRKVDPDRWEFANEGFLRGYK
HsfA1e    KSNNSFVVWNVPEFSRDVLPKYFKHNNFSSFVRQLNIYGYKKVDPDCWEFANEGFLRGQK
           ::.***: : ***::::*******.********** **::**. * *:*:*:**:** *

M1: A1αV42M     M2: A1αR107L     M3: A1aD104G/E109K/A111S
```

Figure 20. Amino acid sequence alignment of DNA-binding domain of HsfA1 members.
Amino acid sequence alignment was done with Clustal Omega. On the top cylinders show α-helices and arrows β strands. Colored amino acids were chosen for mutation analysis.

We initially focused on the Arg residue in the turn between β3 and β4 that could influence β-strands concerning positioning. Interestingly, mutation of the Arg to Leu in HsfA1a (M2), has a significant negative effect on the DNA binding capacity of the protein as judged by GUS repressor assay (Fig. 21). On the other hand, the single amino acid mutation leads to almost complete loss of HsfA1a transactivation activity on PHsp17* reporter but enhanced activity on PHSp70-1 GUS reporter (Fig.21). Thereby, HsfA1a-R107L mutant (M2) has similar activity to HsfA1c which was examined in parallel. To examine whether other neighbouring amino acid residues in the same region can also contribute to the preferential activity of HsfA1a on different promoters, a triple mutant was generated, in which the DPDRWEFA sequence was mutated to GSDLWKFS (mutated aa residues are underlined). Unlikely, this mutation (M3) has no effect on the transactivation activity of HsfA1a on neither Hsp17* nor Hsp70-1 promoters, although the DNA binding capacity of the mutant was reduced as shown by the GUS repressor assay (Fig.21).

Worth mentioning, the reciprocal mutations in HsfA1c did not yield any significant difference (data not shown). Nevertheless, these results point to the crucial role of Arg residue in the turn between β3 and β4 strands of the DBD in the activities of HsfA1a and may explain that difference in transcriptional

activity and specificity toward target genes between HsfA1a and HsfA1c at least for specific sets of genes.

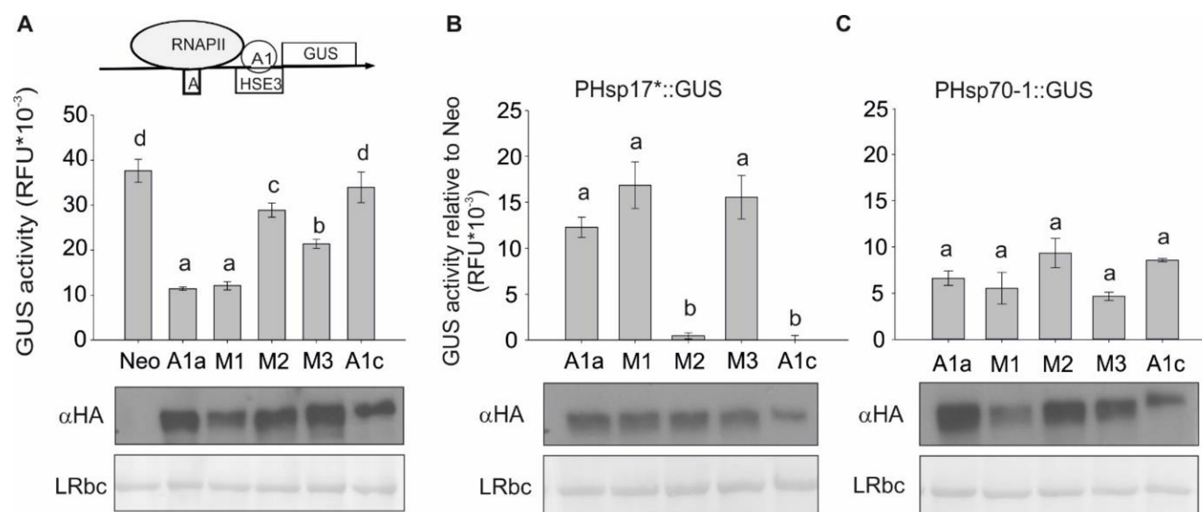

Figure 21. Amino acids contributing to the function specificity of the master regulator HsfA1a.
(A) DNA binding of wild typeHsfA1sand HsfA1a mutants on the HSE containing promoter based on a repressor assay. Bars represent the average GUS activity of the indicated HsfA1s and error bars are SE of three biological replicates. (B-C) Transcriptional activity of HsfA1a mutants compared to wild type HsfA1s on different reporter constructs PHsp17*:: GUS and PHsp70-1::GUS, respectively. Bars represent the average GUS activity of the indicated HsfA1s and error bars are SE of three replicates. The average was normalized to the average of Neo sample (without Hsf). Different letters indicate statistically significant difference ($p < 0.05$) calculated by ANOVA test and Duncan Mutliple Range test. After the GUS fluorescence measurements, samples were subjected to immunodetection of the respective Hsfs using α-HA antibody. Large subunit of RuBisCO (LRbc) serves as a loading control.

