The Role of *Arabidopsis thaliana* Clade A1 Heat Shock Transcription Factors in Growth and Development

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To my parents for supporting me all the way

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Abstract

Arabidopsis thaliana clade A1 heat shock transcription factors function as the master regulators of heat shock and other abiotic stresses. Members of this clade (*HSFA1a/b/d/e*) are highly redundant but have individual and collective functions. Transgenic overexpression of one of its members, *HSFA1b*, in Arabidopsis was shown to have altered developmental traits as well as increased stress and disease tolerance. In this study, plants overexpressing *HSFA1b* had smaller rosettes in seedlings and mature plants, longer inflorescence and early flowering. A number of candidate developmental genes identified to be regulated by *HSFA1b* could not be satisfactorily confirmed using the HSFA1 quadruple knockout due to the mutant being a hybrid of 2 accessions (WS-0 and Col-0). Therefore, a new quadruple mutant was generated using the CRISPR/Cas9 gene editing system to knockout *HSFA1a* from an existing homozygous triple mutant (bdeKO) in a single accession (Col-0). The new quadruple mutant (QK2) was impaired in physiological responses in relation to abiotic stress along with other developmental defects in normal and ambient temperature. Furthermore, an interaction between *HSFA1b* (or HSFA1s) and ARGONAUTE proteins was established suggesting another pathway by which HSFA1s could regulate developmental genes under both normal and stress conditions via the action of microRNAs. Alongside confirming *HSFA1b*-regulated developmental genes, the QK2 was also instrumental in identifying microRNAs precursors that could be regulated by the clade. Together, results show how HSFA1 regulates growth and development during normal and stress conditions.

Abbreviations

AGO	Argonaute
BL	Brassinolide (Synthetic Brassinosteroid)
Cas9	CRISPR associated protein 9
ChIP-SEQ	Chromatin immunoprecipitation sequencing
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
DAG	Developmentally associated genes
DE	Differentially expressed
DNA	Deoxyribonucleic acid
dKO	Double knockout
DSB	Double stranded break
ET	Ethylene
ETI	Effector-triggered immunity
FPKM	Fragments per Kilobase of exon per Million fragments mapped
FW	Fresh weight
GA	Gibberellin
GO	Gene ontology
H_2O_2	Hydrogen peroxide
Hpa	*Hyaloperonospora arabidopsidis*
HS	Heat stress
HSE	Heat shock element
HSFA1	Heat shock transcription factor A1
HSP	Heat shock protein
HSR	Heat stress response
JA	Jasmonic acid
KO	Knockout
LD	Long day
lincRNA	Intergenic long non-coding RNA
lncNAT	Long non-coding Natural antisense transcript

lncRNA	Long non-coding RNA
miRNA	MicroRNA
mRFP	monomeric Red Fluorescent Protein
NHEJ	Nin-homologous end joining
NS	No stress
nt	Nucleotide
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PIC	Picloram (Synthetic auxin)
PPFD	Photosynthetically active photon flux density
PPZ	Propiconazole (Brassinosteroids inhibitor)
RNA	Ribonucleic acid
RNA-SEQ	RNA sequencing
ROS	Reactive oxygen species
SA	Salicylic acid
SD	Short day
QK	Quadruple knockout
qRT-PCR	Quantitative Reverse Transcriptase PCR
sgRNA	Single guide RNA
sRNA	Small RNA
T7EI	T7 Endonuclease I
T-DNA	Transfer DNA
TF	Transcription factor
TKO	Triple knockout
tracrRNA	Trans-activating CRISPR RNA
WT	Wildtype

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CHAPTER 1

General Introduction

1.1 Introduction

Plants live in a constantly fluctuating environment from changes in, for example, temperature and nutrient availability, to a range of pathogen infections and herbivore attack. In order to thrive in eclectic environmental conditions, plants have evolved mechanisms to cope with these changes to maintain growth, development and reproduction (Herms and Mattson, 1992). Due to the inability of plants to physically move to a different location to escape stress, they have developed strategic and efficient responses to tackle unfavourable environmental conditions. These include synthesis of secondary metabolites for protection and defence, differentiation leading to structural re-enforcement of cell walls and initiation of stress-responsive mechanisms (Vinocur and Altman, 2005; Gall *et al.,* 2015; Marco *et al.,* 2015). Given that these defensive strategies or over-accumulation of defensive genes almost often result in a negative effect on growth (Heil *et al*., 2000; Zhang *et al*., 2008a; Yang *et al*., 2012; Heinrich *et al*., 2013; Attaran *et al*., 2014; Leone *et al*., 2014), it was suggested that the resources required to support both growth and stress defence were limiting. Therefore, a resource re-allocation between growth and non-growth processes was inevitable, leading to an eventual "trade-off" with a central message: plants must either grow or defend (Chapin, 1991; Herms and Mattson, 1992; Robert-Seilaniantz *et al.,* 2011; Huot *et al.,* 2014). This trade-off was achieved by changing the levels of different plant hormones, which control the expression of numerous transcription factors and other regulatory proteins upstream and downstream of their signalling pathways which play key roles in all cellular processes (Huot *et al.,* 2014). However, this seesaw relationship between the impact of stress defence on growth and productivity has recently been challenged and suggested to be a complex co-ordination of both physiological processes rather than a simple linear

antagonism (Kliebenstein, 2016; Bechtold *et al.,* 2018). For example, the Arabidopsis C24 accession, despite naturally expressing Salicylic acid (SA)-defences constitutively, was only minimally impacted on growth and developmental characteristics compared to other accessions/mutant with or without stress (Bechtold *et al.,* 2010, 2018).

1.2 Growth and Defence

1.2.1 Hormones

Plant growth processes from cell division, elongation and differentiation are influenced externally by environmental cues and internally by growth regulators i.e. hormones (Santner *et al.,* 2009). Plant hormones (or phytohormones) are small naturally occurring organic compounds that influence physiological processes mainly of growth, development, immunity and reproduction at low concentrations (Santner *et al.,* 2009). They are synthesised in cells or tissues and can also be transported to be used at other locations in the plant. Plant hormones play a crucial role in the way plants grow and develop. Of the hormones implicated for growth responses, auxins, gibberellins, brassinosteroids and cytokinins are considered to be positively essential for plant growth (Depuydt and Hardtke, 2011). These hormones regulate and promote cell elongation and proliferation not only in an independent manner but also collectively as their individual signalling pathways overlap during several cellular processes (Hardtke *et al.,* 2007; Santner *et al.,* 2009; Depuydt and Hardtke, 2011; Robert-Seilaniantz *et al.,* 2011). Ultimately, plant growth hormones are perceived to prompt transcription re-programming of participating cells by actively targeting specific transcription factors (TFs) involved in their signalling (Chapman and Estelle, 2009; Sun, 2011; Zhao and Li, 2012).

Conversely, hormones can have a negative impact on plant growth induced by biotic and abiotic stresses (Wolters and Jürgens, 2009). These stresses trigger the induction of plant defence mechanisms regulated by signalling cascades and hormones leading to the activation of defence-related genes and TFs (Woodrow *et al.,* 2012). Jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) amongst others are important plant defensive hormones and accumulate when a plant undergoes stress (Wolters and Jürgens, 2009). JA is induced during mechanical wounding, insect or herbivore attack (Wasternack and Hause, 2013) while SA and ET are primarily involved in disease resistance (Glazebrook, 2005; Robert-Seilaniantz *et al.,* 2011). These hormones are also induced under abiotic stress (Cheong *et al.,* 2002). Induction of defensive hormones is commonly followed by a slowing down of plant growth (Zhang *et al*., 2008a; Yang *et al*., 2012; Attaran *et al*., 2014). For example, Benzothiadiazole (BTH) is a synthetic SA mimic used to enhance disease resistance by inducing systemic acquired resistance in crops (Görlach *et al.,* 1996). In the absence of pathogens, BTH causes a reduction in plant growth and seed set (Heil *et al.,* 2000). This relationship between growth and defence also occurs *vice versa* when plants with reduced Red: Far-red light ratios were shown to be susceptible to disease pathogens as growth was prioritized over defence (De Wit *et al.,* 2013). The balancing act between growth and stress defence can be achieved by the interaction and cross-talk between defence- and growth-related genes and their signalling pathways (Navarro *et al.,* 2006; Rivas-San Vicente and Plasencia, 2011; Denancé *et al.,* 2013; Huot *et al.,* 2014; Leone *et al.,* 2014).

1.2.2 Abiotic Stress

The fluctuating nature of the environment is often not optimum for plant growth. Abiotic stress like salinity, drought, high temperatures and chemical toxicity are forms of environmental changes detrimental to plant growth, development and productivity. According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC, 2014), the Earth's surface has become warmer leading to increased temperature of land and sea including heavy precipitations. This has in effect increased sea levels and an unprecedented increase in atmospheric temperature. These changes in climate have and will cause widespread impacts on human and natural systems (IPCC, 2014). For example, towards the end of the 21st century, the interaction of increased temperature, increased pollutants during droughts and increased flooding will reduce water quality. A reduction of 50% of the projected average crop yield, would also pose large risks to food security globally, combined with an increase in food demand. However, plants have been able to successfully thrive under these challenging climatic conditions and are "stressed" when these changes are rapid and extreme (global warming). When this stress is perceived, plants adjust internal mechanisms, to maintain homeostasis and survival.

At the cellular level, this range of apparently diverse stresses can be manifested as the common accumulation of toxic reactive oxygen species (ROS), which cause cellular damage by oxidation of cellular macromolecules and inhibition of photosynthesis (Apel and Hirt, 2004; Mittler *et al.,* 2004; Mullineaux *et al.,* 2018). Despite being continually produced predominantly in chloroplasts, mitochondria and peroxisomes, ROS are rapidly removed by scavengers such as superoxide dismutase (SOD) and the glutathione–

ascorbate cycle (Noctor and Foyer, 1998). This tightly controlled production and removal equilibrium can be inhibited by stress causing an over production of ROS and consequent cellular damage (Apel and Hirt, 2004). ROS have also been implicated to act as signalling molecules to activate abiotic stress responsive and defensive pathways. For example, H_2O_2 have been implicated in the signalling of the plant hormone, abscisic acid (ABA), which triggers the closure of the stomata to limit water loss through transpiration during heat and/or drought stress (Iriti *et al.,* 2009) and also in responses to high light (Galvez-Valdivieso *et al.,* 2009; Mittler and Blumwald, 2015; Mullineaux *et al.,* 2018). The dual roles of ROS in toxicity and in signalling mechanisms have enabled plants to develop sophisticated strategies to regulate intracellular ROS concentration during stress defence; avoidance and scavenging mechanisms (Apel and Hirt, 2004; Mullineaux *et al.,* 2018).

To repair damage caused by abiotic stress, plant recruit a complex of defensive mechanisms which alter the cell's biochemical machinery. Responding to abiotic stresses involve complex pathways which overlap and cross-talk to give a specified response. The regulation of these responses requires protein-protein interactions in signal transduction pathways leading to stress-related gene expression (Woodrow *et al.,* 2012). The importance of regulating gene expression and protein turnover by transcriptional and posttranscriptional machinery by plants cannot be over-emphasised during abiotic stress.

1.2.2.1 Abiotic Stress Response

As established above, environmental stress is a limiting factor to plant growth and development and, to combat these effects, plants respond by re-programming important metabolic and regulatory proteins involved in protection and signal transduction leading to altered gene expression (Agarwal *et al.,* 2013). These include those involved in direct

protection i.e. heat stress proteins (HSPs) or chaperones, Late Embryogenesis Abundant (LEA) proteins, enzymes that catalyse the synthesis of osmo-protectants (proline and betaine) and antioxidants (e.g. flavonoids, ascorbate, glutathione), proteins important for the dissipation of excess excitation energy, anti-freezing proteins, free-radical scavengers and detoxification enzymes, those involved in water and ion transport such as aquaporins and ion transporters including those involved in signal transduction and transcriptional control e.g. Mitogen-activated protein kinases (MAPKs), Calcium-dependent protein kinases (CDPK) and SOS kinases, phospholipases and transcription factors (Vinocur and Altman, 2005; Agarwal *et al.,* 2013; Osakabe *et al.,* 2013).

Transcription factors (TFs) alter gene expression by binding to *cis* regulatory elements on promoters and/or enhancers of target genes leading to genetic reprograming and ultimately stress tolerance. While transcription factor binding of *cis* regulatory elements on promoters occurs upstream, few kilobases away from their target genes, transcription factor binding of enhancers can occur both up- or downstream, hundreds of kilobases away from their target genes (Kolovos *et al*., 2012; Sakabe *et al*., 2012). Large families of TFs including basic leucine zipper (bZIP), NAM/ATAF1/CUC2 (NAC), ABA responsive element (ABRE) binding proteins/factors (AREB), DRE-BINDING PROTEIN (DREB), MYC/MYB and WRKY act downstream of regulatory signalling molecules which are ABA-dependent (Saibo *et al.,* 2009). Transcriptome analysis, however, shows that hundreds of genes are implicated in abiotic stress response (Kreps *et al.,* 2002; Gehan *et al.,* 2015; Coolen *et al.,* 2016; Van Veen *et al.,* 2016; Albihlal *et al.,* 2018), some of which have been used to engineer stress tolerance (Vinocur and Altman, 2005; Bechtold *et al.,* 2013; Wang *et al.,* 2016a; Kudo *et al.,* 2017). A number of these stress responsive genes share the same transcription factors; however individual genes of the same family often respond

differently to various environmental stresses due to overlap and gene regulation redundancy during stress (Kreps *et al.,* 2002; Seki *et al.,* 2002). This overlap or crosstalk between different stress responsive genes is crucial for plant survival as plants in the wild experience different forms of stress accompanied by the primary stress (reviewed by Suzuki *et al.,* 2014).

Post-transcriptional modifications mediated by noncoding RNA-dependent mechanisms and post-translational modifications (phosphorylation, ubiquitination and sumoylation) also regulate gene expression by controlling abiotic stress-induced regulatory proteins and TFs (Mazzucotelli *et al.,* 2008). MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are small non-coding RNAs which have been discovered to be regulators of plant abiotic stress defence. miRNAs and siRNAs are small RNA molecules that are processed from hairpin RNA precursors encoded by non-structural genes within the genome and are believed to regulate levels of developmental gene transcripts (Bartel, 2004). In Arabidopsis, miRNAs have been shown to regulate developmental processes by complementary base pairing with target genes which results in cleavage of the messenger RNA (Palatnik *et al.,* 2003; Bartel, 2004). They mediate post-transcriptional gene silencing by guided degradation, translational inhibition or epigenetic modification (Bartel, 2004). In recent years, long noncoding RNAs have been identified and characterised to also regulate gene expression, mRNA translation and chromatin remodelling without being processed (Ariel *et al.,* 2015). These RNA molecules are longer than 200 nucleotides (nt) as opposed to miRNAs and siRNAs (less than 50 nt) and are classified based on their location relative to protein-coding genes (Rinn and Chang, 2012; Ariel *et al.,* 2015). For example, Long noncoding natural antisense transcripts (lncNATs) initiate inside or 3' to a protein-coding gene and are transcribed in the opposite direction (Wunderlich *et al*.,

2014); Intronic lncRNAs initiate inside an intron in either direction without overlapping with an exon (Heo and Sung, 2011); Promoter lncRNAs initiate in a divergent fashion from the promoter of the protein-coding genes (Zheng *et al*., 2013) and intergenic ncRNAs (lincRNAs) initiate in-between protein-coding genes separated by at least 1 kb (Crespi *et al*., 1994). lncRNAs have also been reported to play a role in abiotic stress responses as well as regulation of transcription, development and chromatin modifications (Bonasio and Shiekhattar, 2014; Di *et al.,* 2014; Albihlal *et al.,* 2018).

Phosphorylation is one of the well-studied post-translational protein modifications, tasked with the transmission of extracellular stress signals into the cell (Mazzucotelli *et al*., 2008). Ubiquitination is the addition of ubiquitin to a target protein which is then degraded by proteolysis via the 26S proteasome (Weissman, 2001). Sumoylation is the conjugation of Small Ubiquitin-like modifiers to protein substrates which induces conformational changes or prevent protein degradation by the ubiquitin pathway (Hay, 2005). These modifications may act on the same protein target at different levels during the translational process ultimately determining the amount, activity, sub-cellular location and final fate of the protein. For example, heat shock leads to the hyperphosphorylation of *Chlamydomonas reinhardtii* HSF1, and the extent of phosphorylation correlated with the degree of induction of heat shock genes (Schulz-Raffelt *et al*., 2007). Conversely, kinase-inhibitor treatment delayed *Cr*HSF1 hyperphosphorylation, which correlated with a delayed heat shock response (Schmollinger *et al*., 2013). In Arabidopsis, CALMODULIN-BINDING PROTEIN KINASE 3 (CBK3) and PROTEIN PHOSPHATASE 7 (PP7) have been reported as HSFA1-interacting protein kinase and phosphatase respectively, with their knockout mutants showing a thermosensitive phenotype through the phosphorylation or dephosphorylation of HSFA1

(Liu *et al*., 2007; Liu *et al*., 2008a). Equally, the Arabidopsis E3 ubiquitin ligase DROUGHT TOLERANCE REPRESSOR (DOR) was shown to negatively regulate ABA biosynthesis in guard cells to influence drought tolerance. *dor* knockout mutants show increased cellular ABA levels and are more tolerant to drought stress compared to wildtype due to an increase in stomatal closure (Zhang *et al*., 2008b). Similarly, DREB2A-INTERACTING PROTEIN 1 (DRIP1) and DRIP2 function as E3 ubiquitin ligases to mediate DREB2A ubiquitination and subsequent degradation under non-stress conditions. *drip1/drip2* double knockout mutants results in the accumulation of DREB2A under non-stress conditions (Qin *et al*., 2008). In response to stress, proteins are mainly sumoylated by SAP AND MIZ 1 (SIZ1; Yoo *et al.*, 2006). *siz1* knockout mutants lack basal thermotolerance and show enhanced drought tolerance due to reduced stomatal aperture (Yoo *et al*., 2006; Miura *et al*., 2012). Furthermore, sumoylation have also been shown to decrease HSFA2 activity influencing the heat stress response (Cohen-Peer *et al.*, 2010). Other methods and pathways by which Phosphorylation, Ubiquitination, Sumoylation and noncoding RNAs mediate the regulation of abiotic stress response have been extensively reviewed (Mazzucotelli *et al*., 2008; Khraiwesh *et al*., 2012; Matsui *et al*., 2013; Cabello *et al.*, 2014; Guerra *et al*., 2015; Ohama *et al*., 2017).

1.3 Response to Temperature Changes

Temperature is an important factor that affects plant growth and development. Plants, which are highly plastic, respond differently to changes in growing temperature. While mild temperature increase/warming (22-29°C) results in elongation of plant axes, higher and lower temperatures lead to growth suppression (Mittler *et al.,* 2012). Further to changes in plant growth and development as a result of temperature increase, crop yields

are also affected (Peng *et al.,* 2004; Rizhsky *et al.,* 2004; Vile *et al.,* 2012). Plants respond differently to both growth-promoting and growth-inhibiting temperature changes altering hormones and transcription factors involved in growth and stress defence processes. Therefore, plants response to temperature changes are discussed.