7. Discussion

Despite the high level of sequence conservation, Hsfs represent striking examples for functional diversification among the members of the same family of transcriptional regulators (von Koskull-Döring et al., 2007; Kotak et al., 2007a; Scharf et al., 2012; Ohama et al., 2015). The functional diversity has been demonstrated in numerous studies in which Hsf mutants and transgenic plants have been shown to have altered physiological and cellular responses against various environmental stress conditions and in some cases even growth and developmental alterations (Scharf et al., 2012; Grover et al., 2013). The large number of Hsfs in angiosperms has been proposed to be the result of gene and whole genome duplications during evolution as well as gene loss (Proost et al., 2011). HsfA1 genes are considered as master regulators in *Arabidopsis thaliana* as the essential functions for basal and acquired thermotolerance are shared in at least three members (Lohmann et al., 2004; Liu et al., 2011; Nishizawa-Yokoi et al., 2011; Yoshida et al., 2011). In tomato, HsfA1a and HsfA1c have been proposed to be evolved from gene duplication while independently HsfA1b and HsfA1e are also evolved (Scharf et al. 2012). However, the results presented here, show that despite the high conservation in amino acid sequence, subtle changes have led to a significant functional diversification.

7.1. Regulation of tomato HsfA1s

In general, the expression of Hsfs is not only restricted to stress response, but also to developmental programs. This was explained in several cases where Hsf mutants show phenotypic alterations related to specific developmental processes under stress and non-stress conditions (von Koskull-Döring et al., 2007; Kotak et al., 2007a; Scharf et al., 2012; Fragkostefanakis et al., 2016). In tomato, among four members of subclass A1, HsfA1a was proposed as the master regulator of HSR based on physiological and molecular analysis of HsfA1a co-suppression transgenic lines which are more sensitive to high temperatures than wild-type plants (Mishra et al., 2002). However, no phenotypic alterations were observed in transgenic lines under non-stress conditions. In the light of this, the regulation of the recently identified closely related HsfA1 factors is of great interest.

The basis of the master regulator assumes a constitutively expressed protein that is maintained inactive under non-stress conditions mainly by interaction with molecular chaperones like Hsp70 and Hsp90, and can be only released and activated under stress conditions to induce a set of genes encoding for molecular chaperons, Hsfs and other proteins required for protection against stress (Hahn et al., 2011). This general system of regulation has been previously shown for Hsf1 among four vertebrate Hsfs (Åkerfelt et al., 2010).

From transcriptomic and qRT-PCR analysis, all HsfA1 members are by large constitutively expressed, and only in few cases preferentially induced in specific developmental stages (Fig. 4 and 5). In general, HsfA1c and HsfA1e are expressed in at very low levels as shown by RNAseq data, while HsfA1a is expressed at higher levels (Fig.4). It has been previously shown that HsfA1a protein is detectable in leaves, stems, seedlings and fruits (Mishra et al., 2002), while the lack of antibodies for the other HsfA1s does not allow to judge whether a low but constitutive mRNA synthesis is accompanied by a significant protein accumulation. This would be particularly interesting for HsfA1b which shows the highest variation in transcript abundance in different tissues, and is the highest expressed HsfA1 in ripening fruits and developing seeds (Fig. 4).

The preferential induction of HsfA1b and HsfA1e genes in specific tissues or developmental stages (Fig. 5) might hint to a possible developmental function and in turn explain the lack of an obvious phenotypic alteration of A1CS plants and fruits compared to wild type when grown under non-stress conditions (Mishra et al., 2002). However, further detection of their transcript levels but in A1CS transgenic tissues, especially fruits and seeds, or the individual knock-down of such members is required to support this conclusion.

The developmental regulation of HsfA1s might also be related to the priming of cells in case of an upcoming stress. This has been exemplified for HsfA2 which is pre-synthesized in non-stressed male meiocytes to confer protection in case of a stress in more advanced stages (Fragkostefanakis et al., 2016). In this manner, HsfA1b and HsfA1e could act as priming factors for thermotolerance in fruits and seeds. In support of this, Arabidopsis HsfA1e is preferentially expressed in seeds and is therefore involved in seeds thermotolerance (Liu et al., 2011). In addition, the earlier stages of fruit ripening, and particularly at the mature green pre-climacteric stage, are more sensitive to severe heat stress conditions when compared to more ripening stages (Mishra et al. 2002). The higher tolerance of the ripe fruit might be due to the presence of pre-synthesized HsfA1b and HsfA1e (Fig. 4 and 5).

Among HsfA1 genes, HsfA1b is highly induced in response to heat stress, in a similar manner to the HS-marker gene HsfA2. Interestingly, HsfA1b is rapidly induced, having a peak in expression within 30 minutes of stress, while HsfA1e shows a more late response with a slight increase after 4 hours of stress (Fig. 6). Recently, HsfA1a was shown to acts a positive transcriptional regulator of HsfA1b while HsfB1 as a competitive repressor (Fragkostefanakis et al. 2018). In leaves, suppression of HsfB1 leads to the very strong accumulation of HsfA1b transcripts (Fragkostefanakis et al. 2018). Therefore the developmental or the stress-dependent regulation of HsfA1b might be controlled by the activities of specific Hsfs, like HsfA1a and HsfB1.