1.3.1 Ambient Temperature

Mild changes in growth temperatures which do not trigger a stress response is sometimes termed ambient temperature. This temperature, ranging within ±6°C of the growing temperature (22+1°C), significantly stimulates plant growth and development. This section however focuses solely on increase in growth temperature (+6°C) which induces early flowering, longer hypocotyls and petioles, and leaf hyponasty (Gray *et al.,* 1998; Balasubramanian *et al.,* 2006; Jeong *et al.,* 2008; Koini *et al.,* 2009; van Zanten *et al.,* 2009). Henceforth, '*ambient temperature'* in this section (and study) refers to temperatures within 22-29°C. Due to their sessile nature, plants are able to sense changes in ambient temperature and respond appropriately which often results in changes in plant architecture. For example, an Arabidopsis mutant, *ACTIN RELATED PROTEIN 6 (arp6)*, constitutively expressing ambient temperature-regulated genes, resembles a wildtype plant growing at 28°C (Kumar and Wigge, 2010). These architectural changes owing to temperature or thermo-responsive growth alterations involve the action of hormones and transcription factors. These hormones and transcription factors involved in thermos-responsive growth alterations also often respond to light perception and signalling leading to a complex crosstalk fundamental to the growth and development of plants in natural environments. For example, the red-light-absorbing phytochrome (phyB) have been shown to act redundantly with both blue-light-sensing cry1 and phytochromes A and E to

inhibit internode elongation at temperatures greater than 20°C (Mazzella *et al*., 2000; Halliday and Whitelam, 2003). Furthermore, phyB, the most abundant phytochrome in light-grown seedlings was shown to be important for germination across a range of temperatures especially during warm conditions (Heschel *et al*., 2007). Jung *et al*., (2016) also found phyB directly associating with promoters of key target genes in a temperature-dependent manner. Therefore, light and temperature regulate similar developmental processes and their signals is suggested to converge on hormone signalling pathways, activating common targets to mediate plant elongation (Gray *et al*., 1998; Tao *et al*., 2008).

1.3.1.1 Hormones

Plant hormones play a crucial role in the way plants grow and develop. One example of a developmental trait showing the interplay between hormone and temperature-dependent growth is hypocotyl elongation. Hypocotyl elongation, which involves the expansion of cells, is positively regulated by auxins, brassinosteroids and gibberellin (Stavang *et al.,* 2009; Ibañez *et al.,* 2018). While exogenous application of auxins have long been shown to promote cell elongation in Arabidopsis (Chapman *et al.,* 2012), inhibition of auxin transport using 1-naphthylphthalamic acid (NPA) strongly repressed hypocotyl growth (Jensen *et al.,* 1998). Furthermore, endogenous auxin levels have been correlated with increase in hypocotyl and petiole length typical of plants growing in ambient temperature. For example, wildtype plants growing at 29°C had increased endogenous auxin levels further implicating them in temperature-induced hypocotyl elongation (Gray *et al.,* 1998). The hypocotyls of Arabidopsis mutants defective in auxin signalling or transport failed to elongate at 29°C compared to wildtype (Gray *et al.,* 1998). In the same vein, the gibberellin (GA) pathway is also critical in temperature induced Arabidopsis

hypocotyl elongation. Using GA biosynthesis inhibitor paclobutrazol (PAC) and mutants severely impaired in GA biosynthesis, hypocotyl elongation was significantly reduced in response to temperature increase (Stavang *et al.,* 2009). Other plant species like pea, apple and citrus have also been used to demonstrate the importance of GA in temperature-induced elongation (Steffens and Hedden, 1992; Vidal *et al.,* 2003; Stavang *et al.,* 2007). Brassinosteroids (BR) have also been implicated in temperature-induced hypocotyl elongation. Recent reports place BR downstream of auxin and GA signalling (Ibañez *et al.,* 2018). This was revealed when exogenous application of BR, epi-brassinolide (BL), rescued the hypocotyl elongation defects of Arabidopsis auxin and GA biosynthesis/signalling mutants at 28°C while exogenous application of the synthetic auxin, picloram (PIC), could not rescue the elongation defects of BR biosynthesis/signalling mutants at the same temperature (Ibañez *et al.,* 2018). Furthermore, BR have also been reported to have a synergistic relationship between GA and Auxin in Arabidopsis hypocotyl elongation (Tanaka *et al.,* 2003; Nemhauser *et al.,* 2004). The interaction and crosstalk between these hormones and temperature-induced growth is more evident transcriptionally (Oh *et al.,* 2014a).

1.3.1.2 Transcription Factors

At the molecular level, internal and external cues regulate the expression of a host of target genes which affect development. The phytochrome interacting factors (PIFs), which encode basic helix-loop-helix (bHLH) transcription factors, have been shown to be central integrators (Fig 1.1; Leivar and Quail, 2011; Leivar and Monte, 2014). Of the PIF family of TFs, PIF4 has been linked with ambient temperature responses (Koini *et al*., 2009; Kumar *et al*., 2012; Wigge, 2013). Hypocotyls and petioles of Arabidopsis *pif4* mutants fail to elongate at warming temperatures while WT *PIF4* expression is transiently increased

under the same conditions in hypocotyls and cotyledons (Koini *et al.,* 2009; Stavang *et al.,* 2009). Overexpression of *PIF4* also results in hypocotyl elongation in normal and warming conditions (Stavang *et al.,* 2009; Ibañez *et al.,* 2018).

PIF4 has also been implicated in the regulation of several hormone biosynthesis and signalling genes at ambient temperature. Increased expression of key auxin biosynthesis genes, *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1*, also called *TRANSPORT INHIBITOR RESPONSE 2/TIR2), CYTOCHROME P450, FAMILY 79, SUBFAMILY B, PEPTIDE 2 (CYP79B2), YUCCA8 (YUC8),* as well as auxin-induced genes, SMALL AUXIN UP RNA (SAUR) 19-24, have been reported to be regulated in a *PIF4*-dependent manner in ambient temperature (Franklin *et al.,* 2011; Sun *et al.,* 2012). Direct binding of PIF4 to the promoters of the key auxin biosynthetic genes during ambient temperature was also reported, leading to increase in endogenous auxin levels and cell elongation (Franklin *et al.,* 2011; Sun *et al.,* 2012). As mentioned above, the action of auxin in thermo-responsive growth is dependent on BR. In the same vein, PIF4-mediated hypocotyl elongation is dependent on BR. Treatment of *PIF4* over-expressing plants with BR biosynthesis inhibitor propiconazole (PPZ) supressed the long hypocotyl phenotype of the transgenic plants at 28°C, while treatment of *pif4* mutants with synthetic BR (epi-brassinolide) rescued the hypocotyl elongation at the same temperature (Ibañez *et al.,* 2018). Since BR activity is regulated by its TF, *BRASSINAZOLE-RESISTANT 1 (BZR1,* He *et al.,* 2005), its direct interaction with *PIF4* during ambient temperature responsive growth have been elucidated, synergistically regulating many growth promoting genes (Oh *et al.,* 2012; Oh *et al.,* 2014a; Ibañez *et al.,* 2018).

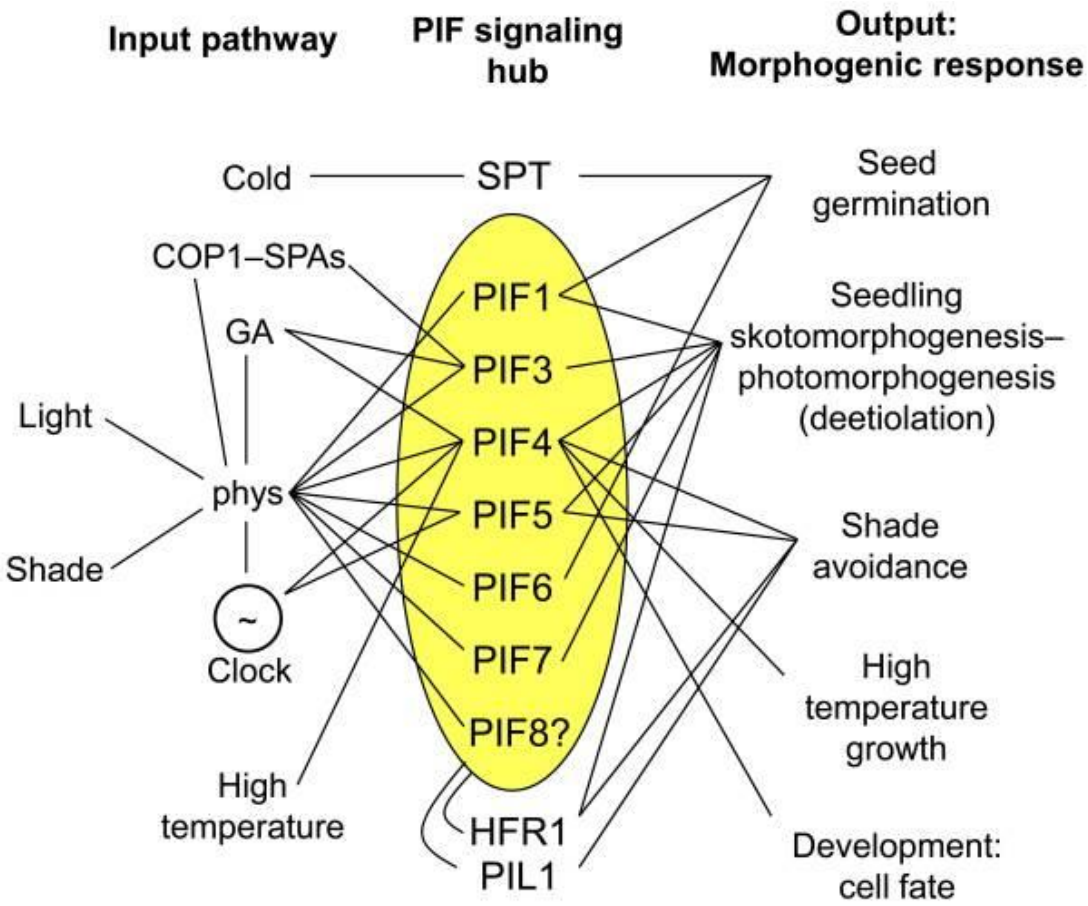


Figure 1. 1. Schematic diagram summarising the integration of environmental and internal signals through different members of PIFs influencing plant growth and development. PIFs act as a hub linking multiple overlapping pathways. For example, PIF4 is regulated by light, circadian clock, shade, GA signalling and temperature which in turn influences plant morphogenesis and architecture. Image from Leivar and Quail, (2011).