Apart from transcriptional regulation, an important control mechanism affecting the fate of Hsfs is protein turnover. This has been shown for tomato HsfA2 (Hu, PhD thesis 2017), HsfB1 (Röth et al., 2016a) and HsfA7 (Mesihovic, PhD thesis 2018), for which the nuclear retention of these Hsfs has been directly related to their degradation rate. The presence of the NES and NLS in the C-terminus of Hsfs facilitate the nucleocytoplasmic shuttling of Hsfs as shown for HsfA1a and confirmed here (Scharf et al., 1998). HsfA1e is the only HsfA1 with no reported NES, and in agreement with this HsfA1e was detected only in the nucleus (Fig. 11). Interestingly, HsfA1b which has both NES and NLS shows also a nuclear retention, while HsfA1a and HsfA1c show nucleocytoplasmic equilibrium (Fig. 11). Both HsfA1b and HsfA1e showed a high turnover rate with half life time of about 3 hours, while HsfA1a and HsfA1c remain stable for the 6 hours of the experiment (Fig. 12). Thereby the accumulation of transcripts of HsfA1b or HsfA1e might not be accompanied by protein accumulation; however it could serve as a priming factor in case of heat stress to allow a faster response in specific tissues and developmental stages. HsfA2 is already involved in such a priming process during pollen development in tomato while other stress-induced Hsfs including HsfA1b might be also involved in such a process (Fragkostefanakis et al., 2016).

The protein stabilization of HsfA1b and HsfA1e might require the presence of co-factors which under specific conditions and developmental stages might allow the protein accumulation of these Hsfs. For example, HsfA1a and HsfA2 interaction in stressed cells leads to the stabilization of both factors, thereby contributing to the stimulation of transcriptional activity of the complex (Scharf et al., 1998). However, co-expression of HsfA1s with HsfA2, HsfA3, HsfA7 or HsfB1 did not lead to significant changes in the abundance of the former, suggesting that are other factors even non-Hsfs might contribute to this. As for example, binding of multiple ubiquitin molecules tags the protein for degradation by 26S proteasome (Muratani and Tansey, 2003). This has been shown for many Hsfs including tomato HsfA2 (Hu, PhD thesis 2017), HsfB1 (Hahn et al., 2011; Röth et al., 2016a) and HsfA7 (Mesihovic, PhD thesis 2018). Further investigations on the protein levels of HsfA1b and HsfA1e in the presence of proteasome inhibitor like MG132 can prove such negative regulatory mechanism. Although, all proteins are targeted by ubiquitin ligase (E3), one of three enzymes that mediate ubiquitin targeting, and consequential proteasome-mediated turnover, the intracellular localization of the Hsf is an important determinant factor for such regulatory mechanism as cytosolic Hsfs can escape it. Another determinant factor is the structure and the presence of a degradation signal, specific sequence in the target protein, that signals proteolysis by representing a site for ubiquitin ligase (E3) binding (Muratani and Tansey, 2003). Whether, HsfA1b and HsfA1e have such degradation signals still needs to be further investigated.

All in all, these different regulation mechanisms, developmental or stress dependent, positive or negative, among HsfA1-members might be in part responsible for such functional diversity in tomato subclass A1 Hsfs.

7.2. HsfA1a has a broad range of transactivation activity

The onset of heat stress response requires the activity of pre-synthesized one or more transcription factors which serve as single or multiple master regulators. Although tomato HsfA1 members share high protein sequence similarity, the transcriptional transactivation activity assays point to a major divergence in activity potential regarding downstream genes. The GUS reporter assay revealed a higher range of activity of HsfA1a compared to the other members of the subfamily on HSE containing Hsf and Hsp promoters (Fig.7). These results were further confirmed by the upregulated transcript level of the endogenous genes encoding for Hsfs and Hsps in HsfA1-expressing protoplasts (Fig. 9).

Interestingly, HsfA1c expression led to the stronger upregulation of several *Hsps*, when compared to HsfA1b or HsfA1e. Particularly interesting is the similar induction of *Hsp70-1* expression by HsfA1c when compared to HsfA1a, suggesting that HsfA1c might specifically contribute to the upregulation of a subset of stress induced genes. Instead, no basal activity was detected for HsfA1e in any of the examined genes with the exception of HsfA7. HsfA7 is considered as an early induced Hsf, which is particularly abundant at mildly elevated temperatures when compared to HsfA2 (Mesihovic, PhD thesis 2018). Thereby, the strong induction of HsfA7 might be due to the activities of the HsfA1 factors.

In the opposite manner, *Arabidopsis* HsfA1a has the lowest transcriptional transactivation activity among HsfA1-members where all of them are responsible for Hsfs and Hsps induced expression (Yoshida et al., 2011). This might explain the function redundancy among HsfA1-members in Arabidopsis but functional diversity among tomato HsfA1s.

The upregulation of HS-induced genes is also dependent on the complex formation of different Hsfs which leads to the synergistic activity as previously shown for HsfA1a with HsfA2 (Chan-Schaminet 2002), HsfA7 (Mesihovic, PhD thesis 2018) and HsfB1 (Bharti 2004). In current study, co-expression of HsfA1b, HsfA1c or HsfA1e with HsfA2 has a stronger synergistic effect than that of HsfA1a, however due to the higher basal activity of HsfA1a, the HsfA1a-HsfA2 complex has the highest GUS activity (Fig. 10). The co-activity of HsfA1s with partner Hsfs is specific, as HsfA1b, HsfA1c and HsfA1e do not cause any significant induction of Hsp17-GUS reporter when co-expressed with HsfA7 or HsfB1 (Fig. 10). These findings support the idea that the large family of plant Hsfs or even the small subfamily of plant

Hsfs is organized in a network of specially interacting members with distinct functions to ensure tight regulation of Hsf-dependent gene expression under stress conditions.