PIF4 is also transcriptionally regulated by the circadian clock. A negative regulation by *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4 (ELF4),* and *LUXARRHYTHMO (LUX)*, members of the evening complex (EC; Nusinow *et al.,* 2011; Box *et al.,* 2015), have been

reported although, the direct repression of *PIF4* by *ELF3* is independent of the EC during thermo-responsive hypocotyl growth (Nieto *et al.,* 2015). PIF4 has also been implicated in flowering (Brock *et al.,* 2010) by binding to the *FLOWERING LOCUS T (FT)* promoter in a temperature dependent manner, gated by the thermo-sensory H2A.Z-nucleosomes in Arabidopsis (Kumar and Wigge, 2010; Kumar *et al.,* 2012). The eviction of H2A.Z-nucleosomes by ambient temperature led to a stronger binding of PIF4 to the *FT* promoter (Kumar *et al.,* 2012). PIF4 is also regulated by other factors like light, shade and GA signalling affecting cell elongation (Fig 1.1; Nozue *et al.,* 2007; De Lucas *et al.,* 2008; Niwa *et al.,* 2009; Leivar and Quail, 2011; Leivar and Monte, 2014) which are important but beyond the scope of this chapter.

1.3.2 Heat Stress

Unlike ambient temperature, heat stress (+10-15°C above growing temperature) is detrimental to plant growth and development. Amongst other biological processes, reproduction is mostly affected by high temperatures resulting in reduced seed yield (Zinn *et al.,* 2010). The detrimental effect of heat stress on growth and development is dependent on temperature intensity, timing, duration and rate of increase (Larkindale *et al.,* 2005; Wahid *et al.,* 2007; Larkindale and Vierling, 2008; Echevarría-Zomeño *et al.,* 2016). Heat stress is often accompanied by drought in the field and their combined effects severely affects plant growth and reproduction (Rizhsky *et al.,* 2004; Mittler and Blumwald, 2010; Vile *et al.,* 2012; Suzuki *et al.,* 2014). While plant can cope with elevated temperatures to a degree, prolonged heat stress impairs photosynthesis negatively affecting plant growth, development and productivity (Wheeler *et al.,* 2000; Wise *et al.,* 2004; Lobell *et al.,* 2011).

1.3.2.1 Heat Stress Response (HSR)

At the cellular level, higher temperatures results in increased membrane fluidity, protein denaturation and aggregation, enzyme inactivation, inhibition of protein synthesis and loss of membrane integrity leading to growth inhibition and cell death when prolonged (Wahid *et al.,* 2007; Mittler *et al.,* 2012; Echevarría-Zomeño *et al.,* 2016). This is due to the over accumulation of reactive oxygen species (ROS) causing oxidative stress damage owing to increased cellular toxicity. High temperatures induces ROS production such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and superoxide anion (O_2^-) which are neutralized by ROS scavengers (Apel and Hirt, 2004; Mullineaux *et al.,* 2018). Among ROS, H_2O_2 have a longer half-life, more stable and permeable as it shares many physical features with water (Bienert *et al.,* 2006). Oxidative bursts following heat stress also serve as signalling molecules which alter gene expression leading to the appropriate response (Mittler *et al.,* 2012). For example, it was shown that heat stress-induced H_2O_2 was necessary for the expression of heat stress genes (Volkov *et al.,* 2006). A link between oxidative stress and heat stress response has been established. Panchuk *et al.,* (2002), showed that, *ASCORBATE PEROXIDASE2* (*APX2)*, an antioxidant enzyme that catalyses the reduction of H_2O_2 using ascorbate as the electron donor in plants was induced under heat stress. They also showed an increase in *APX2* expression in transgenic plants overexpressing *HSF3* (*HSFA1b*) compared to wildtype under normal conditions. These transgenic plants, which were heat tolerant, also coped better with oxidative stress compared to wildtype plants (Prändl *et al.,* 1998). Overexpression of other HSFs, *AtHSFA3* and *AtHSFA4,* also gave similar results in oxidative stress tolerance (Perez-Salamo *et al.,* 2014; Song *et al.,* 2016a). Furthermore, high light stress contributes to cellular damage during high temperatures equally increasing H_2O_2 production (Apel and Hirt, 2004). A

change in redox state was observed in high light treated Arabidopsis plants causing the expression of hundreds of genes with heat shock elements on their promoters (Jung *et al.,* 2013). Among them was *APX2* of which its expression was regulated by *HEAT SHOCK TRANSCRIPTION FACTORs A1d, A2* and *A3* (*HSFA1d, HSFA2* and *HSFA3;* Jung *et al.,* 2013).

At the molecular level, all organisms respond to a variety of stresses by rapidly synthesizing a highly conserved set of polypeptides termed heat shock proteins (HSPs; Vierling, 1991). HSPs are molecular chaperones that assist in stabilizing partially folded proteins and preventing aggregation of denatured proteins in the cell during a stress episode (Baniwal *et al.,* 2004). These proteins are essential for survival at both normal and elevated temperatures and are also crucial in the development of thermotolerance and protection from stress-induced cellular damage. HSPs are involved in many regulatory pathways. They recognize and bind to other cellular proteins that are in an unstable or inactive state. HSPs are located in both the cytoplasm and organelles, such as the nucleus, mitochondria, chloroplasts and ER (Vierling, 1991). Five major families of HSPs are recognized based on their molecular weights: The Hsp70 family, Hsp60 family, Hsp90 family, the Hsp100 family, and the small HSP (sHSP) family (Wang *et al.,* 2004). Not all HSPs are stress-inducible, but those that are respond to a wide range of other abiotic stresses, such as drought, salinity, osmotic, cold, heavy-metal and oxidative stress (Schöffl *et al.,* 1998). Typically, HSPs function as oligomers and are responsible for maintaining proteins in a folding, folded, or unfolded state; import, and/or export of partially folded proteins into mitochondria or plastids; minimizing the aggregation of non-native proteins and targeting non-native or aggregated proteins for degradation and cell removal (Feder and Hofmann, 1999). In eukaryotes, the expression of HSPs is not only triggered by a

number of environmental stresses but also by developmental cues (Vierling, 1991; Wehmeyer *et al.,* 1996; Kotak *et al.,* 2007).

HSPs are transcriptionally regulated by multiple HSFs that possess distinct and overlapping roles (Wang *et al.,* 2004). Some HSFs in plants are themselves expressed in a stress-dependant manner. However, studies have established the involvement of HSFs in the regulation of cellular response to other forms of biotic and abiotic stress (Miller and Mittler, 2006; Bechtold *et al.,* 2013; Perez-Salamo *et al.,* 2014). HSFs have now become widely known as major regulators of general stress response and not only heat (Liu *et al.,* 2011; Bechtold *et al.,* 2013). HSPs can play a key role in the regulation of HSF activity under different conditions. In humans, HSP90 can have an inhibitory effect on *HSF1* under no stress condition by binding to their oligomerisation domain maintaining it in an inactive monomeric state (Zou *et al.,* 1998). Similarly, hHSP70 had the same effect on *hHSF1*, blocking *hHSF1* from binding to the DNA (Abravaya *et al.,* 1992).

1.3.2.2 Heat Shock Transcription Factors

Heat Shock Transcription Factors (HSFs) are central to the control of the heat stress response in eukaryotes with a conserved basic structure (Baniwal *et al.,* 2004; Scharf *et al.,* 2012). A typical eukaryotic HSF is characterised by an N-terminal DNA-binding domain (DBD), an oligomerization domain (OD or HR-A/B region), Nuclear Localisation Signal (NLS), Nuclear Export Signal (NES) and the Activation Domain (AD) (Fig 1.2A; Morimoto, 1998; Nover *et al.,* 2001; Baniwal *et al.,* 2004).

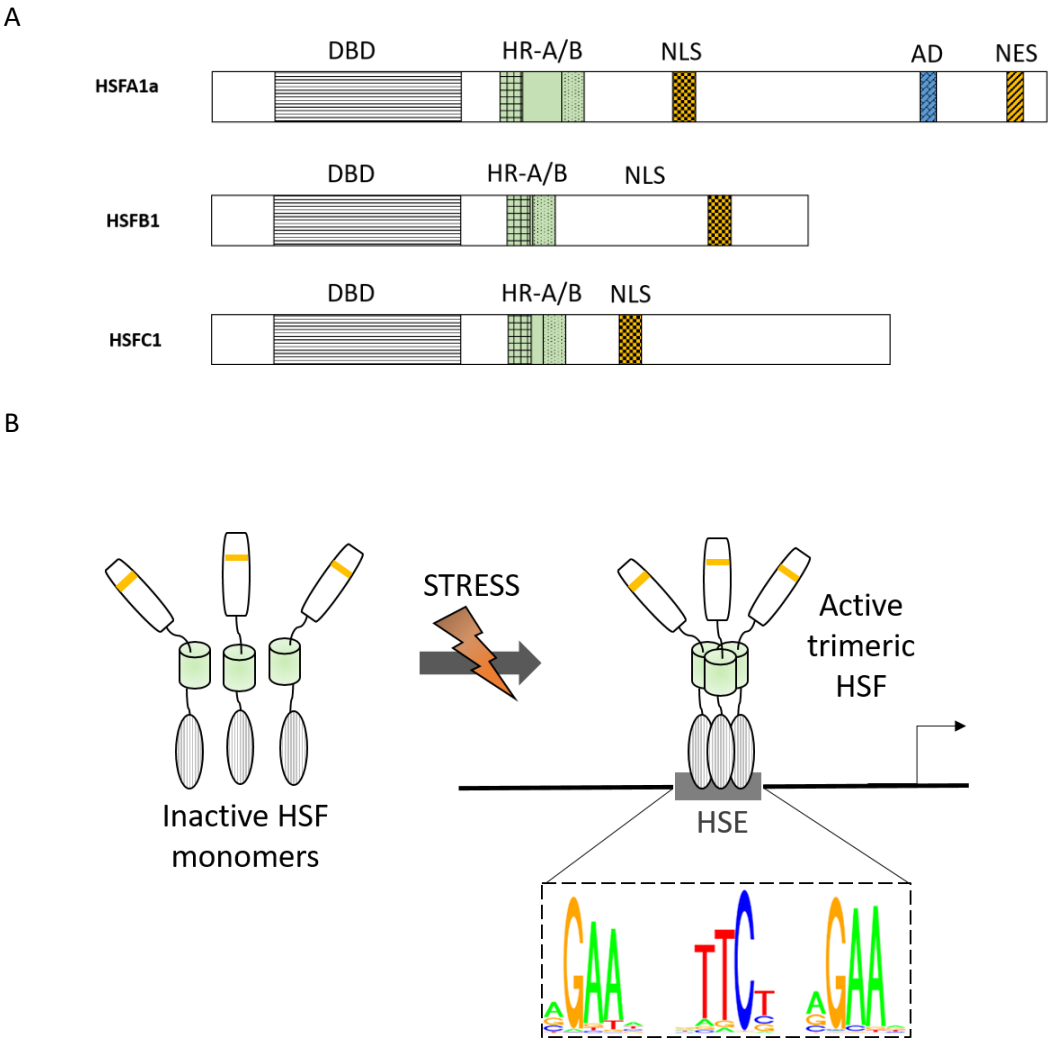


Figure 1. 2. Representative structure of Arabidopsis HSF Class A, B and C. A) The DBD is made up of a hydrophobic core with a conserved Helix-Turn-Helix which is essential for the recognition and precise binding to the promoters of *cis*-regulatory Heat shock elements (HSE). The oligomerization domain HR-A/B is characterized by the heptad pattern of hydrophobic residues (crisscross lines, dots). The insertion of additional amino acid residues between parts A and B are marked in Green. The NLS and NES region allows the import and export of HSF into the nucleus and cytoplasm respectively (Orange). Class A HSFs possesses AD (Blue) used for transcription activation while other classes lack this domain and are not directly involved in *de novo* transcription. **B)** HSFs are present as inactive monomers in the cytosol but when stress is encountered HSFs are then activated, trimerise and compartmentalised to the nucleus where the DBD binds to HSEs with the consensus sequence 5'-nGAAnnTTCnnGAAn-3' to initiate transcription. DBD; DNA-binding domain, HR-A/B; oligomerization domain, NLS; Nuclear Localisation Signal, NES; Nuclear Export Signal, AD; Activation Domain and HSE; Heat shock element. Image not drawn to scale and adapted from (Nover *et al.,* 2001).

The DBD is made up of three-helical bundles and four antiparallel β sheet. The hydrophobic core of the DBD is a conserved Helix-Turn-Helix which is essential for the recognition and precise binding to the promoters of *cis*-regulatory Heat Shock Elements (HSE) which confer their thermo-inducibility (Schöffl *et al.,* 1998; Ahn *et al.,* 2001). The HR-A/B region is next to the DBD connected by a flexible linker of 15 to 80 amino acid residues. A heptad pattern of hydrophobic amino acid residues in the HR-A/B region leads to the formation of a coiled-coiled structure essential for homotrimer formation (Peteranderl and Nelson, 1992). The NLS and NES region allows the import and export of HSF into the nucleus and cytoplasm respectively (Lyck *et al.,* 1997). The AD, as well as NLS and NES, are located towards the C-terminus of the HSF. HSFs which have the AD domain act as transcription activators while those that lack this domain are not involved directly in *de novo* transcription (Scharf *et al.,* 2012). However, some HSFs (Class B) have a repressor domain which act as a binding site for co-repressors and also function as repressors of transcription (Fig 1.2A; Scharf *et al.,* 2012).

The HSEs are present in multiple copies upstream of the HSP genes. HSEs are formed by conserved repetitive consensus sequences 5'-nGAAn-3' (Littlefield and Nelson, 1999). Heat Shock Transcription Factors (HSFs) bind to at least three inverted units of the consensus sequence resulting in 5'-nGAAnnTTCnnGAAn-3' / 5'-nTTCnnGAAnnTTCn-3' consensus sequence in response to stress which activates HSP expression (Fig 1.2B; Littlefield and Nelson, 1999; Ahn *et al.,* 2001). When stress is encountered, HSPs are recruited to chaperone and maintain protein homeostasis. HSF is then activated, trimerised and compartmentalised to the nucleus to initiate transcription of an array of stress-responsive genes (Fig 1.2B; Wu *et al.,* 1994; Schöffl *et al.,* 1998).

Activation and translocation of HSFs to the nucleus has been shown to be redox-dependent. For example, the cysteine residues of mammalian HSF1 forms a disulfide bond upon exposure to heat stress, which leads to the trimerisation of HSF monomers, translocation of the complex to the nucleus and transcription of stress-responsive genes (Ahn and Thiele, 2003). This redox regulation of HSF activation has also been shown in Arabidopsis *HSFA8* (Giesguth *et al.,* 2015). It occurred following the reversible redox regulation of *HSFA8* from an inactive monomer to the active homotrimer involving two cysteine residues located within or near the DBD. Mutation of the 2 cysteine residues Cys24 and Cys269 blocked translocation of *HSFA8* to the nucleus in Arabidopsis protoplasts during oxidative stress (Giesguth *et al.,* 2015). A similar block was also observed when the 3 conserved cysteine residues in *HSFA4A* were mutated (Perez-Salamo *et al.,* 2014).

1.4 HSF and Development

In conjunction with abiotic stress response, HSFs have also been implicated in other cellular processes including development. For example, baker's yeast (*Saccharomyces cerevisiae*) possesses only one HSF gene (*yHSF*), whose protein is constitutively in an active form under all conditions (Jakobsen and Pelham, 1988). It is the only regulator of HSR in yeast and it undergoes intrinsic conformational changes in response to elevated temperature. The expression of HSPs in yeast is solely controlled by this HSF under normal growth condition and stress (Liu *et al.,* 1997). Knockout studies have shown that loss of *yHSF* is lethal under normal growth conditions (Jakobsen and Pelham, 1988). These results established the important role of *yHSF* for yeast cell viability and survival. Similarly, the

fruit-fly (*Drosophila melanogaster*) possesses only one HSF (*dmHSF*). *In vitro* analysis of *dmHSF* showed that it is subject to intrinsic conformational changes in response to elevated temperature (Zhong *et al.,* 1998). Unlike yeast, the loss of *dmHSF* is not lethal but leads to hyper-sensitivity to elevated temperatures (Jedlicka *et al.,* 1997). Developmentally, it results in impaired growth of the fruit-fly larvae when exposed to elevated temperature. Loss of *dmHSF* also leads to defective oogenesis development (Jedlicka *et al.,* 1997).

In vertebrates, there are 4 known HSF genes and corresponding proteins; *HSF1, HSF2, HSF3* and *HSF4*. *HSF1* and *HSF2* are constitutively expressed in all tissues and cell types (Åkerfelt *et al.,* 2010). The expression of *HSF4* is limited to eye and brain tissue and *HSF3* is found in avian species (Pirkkala *et al.,* 2001). *HSF1* is considered the sole master regulator of HSR in vertebrates (Åkerfelt *et al.,* 2010). In terms of development, single knockout of *HSF1, HSF2* or *HSF4*, or double knockout of *HSF1/HSF2* or *HSF1/HSF4* is not lethal in mice but have their specific developmental functions (Christians and Benjamin, 2006; Jin *et al.,* 2011b). Loss of mouse HSF1 (*MmHSF1*) led to a severe developmental impairment including neurodegeneration and development of muscle atrophy (Kondo *et al.,* 2013), increased prenatal lethality, growth retardation and female infertility (Xiao *et al.,* 1999). Loss of *MmHSF2*, on the other hand, results in increased embryonic lethality, mental retardation and defective spermatogenesis (Wang *et al.,* 2003). Furthermore, it was shown that *MmHSF4* is required for cell differentiation in eye lens and for proper eye development (Min *et al.,* 2004). HSFs have also be implicated in tumorigenesis. For example, loss of *HSF1* in mice has been shown to reduced tumour growth compared with those with intact *HSF1* (Dai *et al.,* 2007, 2012; Min *et al.,* 2007). It was suggested to be due to cancer cells actively expressing *HSF1* to manage the imbalances in protein homeostasis

that occur during tumorigenesis, via HSPs, to maintain survival (Jin *et al.,* 2011b). In addition, *HSF1* was shown to actively drive a distinct transcriptional program from heat stress in different cancer cells to support growth and not only through the actions of molecular chaperones like HSP90 and HSP70 on client proteins (Mendillo *et al.,* 2012). In direct contrast to *HSF1*, *HSF2* has been shown to be a tumour suppressor. A decrease in *HSF2* expression was identified in a wide range of human cancer types and a knockdown led to tumour invasiveness of prostate cancer cells (Björk *et al.,* 2016). These few studies reveal that the role of HSF might not be limited to stress response but also in regulation of important cellular processes like growth and development under non-stress conditions.