Remarkably, HsfA1b showed a strong transactivation activity when co-expressed with HsfA3 (Fig. 10). The latter is highly upregulated under HS and drought stress where it activates the expression of many downstream genes involved in stress tolerance (Yoshida et al., 2011 and references therein). Thereby, HsfA1b is likely to participate in this function through complex formation with HsfA3 enhancing their transcriptional activation activities under stress conditions.

In conclusion, only HsfA1a has the capacity to induce the transcription of a wide range of HS-induced genes by its own, while all of HsfA1 members can activate further transcriptional activities in cooperation with well characterized partner Hsfs, like HsfA2, HsfA3, HsfA7 and HsfB1 which in turn strongly support the original proposed model, for a single master regulator in tomato (Mishra et al. 2002).

The lower basal activity of HsfA1b and HsfA1e might be due to their lower protein abundance; however transformation of protoplasts with higher amounts of HsfA1b or HsfA1e-encoding plasmids did not result in higher activities (data not shown). Furthermore, the lower activity could be due to the lower DNA binding capacity of the factors as judged by the GUS repressor assay (Fig. 13). However, it should be noted that the GUS repressor assay is based on a promoter containing consecutive HSE elements mostly representing strongly HS-induced genes such as sHsps (Treuter et al., 1993). Whether this is general or it applies on specific HSE structure as found in different Hsf dependent genes still needs to be explored further in the future.

7.3. Functional diversification based on DNA binding domain

The unique activity properties of HsfA1a were further examined by generating swapping mutants with HsfA1c as both of them show the highest sequence similarity, similar nucleocytoplasmic equilibrium and protein stability but divergent activity and coactivity (Supplemental Fig 1, Fig. 11 and 12). As well known, the synergistic activity of HsfA1a with HsfA2 is dependent on the OD-mediated interaction of the two Hsfs and the special combination of different AHA motifs contact with the basic initiation complex subunits (Chan-Schaminet et al., 2009). Instead, HsfA1a and HsfB1 build activator ternary complexes with the binding protein HAC1 that binds specifically to their C-termini when they are bound to the DNA (Bharti, 2004).

Firstly, swapping of either C-terminal domain or oligomerization domain between HsfA1a and HsfA1c did not yield any change regarding transcriptional activities either when expressed alone or in

combination with HsfA2 and HsfB1 (Fig. 15 and 16). The abolished synergistic activity of *CCA* hybrid with HsfA2 and HsfB1 might be due to some conformational changes derived by swapping that result in a less active protein. Rather than we can assume that the difference in transactivation activity between HsfA1a and HsfA1c is not due to the dissimilarities in the oligomerization domain or the C-terminal domain containing the activation motifs.

The retained higher activity of HsfA1 hybrids (*AAC* and *ACA*) similar to HsfA1a while the same lower activity of HsfA1c hybrids (CAC and CCA) similar to HsfA1c points to the crucial role of the molecular context (Fig. 15 and 16). Supporting this, swapping of the N-terminus between HsfA1a and HsfA1c had a major impact on their transactivation activity and synergistic activity, as *CAA* behaves now typically like HsfA1c while *ACC* like HsfA1a (Fig. 17). In addition, *CAA* showed a lower stimulatory effect on the majority of the examined target genes and impaired DNA binding capacity compared to HsfA1a (Fig. 18 and 19). Thereby, such dramatic difference in the activities of HsfA1a and HsfA1c regarding transcriptional activity and DNA-binding is likely driven by N-terminus. Interestingly, function specificity is also driven by the N-terminus as the *CAA* hybrid showed the same enhanced stimulatory effect on *Hsp70-1* gene similar to HsfA1c (Fig. 19). All in all, these finding support the concept of promoter specificity of Hsfs pointing to the indispensable role of N-terminus in function specificity of Hsfs.

The gained transcriptional activity of *ACC* hybrid which even higher than that of intact HsfA1a (fig. 17), suggests that domain swapping in this case affects the conformational structure of the hybrid resulting in a more functionally active protein. The activity of HsfA1a is known to be self-regulated by intramolecular interaction between N-terminus and C-terminus (Scharf et al., 1990; Scharf et al., 1998). It is likely that the replacement of the C-terminus of HsfA1a including the oligomerization domain with that of HsfA1c in the *ACC* hybrid disrupts such intramolecular interactions resulting in a super active protein. Furthermore, the gained transcriptional activity might be due to the enhanced DNA- binding capacity (Fig. 18).

The N-terminus of the Hsfs contains not only the DBD but also the linker between DBD and the oligomerization domain OD. This linker is variable in HsfA1 subfamily, being extended in HsfA1a by 10-11 amino acid residues compared to the other HsfA1 proteins (Supplemental fig. 1). However, it is unlikely that the activity diversification derives from differences in the linker, as *Arabidopsis thaliana* HsfA1 members have similar deviations but show functional redundancy (Liu et al., 2011).

The DBD-independent interaction of HsfA1a with HsfA2 and subsequent synergistic activity(Chan-Schaminet et al., 2009) might explain the common cooperation capacity of N-terminus swapping mutants with HsfA2 (Fig. 17). However, the cooperation with HsfB1 is DBD- these findings highlight the

crucial role of DBD not only in highly specific activity but also in cooperation capacity with other Hsf partners. Thereby, none of HsfA1 members can compensate the function of HsfA1a in that proposed triad responsible for the onset of HSR in tomato.