With respect to plants, Arabidopsis in particular, only a small subset of HSFs have been individually implicated in growth and development. *HSFB2a* was shown to be involved in gametophyte development when a homozygous T-DNA knockout line could not be isolated (Wunderlich *et al.,* 2014). Heterozygous mutants however were viable but had 45% sterile ovules linking *HSFB2a* with female gametophyte development. *HSFB2b* was also shown to regulate hypocotyl growth and flowering time. Under normal conditions, plants overexpressing *HSFB2b* had longer hypocotyls compared to control in short day (SD); while in long day (LD), flowering was significantly delayed (Kolmos *et al.,* 2014). The high expression of *HSFB4* in Arabidopsis roots led to the investigation of the HSF in root development. As a result, transgenic overexpression of *HSFB4* caused retardation of root growth compared to wildtype due to morphological differences at the root surface (Begum *et al.,* 2013). Seed development has also been shown to be regulated by *HSFA9.* Its expression was detected in the late stages of seed maturation and regulated by the seed specific TF *ABSCISIC ACID–INSENSITIVE3 (ABI3*; Kotak *et al.,* 2007). *ABI3* knockout lines lacked detectable levels of *HSFA9* transcript while *HSFA9* Knockout mutants have not

been isolated suggesting embryo lethality (Kotak *et al.,* 2007). Furthermore, *HSFA2* has been shown to play an important role in cell proliferation when plants overexpressing the HSF had accelerated callus growth from roots compared with wild type (Ogawa *et al.,* 2007). Rosette size of *HSFA2* transgenic plants were also retarded albeit dose dependent. Overexpressing *HSFA1b,* a member of the clade A1 HSFs, improved seed yield and harvest index in Arabidopsis and Brassica achieved by increased branching of flowering stems and redistribution of biomass of reproductive structures at the expense of shoot growth (Bechtold *et al.,* 2013). However, quadruple knockout mutants of Arabidopsis clade A1 HSFs (QK) resulted in a plant with severe developmental defects including aberrant seeds, poor germination and small size (Liu *et al.,* 2011). Despite these altered morphologies, the quadruple HSF knockout was not lethal under normal conditions suggesting a developmental role for the clade A1 HSF's.

1.4.1 Arabidopsis class A1 HSFs

Plants have large HSF gene families and in Arabidopsis, it accounts for 21 HSF genes whose member TFs are sub-divided structurally into class A, B and C based on the number of amino acid residues of their oligomerisation domain (HR-A/B) (Fig 1.2; Kotak *et al.,* 2004; Nover *et al.,* 2001). Class A HSFs have with 21 amino acids between part A and B, Class C HSFs have 7 while Class B is compact (Scharf *et al.,* 2012). Class A HSFs consists of 15 HSFs and are believed to be transcription activators, Class B (5 HSFs) functions as transcription repressors and/or co-activators and Class C (1 HSF) has no known function ascribed to them (Nover *et al.,* 2001; Czarnecka-Verner *et al.,* 2004). The Class A HSFs are further divided into clades; A1, A2, A3, A4, A7 and A9. Arabidopsis class A HSFs, have been suggested to not only be involved in thermotolerance but also in other abiotic stresses

like drought, salinity, chilling, osmotic and oxidative stress (Li *et al.,* 2003; Li *et al.,* 2005a; Bechtold *et al.,* 2013; Liu and Charng, 2013; Perez-Salamo *et al.,* 2014; Song *et al.,* 2016a).

Clade A1 members in Arabidopsis (*HSFA1a, HSFA1b, HSFA1d* and *HSFA1e*) are key regulators of early and late response to heat stress and collectively have been shown to work redundantly as master regulators of not only heat stress responses but also a range of environmental stress defences (Liu *et al.,* 2011). For example, plants over-expressing the *HSFA1b* transcription factor are not only heat tolerant (Prändl *et al.,* 1998) but also show increased seed yield, drought and disease resistance though at the expense of reduced shoot growth (Bechtold *et al.,* 2013). Expression profiling showed that some Arabidopsis clade A1 HSFs are constitutively expressed and their expression levels do not change in response to changes in growth conditions (Miller and Mittler, 2006; Swindell *et al.,* 2007). The role of clade A1 HSFs in Arabidopsis have been extensively studied in relation to heat stress as well as other stresses due to the availability of transgenic and T-DNA knockout lines. For instance, overexpression of *HSFA1a*, resulted in the activation of HSPs which in turn enhanced basal thermotolerance under normal conditions as well as tolerance to a wide range of pH changes and hydrogen peroxide treatment (Qian *et al.,* 2014). In the same vein, overexpression of *HSFA1b* resulted in a high plant survival rate under extreme heat stress treatments (Prändl *et al.,* 1998) and also enhanced basal resistance to biotrophic pathogens compared to wild type (Bechtold *et al.,* 2013). *HSFA1a* and *HSFA1b* have also been shown to act redundantly in the early phase of HSR but double knockout of both TFs did not show any substantial defects in thermotolerance but only in heat stress gene expression (Lohmann *et al.,* 2004). On the other hand, double knockout of *hsfA1d/hsfA1e* resulted in an impairment to heat stress and high light (Nishizawa-Yokoi *et al.,* 2011). Due to the high level of redundancy between the clade A1 HSFs, studies

were focused on knocking out 3 of all 4 clade A1s in different combinations as well as all 4 which resulted in a more sensitive plant to abiotic stress (Liu *et al.,* 2011; Yoshida *et al.,* 2011). Results revealed that the triple knockouts mutants with either of *HSFA1a, HSFA1b* and *HSFA1d* as the only functional member could confer thermotolerance to a degree while that of *HSFA1e* and quadruple knockout (*QK*) mutants showed dramatic defects in heat stress tolerance compared to wild type. It was also revealed that expression of HSP genes under heat stress was extremely reduced in QK plants which suggested that *HSFA1a, HSFA1b* and *HSFA1d* were the master regulators of HSR in Arabidopsis and also a partial redundancy between the clade A1 HSFs.

1.5 Aims and Objectives

The main goal of this research was to identify and characterise the genes regulated by the clade A1 HSFs involved in the proper functioning of Arabidopsis growth and development under normal conditions as well as under stress. It was suggested that the phenotype of the QK was due to low HSP90 levels but other genes actively involved in causing the phenotype was not ruled out (Liu *et al.,* 2011). To this effect, the objectives of this study include;

- Identifying developmental genes that are regulated by *HSFA1b* and other members of clade A1.
- Explore how HSFA1s could regulate the developmental genes identified post-transcriptionally.
- Generating a new quadruple knockout (QK) mutant because the available QK was a hybrid of 2 Arabidopsis accessions (Liu *et al.,* 2011).
- Examine transcriptomic profile of the new quadruple mutant to confirm and/or uncover other developmental targets.

CHAPTER 2

Materials and Methods

2.1 Plant Material

2.1.1 *Arabidopsis thaliana*

Arabidopsis thaliana genotypes used in this study include the following;

Wildtypes: Wassilewskija (Ws-0) and Columbia (Col-0); transgenic plants overexpressing *HSFA1b* under the control of 35S promoter fused to a monomeric Red Fluorescent Protein (*35S:HSFA1b*; Bechtold *et al.,* 2013); *hsfa1a/hsfa1b* double knockout mutant in the Ws-0 background (*hsfa1a/a1b*; Lohmann *et al.,* 2004); *hsfa1b/hsfa1d/hsfa1e* Triple knockout (TKO) mutant lines in a Col-0 background (*hsfa1b/d/e;* Persad, 2015), HSFA1 quadruple knockout (QK) mutant which is a combination of Col-0 and Ws-0 genotype (Liu *et al.,* 2011); and *ago1/ago2* double knockout (*ago1/2 dko;* Garcia-Ruiz *et al.,* 2015).

2.2 Arabidopsis Growth and Stress Conditions

2.2.1 Growth Conditions

Arabidopsis thaliana seeds were grown on commercial compost mixture (Levington F2+S, The Scotts Company, Ipswich, UK), stratified for 3 days at 4°C and placed in a controlled environment under short day conditions (8-hour-light/16-hour-dark cycle) or long day (16-hour-light/8-hour-dark cycle) at a photosynthetically active photon flux density (PPFD) of 120 µmol m^{-2} s^{-1} (250W Double Ended Quartz Metal Halide bulb, GE) measured with LI-250 light meter (LI-COR, USA), 22°C ± 1 temperature and 59 ± 1 % relative humidity. Arabidopsis seeds were also sown on ½ strength Murashige and Skoog (MS) agar plates (Murashige and Skoog mineral salts, pH 5.9) with or without sucrose and stratified for 3 days at 4°C to encourage uniform germination, before being transferred to growth chambers (Fitotron PG660, Sanyo Gallenkamp, UK). Plants were harvested around noon (±1 hour) in all experiments except otherwise stated.

2.2.2 Growth Measurements

All genotypes were grown as outlined in section 2.2.1. Measurements of germination rate was carried out according to Boyes *et al.,* (2001). Imbibed seed were sown on ½ MS plates with 1% sucrose and put in long day condition. Germination was scored when the radicle emerged from the seed. Rosette area of five week old plants grown in short day conditions was determined from an image captured using a digital camera and processed using ImageJ (https://imagej.nih.gov/ij/). Rosette area in mm^2 over time was determined using the FluorImager chlorophyll fluorescence instrument (Technologica Ltd, Colchester, UK). This was estimated from the area of chlorophyll fluorescence emission using the *Fm* parameter (Baker, 2008). The fresh weight of rosettes grown in short day conditions was determined by immediately weighing freshly detached rosettes on a scale. Flowering time (in days) was measured when the emerging flower bolt was ≥ 1 cm. Inflorescence height was measured with a ruler when half the number of samples had open flowers.

For seed size, distribution and weight, *n* amount of seeds were spread on a white sheet of paper and images were taken. The images were processed with ImageJ to determine the number of seeds dispersed. The seeds were then put in an Eppendorf tube and weighed. The number and weight of the seeds were then used to calculate the 1000-seed weight. The size of each seed were also extracted from the images taken and used to determine the seed distribution and average seed size. Seed measurements were repeated 3 times.

2.2.3 Heat Stress Experiment

Heat stress treatments were carried out on all wildtype and mutant plants in a growth cabinet (Sanyo Electric Co., Ltd, Watford, UK) at 37°C for 30 minutes except otherwise stated under the same growth conditions and PPFD but with an 83% relative humidity

(RH) to eliminate dehydration effects due to changes in vapour pressure deficit (VPD). At 22°C and 60% RH, VPD was 1.058 KPa. At 37°C and 83% RH, VPD was 1.068 KPa. VPD would have been 2.512 KPa at 37°C if RH remained unchanged at 60%.

2.3 Methods

2.3.1 DNA Extraction

Fresh plant material (2cm leaves) was homogenised in a 1.5ml microcentrifuge tube containing 400 µl of extraction buffer (0.1M Tris pH 8.0, 0.05M EDTA, 1.25% w/v SDS) with a sterile plastic pestle. Samples were centrifuged at 12470 xg for 10 minutes and the supernatant transferred into a new 1.5ml microcentrifuge tube. DNA was precipitated by adding 1 volume (V) of isopropanol and samples incubated at RT for 10 minutes. To pellet the DNA, samples were centrifuged for 20 minutes at 12470 xg. The supernatant was discarded and the DNA pellet washed with 750µl cold 70% ethanol. The pellet was dried in a flow hood and resuspended in 100 µl of RO water. The samples were stored in -20°C for use in further experiments. DNA concentration was determined via spectrophotometry (section 2.3.10).

2.3.2 Polymerase Chain Reaction (PCR)

All PCR was performed in a thermal cycler for 35 cycles with varied cycle conditions depending on the size of the amplicon, G: C content of the primer and DNA polymerase enzyme used. 2 types of DNA polymerase was used throughout this study; Taq DNA Polymerase made in-house and Phusion High-fidelity DNA polymerase (Thermo Scientific # F-530S).

For PCR using in-house Taq polymerase, 20 µl reactions was prepared containing 2µl 10x Dream Taq buffer (Thermo Scientific), 0.4 µl of 10 mM dNTPs, 0.4 µl of 10µM specific forward and reverse primers, DNA within 50-150 ng, 0.2 µl Taq Polymerase and milli-Q water to make up the total volume. Cycling conditions include an initial denaturation step of 5 mins at 94°C; 35 cycles of 30 secs denaturation at 94°C, annealing temperature of 60°C (depending on the primer melting temperature) for 30 secs and extension at 72°C at 60secs per kilobase; and a final extension at 72°C for 5mins with a further cooling step at 20°C.

For PCR using Phusion High-fidelity DNA polymerase, 20 µl reactions was prepared following the manufacturer's instructions. Positive controls were added using DNA template known to produce a band with the primer combinations used for the same PCR experiment. A negative control was also added by replacing the DNA template with sterile milli-Q water.

2.3.3 Blunt End Cloning of PCR Products

Phusion high fidelity PCR products were cloned using the CloneJET PCR cloning kit (#K1232) supplied by Thermo Scientific following the manufacturer's instructions. The kit features a novel positive selection pJET 1.2/blunt end vector with a lethal gene which is disrupted by ligation of the DNA insert. 5µl of the ligation reaction was used for the transformation of chemically competent *E.coli* cells by the heat shock method described in Section 2.3.5.

2.3.4 Preparation of Chemically Competent *E.coli* Cells

Two chemically competent *Escherichia coli* (*E.coli)* cells were used in this study; DB3.1 (F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1) with the *ccdB* lethal gene and TOP10 One Shot Chemically competent cells (F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-; Invitrogen). A glycerol stock of each *E.coli* strain was streaked onto separate Luria-Bertani (LB) agar plates with antibiotic resistance and incubated overnight at 37°C. A single colony from each *E.coli* strain was used to inoculate 5ml of LB broth (0.5% (w/v) NaCl, 1.0% (w/v) Bacto™ Tryptone, 0.5% (w/v) Bacto™ Tryptone Yeast Extract, 0.1% (w/v) glucose, pH 7.0) and incubated at 37°C with shaking at 250rpm. 100µl of this bacterial culture was used to inoculate 100 ml of LB media and then incubated on a 250 rpm shaker at 37°C until the A600 O.D reading was 0.4. The cells were then placed on ice for 10mins and centrifuged at 2700 xg for 10mins at 4°C. The LB broth was discarded and the pellet washed in cold 100mM $CaCl_2$ by swirling gently and incubated on ice for 30mins. The cells were then centrifuged at 2700 xg for 10mins at 4°C. The wash process was repeated twice and then finally resuspended in cold 80% glycerol. The cells were aliquoted and stored at -80°C.

2.3.5 Transformation of Chemically Competent *E.coli* Cells

E.coli transformations was done by the heat shock method. 5µl of a ligation reaction was added to 50µl of thawed competent cells in a 1.5 microcentrifuge tube without mixing and incubated for 30 minutes on ice. The cells were heat shocked at 42°C for 45 seconds in a water bath and immediately transferred on ice for 2 minutes. 750 µl of room temperature LB broth (0.5% (w/v) NaCl, 1.0% (w/v) Bacto™ Tryptone, 0.5% (w/v) Bacto™

Tryptone Yeast Extract, 0.1% (w/v) glucose, pH 7.0) was added to the heat shocked cells and incubated on a 200 rpm shaker at 37 ^{o}C for 1 hour. After incubation, 100µl of the transformed cells was spread onto LB agar plates containing appropriate antibiotic for selection and placed in a 37 °C oven and kept overnight.

2.3.6 Colony PCR

A colony PCR is similar to the traditional PCR with the DNA template replaced by a bacterial colony. For a single colony PCR, the bacterial colony was picked from an LB agar plate using a sterile pipette tip and mixed (pipetting up and down) into a PCR master mix containing 2µl 10x Dream Taq buffer (Thermo Scientific), 0.4 µl of 10 mM dNTPs, 0.4 µl of 10µM specific forward and reverse primers, 0.2 µl Taq Polymerase and milli-Q water to make up 20 µl total volume. The PCR was carried out in a thermo cycler with the following cycling conditions. An initial denaturation step of 5 mins at 94°C; 30 cycles of 30 secs denaturation at 94°C, annealing temperature of 60°C (depending on the primer melting temperature) for 30 secs and extension at 72°C at 60secs per kilobase; and a final extension at 72°C for 5mins with a further cooling step at 20°C. The samples were then stored or used for further experiments.

2.3.7 Gel Electrophoresis and Visualisation of DNA Fragments

Nucleic acid were separated using agarose/TAE gels. 1% (w/v) agarose/TAE gels was mostly used in this study for DNA separation although 1.5% (w/v) agarose/TAE gels was used for RNA separation. 7µl GeneRuler™ DNA ladder Mix (#SM0331) ready-to-use high molecular weight size marker supplied by Thermo Scientific was used as the reference size marker in all gel types. 10% of the total sample volume of 6X DNA loading buffer supplied by ThermoFisher (#R0611; 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll

Type 400) was mixed with the DNA sample and loaded into the gel (Loading dye for RNA gels contained formaldehyde). Gels were run in 1x TAE buffer (4.84 g Tris, 1.14ml Acetic acid, 0.37g Di sodium EDTA (pH 8.0) per litre) at 100V for 30 minutes (85V for 45 minutes for RNA). For DNA and RNA visualization, SafeView (NBS Biologicals: # NBS-SV5) was added to the agarose mixture before the gel was cast or by staining the gel for 30 minutes in a solution containing ethidium bromide of approximately 0.25–1 µg/ml after the electrophoresis run. Nucleic acid bands were visualised under a blue LED trans-illuminator (Syngene, UK).

2.3.8 PCR Clean-up, Gel Extraction and Purification

PCR Clean-up means to purify the DNA in the completed PCR reaction by removing dNTPs, primers, Taq, and Mg^{2+} ion leaving a clean DNA to use for downstream experiments. First, 5 volumes of buffer PB (5M Guanidine Hydrochloride + 30% Isopropanol) was added to the PCR sample, mixed and loaded into a spin column (NBS biologicals #SD5005) with a collection tube. The loaded spin column was then centrifuged for 30-60 secs at 13447 xg. The flow through was discarded and the column washed with 750 µl of 75% ethanol and centrifuged for 30-60 secs at 13447 xg. The flow through was discarded and the column spun for a further 120 secs to remove residual ethanol in the column. To elute the DNA, the column was then put in a clean 1.5ml microcentrifuge tube with 50 µl of nuclease-free water added to the membrane and centrifuged for 60 secs at 13447 xg. The flow through was then stored in -20°C. For gel extraction and purification, the QIAquick gel extraction kit (Qiagen #28704) was used following the manufacturer's instructions.