The interaction of the DBD with DNA is mainly mediated by the hydrogen bonds between the Arg residue in the highly conserved helix three (H3) of the DBD and the invariant G residue of the HSE in the promoters of HS-genes (Littlefield and Nelson, 1999). However, the neighboring conserved residues like Ser, Gln, Asn, and Tyr are participating in this interaction by providing hydrogen bonds and van der Waals interactions with the phosphate backbone of the DNA (Littlefield and Nelson, 1999; Neudegger et al., 2016). In addition, the cluster of basic residues (Arg and Lys) in the C-terminus of the DBD can give other contacts across the major groove of the DNA (Neudegger et al., 2016). However, these lysine residues are accessible for modification by acetylation which giving rise to a platform of DNA-binding regulation (Westerheide et al., 2009; Raychaudhuri et al., 2014). Acetylation removes the positive charge from Lys residues, which is required for ionic interactions with the DNA backbone, and thus lowers overall affinity to DNA (Zelin et al., 2012).

Sequence comparison among tomato HsfA1 DBDs revealed a striking low conservation in the C-terminus region, particularly the β3-turn-β4 region, in HsfA1c and HsfA1e when compared to HsfA1a and HsfA1b (Fig. 20). Interestingly, among different mutations, mutation of the Arg residue (R107) in the turn between β3 and β4 sheets to the Leu residue (L93 in HsfA1c) led to a drastic alteration of HsfA1a specific activities on different promoters. The specific activity of HsfA1a on Hsp17* promoter was completely abolished while the activity on Hsp70-1 promoter was enhanced (Fig. 21). The complete loss of activity on the Hsp17* promoter might be due to the impaired DNA binding capacity observed in repressor assay (Fig. 21). Accordingly, we assumed that the Arg residue at this position might contribute directly to the binding of the Hsf to the DNA with consecutive HSEs. However, the retained activity of the HsfA1a-R107L mutant on Hsp70-1 promoter or even enhanced like that of HsfA1c points to another factor which is promoter specificity. Therefore, the Arg residue (R107) in the β3-turn-β4 region is crucial for the function specificity of HsfA1a rather than absolute DNA-binding capacity. It is likely to be involved in the structural confirmations required for the optimum positioning and stabilization of the Hsf oligomer complex on certain promoters.

Interestingly, mutations of other neighboring amino acid residues present in the same the β3-turn-β4 region of HsfA1a DBD (Fig. 20), had no significant effect on function specificity but resulted in impaired DNA binding capacity (Fig. 21). From this, it can be concluded that the whole β3-turn-β4 region or the C-terminus of the DBD is involved in the DNA binding and functional specificity. Furthermore, the reciprocal mutation (L109R) in HsfA1c did not yield any significant difference in transcriptional activity

or DNA binding capacity (data not shown), supporting that neighboring amino acid residues in in β3 and β4 strands are also crucial.

Like HsfA1a, HsfA1b has an Arg residue at this position while HsfA1e has a Cys residue instead (Fig. 20). This in turn might explain the absence of HsfA1e activity for the majority of genes with the exception of HsfA7, and the wider activity of HsfA1b for several genes (Fig. 9). It is likely that the conservation of this region, as for example in Arabidopsis HsfA1s, or the divergence in this region, as in tomato HsfA1s, might lead to functional redundancy or functional diversification, respectively. This notion might be applied for other species as well, particularly potato and other Solanaceae, where HsfA1s share such amino acid variation in the C-terminus of the DBD (Supplemental fig. 2).

Interestingly, the turn between β3 and β4 strands in non-plant Hsfs is completely different forming a wing loop that involved in DBD-DBD interactions of the neighboring Hsfs bound to the DNA (Schultheiss et al., 1996)HS. It is seemingly that this region in both cases functions for the stabilization of Hsf oligomer on the DNA but in different mechanisms among eukaryotes.

Although DBD is likely responsible for the functional diversification of Hsfs as exemplified here for HsfA1s, the different levels of the upregulated target genes is probably due to the variation in the number, sequence and organization of HSEs in the promoter of such genes. Many genes contain at least a basic potentially active HSE, and interestingly, many of the HSE containing genes are not stress induced (Fragkostefanakis et al., 2015a) posing the question how many of these genes are actually transcriptionally controlled by Hsfs. HsfB1 has been shown to regulate the expression of cognate Hsp70 and Hsp90 genes but also different housekeeping genes which are not directly controlled by HsfA1a (Fragkostefanakis et al.,2018; Bharti et al. 2004). The latter function has been proposed to include other non-Hsf factors with which HsfB1 might have synergistic or even antagonistic functions. A specific HSE architecture might control the binding of Hsf complex as shown for different complexes with promoter specific activity (Bharti et al. 2004; Mesihovic, PhD thesis 2018), however this is not well understood up to now.

8. Conclusion and outlook

In tomato, among four members of subclass A1, only HsfA1a has the unique criteria required for the master regulator function. HsfA1a is constitutively expressed in all tissues with high stability and wide transcriptional activation activity on several stress induced genes required for protection and survival under stress. By its own, HsfA1a can trigger the onset of heat stress response by inducing the expression of HsfA2, HsfB1 and Hsps function as molecular chaperons. Instead, other HsfA1-members (HsfA1b, HsfA1c and HsfA1e) with lower and narrower transcriptional activation activity on specific set of genes can't initiate such primary line of defense except in some tissues or developmental stages where their expression is upregulated under non stress conditions e.g. HsfA1b and HsfA1e in fruits and seeds. However, all of them can participate in the latter response by cooperation with the newly synthesized HsfA2 or HsfA3 where they compromise their lower activity.

Despite considerable similarity in the sequence and the basic structure of the DNA-binding domain among HsfA1 members, there are evidently subtle but decisive differences in the amino acid sequence, particularly in the C-terminus region, leading to functional diversity among HsfA1 members. The DBD of HsfA1a is likely more conformationally flexible than that of other members and thereby allow a wide range of distinct interactions with HSEs. The finding that a single amino acid substitution in the DBD C-terminus of HsfA1a alters the function specificity toward different promoters is consistent with this idea.

We identified here, the Arg residue (R107) in the turn between β3 and β4 sheets in the DBD as a crucial element for the efficient DNA-binding and specific transcriptional activity of HsfA1a on different promoters. The Arg residue is present in HsfA1a and HsfA1b but is replaced by Leu or Cys resides in HsfA1c and HsfA1e, respectively. This tight turn in most of Hsfs is shorter in plant Hsfs compared to metazoan and yeast which forms an extended wing (Schultheiss et al., 1996). The wing is important for the interaction of neighboring Hsfs, thereby contributing to DNA binding but also to preferential target gene activation (Ahn et al., 2001). At stage we cannot conclude for the possible function of the turn in the β3-β4 sheet, but due to the short length and based on the human Hsf model, it is not likely that the turn is involved in interactions between adjacent DBDs. We assume that either the Arg is involved directly in the binding to the DNA or that it is important for the overall conformational structure of the DBD.

The Arg residue at this position is highly conserved in several plant HsfA1 proteins supporting the notion for an important role in the function of the DBD. From the analyzed species, the sequence discrepancy is consistent for tomato and potato HsfA1c and HsfA1e, suggesting that the functional diversification might have appeared during the evolution of Solanaceae species (Supplemental Fig.2).

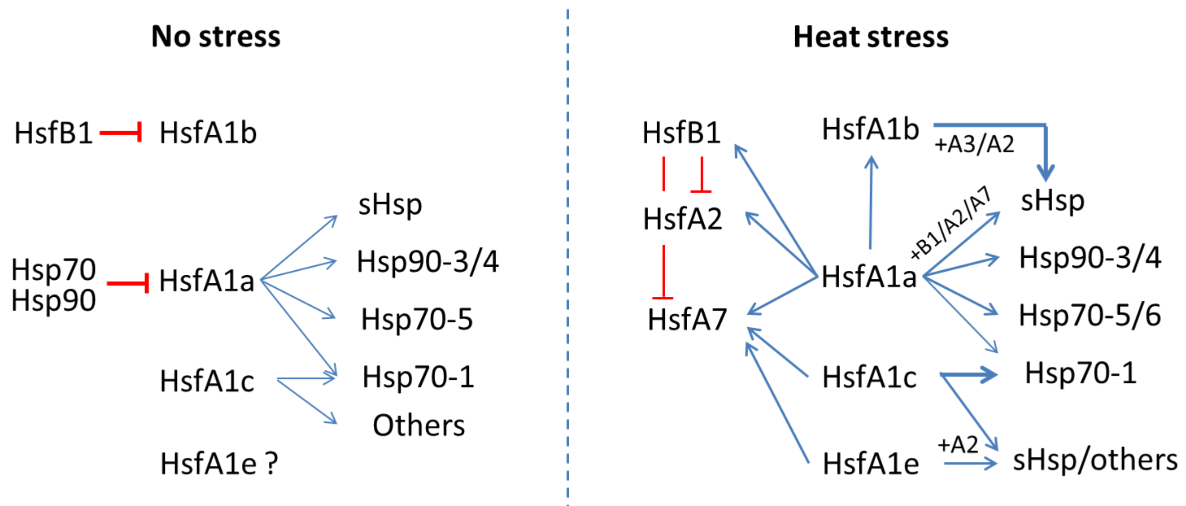

Figure 22. Working model for the role of HsfA1 subfamily in regulation of HS-related genes in vegetative cells. Under non-stress conditions HsfA1a has very low activity due to the interaction with high molecular weight chaperones (Hahn et al., 2011). HsfA1b is weakly expressed due to the repressor activity of HsfB1 (Fragkostefanakis et al. 2018), while HsfA1c has only weak activity on Hsp70-1 and possibly other yet to be identified genes. HsfA1e is also weakly expressed, and putative translation is followed by the rapid protein degradation. Therefore HsfA1e is not involved in transcription under non stress conditions. Under heat stress, HsfA1a is relieved by the inhibitory activity of Hsp70 and Hsp90, and activates the transcription of Hsps and Hsfs like HsfA2, HsfA7 and HsfB1, as well as HsfA1b. HsfA7 induction is further supported by HsfA1c and HsfA1e which can only regulate a small subset of HS-genes, including Hsp70-1 by HsfA1c. Instead, the majority of Hsps is strongly upregulated due to the synergistic activity of HsfA1a with the newly synthesized HsfA2, HsfA7 and HsfB1. HsfA1b and HsfA3 can also form putative highly active complexes to further induce HS-genes like sHsps. Likely, HsfA2 cooperates with all HsfA1 members to further induce HS-genes like sHsps. In conclusion, we propose that only HsfA1a has a master regulator function for heat stress response and thermotolerance. The other HsfA1 members have supporting role. The preferential activity of HsfA1 on specific genes are probably due to the structural differences in their DNA binding domains.

The loss of functional redundancy within HsfA1 subfamily could allow a more refined regulation at a tissue or even cell-type specific level however this requires further investigation in the future. Although the knowledge about Hsfs is growing rapidly, much still remains to be investigated, especially with respect to structural function aspects. Research on Hsfs transcription factors is not only of interest to basic research in plant science, but also to plant breeding with the aim of obtaining plants with improved ability to withstand stress exposure. Detailed understanding of both protein-protein and protein-DNA interactions of the molecular networks of Hsfs implicated in stress responses and senescence thus represents a necessary platform for engineering Hsfs. Translation of the results obtained from basic research in the plant model Tomato to other crops also represents a significant future breeding potential for agriculture.

9. References

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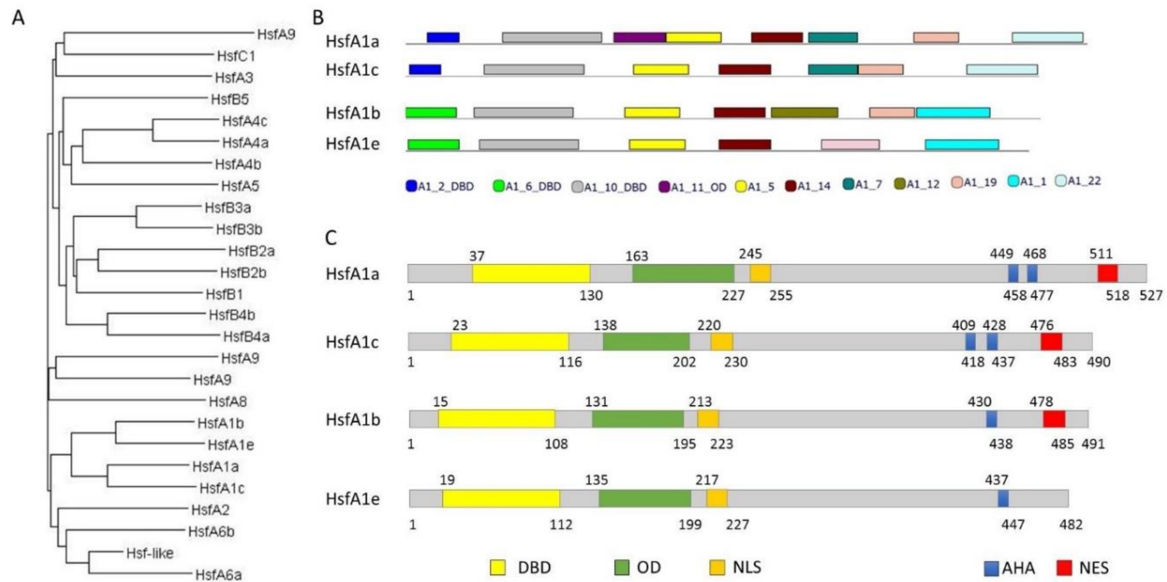
10. Supplemental data



Supplemental Figure 1. Protein similarity of HsfA1 members in tomato.
(A) Phylogenetic tree generated by Clustal Omega analysis of the amino acid sequences of tomato Hsfs. (B) Motifs found in HsfA1 proteins based on HEATSTER database (Scharf et al., 2012). Each motif is depicted by a different color. (C) Domain structure of HsfA1 proteins obtained by HEATSTER database. DBD: DNA binding domain; OD: oligomerization domain; NLS: nuclear localization signal; AHA: activator motif consisted of aromatic, hydrophobic and acidic amino acid residues; NES: nuclear export signal.

BraraHsfA1a	GFRKVDPDRWEFAN	PhavuHsfA1d	GFRKVDPDRWEFAN	CapruHsfA1b	GFRKVDPDRWEFAN
CapruHsfA1a	GFRKVDQGRQEYAN	LotjaHsfA1c	GFRKVDPDRWEFAN	AralyHsfA1b	GFRKVDPDRWEFAN
ThhalHsfA1a	GFRKVDPDRWEFAN	PhavuHsfA1c	GFRKVDPDRWEFAN	ArathHsfA1b	GFRKVDPDRWEFAN
AralyHsfA1a	GFRKVDPDRWEFAN	GlymaHsfA1a	GFRKVDPDRWEFAN	SolycHsfA1e	GYKKVDPDCWEFAN
ArathHsfA1a	GFRKVDPDRWEFAN	GlymaHsfA1c	GFRKVDPDRWEFAN	SoltuHsfA1e	GYKKVDPDCWEFAN
ZeamaHsfA1c	GFRKVDPDRWEFAN	LinusHsfA1a	GFRKVDPDRWEFAN	SolycHsfA1b	GFRKVDPDRWEFAN
SetitHsfA1a	GFRKVDPDRWEFAN	LinusHsfA1c	GFRKVDPDRWEFAN	SoltuHsfA1b	GFRKVDPDRWEFAN
PanviHsfA1a	GFRKVDPDRWEFAN	EucgrHsfA1a	GFRKVDPDRWEFAN	LotjaHsfA1b	GFRKVDPDRWEFAH
PanviHsfA1c	GFRKVDPDRWEFAN	CucsaHsfA1a	GFRKVDPDRWEFAN	PhavuHsfA1b	GFRKVDPDQWEFAN
SorbiHsfA1a	GFRKVDPDRWEFAN	VitviHsfA1a	GFRKVDPDRWEFAN	GlymaHsfA1b	GFRKVDPDRWEFAN
ZeamaHsfA1a	GFRKVDPDRWEFAN	CitsiHsfA1a	GFRKVDPDRWEFAN	GlymaHsfA1e	GFRKVDPDRWEFAN
HorvuHsfA1a	GFRKVDPDKWEFAN	CarpaHsfA1a	GFRKVDPDRWEFAN	LinusHsfA1e	GFRKVDPDRFEFAN
BradiHsfA1a	GFRKVDPDRWEFAN	GosraHsfA1f	GFRKVDSDRWEFAN	MimguHsfA1b	GFRKVDPDHWEFAN
OrysaHsfA1a	GFRKVDPDRWEFAN	GosraHsfA1d	GFRKVDPDRWEFAN	AqucoHsfA1a	GFRKVDPDRWEFAN
SolycHsfA1a	GFRKVDPDRWEFAN	GosraHsfA1e	GFRKVDPDRWEFAN	FraveHsfA1b	GFKKVHPDRWEFAN
SolycHsfA1c	GFRKVGSDLWKFSN	ThecaHsfA1a	GFRKVDPDRWEFAN	MaldoHsfA1b	GFRKVDPDRWEFAN
SoltuHsfA1c	GFRKVGSDNWKFSN	PoptrHsfA1a	GFRKVDPDRWEFAN	MaldoHsfA1d	GFRKIDPDRWEFAN
CapruHsfA1d	GFRKVDPDRWEFAN	PoptrHsfA1c	GFRKVDPDRWEFAN	PrupeHsfA1b	GFRKVDPDRWEFAN
AralyHsfA1d	GFRKVDPDRWEFAN	ManesHsfA1a	GFRKVDPDRWEFAN	VitviHsfA1b	GFRKVDPDRWEFAN
ArathHsfA1d	GFRKVDPDRWEFAN	RicomHsfA1a	GFRKVDPDRWEFAN	PoptrHsfA1b	GFRKVDPDRFEFAN
BraraHsfA1d	GFRKVDPDKWEFAN	BraraHsfA1e	GFRKVDPERWEFAN	ManesHsfA1b	GFRKVDPDRYEFAN
ThhalHsfA1d	GFRKVDPDRWEFAN	BraraHsfA1f	GFRKVDPDRWEFAN	RicomHsfA1b	GFRKVDPDRYEFAN
MimguHsfA1a	GFRKVDPDRWEFAN	BraraHsfA1h	GFRKVDPDRWEFAN	CarpaHsfA1b	GFRKVDPDRWEFAN
FraveHsfA1a	GFRKVDPDRWEFAN	ThhalHsfA1e	GFRKVDPDRWEFAN	CitsiHsfA1b	GFRKVDPDRYEFAN
PrupeHsfA1a	GFRKVDPDRWEFAN	CapruHsfA1e	GFRKVDPDRWEFAN	GosraHsfA1b	GFKKVDPDRWEFAN
MaldoHsfA1a	GFRKIDPDRWEFAN	AralyHsfA1e	GFRKVDPDRWEFAN	GosraHsfA1c	GFRKVDPDRWEFAN
MaldoHsfA1c	GFRKVDPDRWEFAH	ArathHsfA1e	GFRKVDPDRWEFAN	GosraHsfA1a	GFRKVDPDRWEFAN
LinusHsfA1b	GFRKVDPDRWEFAN	BraraHsfA1g	GFRKVDPDRWEFAN	ThecaHsfA1c	GFRKVDPDRWEFAN
LinusHsfA1d	GFRKVDPDRWEFAN	BraraHsfA1b	GFRKVDPDQWEFAN		
GlymaHsfA1d	GFRKVDPDRWEFAN	ThhalHsfA1b	GFRKVDPDRWEFAN		

Supplemental Figure 2. Amino acid sequence alignment of β3-β4 sheet in HsfA1 proteins from various plant species.

In green *A. thaliana*, red *S. lycopersicum* and blue *S. tuberosum* are shown. Divergence from the conserved Arg in amino acid residue 9 shown here, is highlighted in dark background. Species names and sequence are taken from HEATSTER database (Scharf et al., 2012).

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12. *Curriculum Vitae*

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Publications

Fragkostefanakis S, Simm S, El-Shershaby A, Hu Y, Bublak D, Mesihovic A, Darm K, Mishra SK, Tschiersch B, Theres K, Scharf C, Schleiff E, Scharf KD (2018) The repressor and co-activator HsfB1 regulates the major heat stress transcription factors in tomato. Plant, Cell Environ. doi: 10.1111/pce.13434

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Manuscripts

El-shershaby A, Ulrich S, Simm S, Scharf KD, Schleiff E, Fragkostefanakis S (2018) Functional diversification of HsfA1 factors in tomato based on DNA binding domain properties.

Erklärung

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung im Mathematisch-Naturwissenschaftlichen Bereich unterzogen habe.

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Asmaa El-shershaby

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Ich erkläre hiermit, dass ich die vorgelegte Dissertation über

„ Characterization of subclass A1 heat stress transcription factors in tomato"

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Asmaa El-shershaby