2.3.9 Plasmid DNA Isolation

2ml of overnight *E.coli* bacterial cultures picked from LB agar plates containing appropriate antibiotics was centrifuged at 13500 rpm for 2 min in a 2ml microcentrifuge tube. The supernatant was discarded and the pellet resuspended in 250 µl of buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA and 100µg/mL RNase A). 250 µl of lysis buffer (200mM NaOH and 1% SDS) was added and mixed by inverting. 350 µl of neutralisation buffer (4.2M Guanidine Hydrochloride and 0.9M Potassium acetate pH 4.8) was also added, mixed by inversion and centrifuged at maximum speed for 10 minutes. 700 µl supernatant was then loaded into a spin column and centrifuged for 30-60 secs at 12470 x g. The flow through was discarded and the column washed with 750 µl of 75% ethanol and centrifuged for 30-60 secs at 12470 x g. The flow through was discarded and the column spun for a further 120 secs to remove residual ethanol in the column. To elute the plasmid DNA, the column was then put in a clean 1.5ml microcentrifuge tube with 30 µl of nuclease-free water added to the membrane and centrifuged for 60 secs at 12470 x g. The flow through was then stored in -20°C

2.3.10 Nucleic Acid Quantification

DNA and RNA were quantified by spectrophotometry using a NanoDrop® ND-1000 UV-Vis Spectrophotometer and the NanoDrop® V3.1 software (Labtech International Ltd, United Kingdom). The purity of the measured nucleic acid was determined by values obtained for the ratio of OD_{260}: OD_{280} and OD_{260}: OD_{230}. Quantification of nucleic acids was determined in ng/µl according to Beer-Lambert Law which states that absorbance is proportional to the concentrations of the attenuating species in the material sample or $A = \varepsilon cl$ where A is the absorbance of radiation by the sample; c is the concentration of the substance and l

is length of the path of light through the sample. Therefore the concentration of nucleic acids was quantified based on how much light was absorbed by the sample.

2.3.11 Restriction Enzyme Digest

Restriction digests was carried out using restriction enzymes in their specific restriction buffers supplied by Thermo Scientific and New England BioLabs. For both single and double restriction enzyme digest 1 µg of DNA was digested in a 10 µl reaction. Typically 5-10 units per µg of DNA was digested for 1 hour. For double digests, the enzyme activity in their respective buffers was determined for both enzymes following the manufacturer's instructions. The reaction mixture was incubated at 37°C (except otherwise stated) in a thermo cycler and the enzyme deactivated according to the manufacturer's instructions. The digested sample were stored or used for downstream applications.

2.3.12 Glycerol Stocks

Overnight cultures of *E. coli* containing plasmids were grown in 5ml LB broth with their respective antibiotic at 37°C in 200rpm shaker and glycerol stocks were prepared in a flow cabinet by adding 500 µl of culture to 500 µl of sterile 80% (v/v) glycerol. Cells were mixed briefly by pipetting and stored at -80°C.

2.3.13 DNA Sequencing and Sequence Analysis

DNA was sequenced by Source Bioscience (http://www.sourcebioscience.com) Sanger sequencing company and sequences were analysed using the Snap Gene programme. For sequence alignments MEGA 7 software was employed.

2.3.14 Preparation of Electro-competent *Agrobacterium* Cells

Three different *Agrobacterium tumefaciens* strains were used in this study (GV3101, AGL1 and LBA4404) and were all prepared in the same way. One colony was streaked on LB agar plate containing the respective antibiotic and grown at 28°C for 48 hours. A single colony was then used to inoculate 10ml of LB broth containing the same antibiotic and incubated for 48 hours at 28°C. Cells were quickly chilled on ice and spun for 15 minutes, 4°C at 3000 xg. The supernatant was decanted and cells were washed by resuspending in 10ml of cold sterile milli-Q water. The wash step was repeated three times and cells were finally resuspended in 200µl of sterile 10% glycerol. 50µl of electro-competent cells were aliquot in 1.5ml tubes and stored at -80°C.

2.3.15 *Agrobacterium* Transformation by Electroporation

Electro-competent *Agrobacterium tumefaciens* strains were thawed on ice and 100 – 150 ng of plasmid DNA was added without mixing. The cells were then transferred to pre-chilled electroporation cuvettes (Equibio, Boughton Monchelsea, Kent, UK) and electroporation was done using the EasyJecT Prima Electroporator (Equibio). A pulse of 1800V was applied to the cuvette and immediately after, 750 µl LB Broth was added to the cuvette. The cells were then transferred into a 1.5 ml microcentrifuge tube and incubated for 3 hours at 28°C. After incubation 100 µl of cells were plated onto LB agar plates containing 25 µg/ml rifampicin and the appropriate antibiotic for plasmid DNA selection. Plates were incubated for 2-3 days at 28°C depending on the *Agrobacterium* strain.

2.3.16 Agrobacterium-mediated Transformation (Floral inoculation)

Arabidopsis plant transformation was carried out based on the floral inoculation method by (Narusaka *et al.,* 2010) with modifications in plant growth conditions already outlined in Section 2.2.1.

2.3.17 Seed Sterilization

Seeds used in this study were surface sterilised under the flow hood using ethanol. A few Arabidopsis seeds were placed in a 1.5ml microcentrifuge tube and sterilized by adding 500 µl of 95% Ethanol + 0.1% Tween 20 solution. The microcentrifuge tube was then put in a rotator wheel (FINEPCR, Korea) for 3 to 5 minutes. The ethanol solution was then carefully decanted and seeds washed three times with 750 µl 70% Ethanol. Seeds were then collect using a pasture pipette and placed onto sterile filter paper to air-dry under the flow hood. After drying seeds were sown onto MS agar plates.

2.3.18 Transgenic Plant Screening and Selection

This process involved using either antibiotic resistance or herbicide resistance marker genes to select transgenic plants harbouring T-DNA of interest. In this study, only Hygromycin, Kanamycin and Basta (Glufosinate ammonium) were used in the screening/selection of positive transgenic plants. With selection using Hygromycin and Kanamycin, 20 and 30 µg/ml was added respectively to MS agar plates. Sterilized Arabidopsis seeds were spread across square MS agar plates with Hygromycin or Kanamycin, sealed, stratified for 3 days at 4°C and placed in a controlled environment according to Section 2.2.1. Screening/ selection with Basta was done by spotting a diluted Basta solution (0.04% V/V) on 3 week old Arabidopsis leaves. Basta treated plants without

the resistance showed yellowing of leaves while those with resistance remained green. These plants were then tested further to confirm presence of T-DNA of interest.

2.3.19 RNA Isolation

Total RNA was extracted from plant materials either under heat stress and no stress from 3 biological replicates. Inner leaves including the shoot apical meristem were harvested, flash frozen and grounded in liquid nitrogen to maintain samples as cold as possible to avoid degradation in a mortar and pestle. The RNA extraction was carried out using TRI-reagent® (Life technologies Inc., USA). Samples were homogenised in 1ml TRI-reagent, vortexed for 30 seconds placed on ice for 1 minute. 200µl chloroform was then added to the samples, vortexed for 30 seconds and centrifuged at 12470 x g for 15 minutes at 4°C. 400µl of the upper aqueous phase was transferred into another 1.5 ml Eppendorf tube and the nucleic acid precipitated with 1 volume isopropanol, mixed by inversion and kept at room temperature for 10 minutes. The sample was the centrifuged at 12470 x g for 15 minutes at 4°C to pellet the nucleic acid. The supernatant was decanted and the pellet washed with 1 ml 75% (v/v) ethanol. The pellet was air-dried and re-suspended in 26 µl nuclease free water. The RNA was further treated in the event of genomic DNA contamination. This was done by treating the RNA with the DNA-free™ kit (Ambion Inc.). 3µl of 10X DNase buffer and 1µl DNase1 enzyme was added to the 26µl RNA and incubated at 37°C for 30 minutes. DNase1 was inactivated by adding 1.8µl of 50mM EDTA to the sample and incubated at 65°C for 10mins. RNA was re-precipitated by adding 2 V of 100% ethanol and 0.25 V of 10 mM ammonium acetate followed by overnight incubation at -20°C. The RNA was then pelleted by centrifugation at 12470 x g for 20 minutes at 4°C. RNA pellet was washed with 1ml cold 75% ethanol, air dried at room temperature then re-

suspended in 20 µl of sterile nuclease-free water. RNA was quantified using a Nanodrop (See section 2.3.10) and quality checked on 1.5% (w/v) TAE agarose gel subjected to electrophoresis and visualised using SyberSafe (See section 2.3.7).

2.3.20 cDNA Synthesis and qRT-PCR Analysis

cDNA was synthesised from 3µg of total RNA in all samples diluted to 11.5 µl in sterile water. The reverse transcriptase (RT) reaction was carried out in 20 µl reaction volume containing 4µl 5X RT reaction buffer, 2 µl 10 mM dNTPs, 0.5 µl of reverse transcriptase enzyme, 1µl of 24µg random hexamer primers (#SO142; Thermo Fisher Scientific Inc.) and 1 µl sterile nuclease-free water. The reaction was incubated in a thermal cycler with conditions 25°C for 10 minutes, 42°C for 1 hour followed by 72°C for 10 minutes. The resulting cDNA were then stored at -20°C.

Quantitative RT-PCR analysis was conducted in a Biorad CFX96 thermal cycler (Biorad Laboratories, Inc., USA) using SYBR Green chemistry. Each reactions were carried out in 20 µl volumes following a three-step program, 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. The expression levels of gene of interest was normalised against Protein phosphatase 2A subunit A3 (*PP2AA3*) except otherwise stated. This reference gene was selected because the transcript level was more stable, amongst 2 others, after heat stress via qPCR. Relative quantification was then calculate using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3.21 Arabidopsis Crossing

35S:HSFA1b Arabidopsis mutant was crossed with *ago1-27/2-1* double knockout (hereby called *ago1/2 dko*) under a binocular dissecting microscope. Curved tip tweezers were

sterilized in 95 % ethanol, rinsed in sterile RO water and dried before using to emasculate flowers. Flower emasculation was done by removing sepals, petals and immature stamen leaving the intact pistil from an individual flower bud of the *ago1/2* DKO. Pollen of a mature anther from *35S:HSFA1b* mutant was then dabbed on the stigma of the emasculated pistil of the *ago1/2* DKO mutant to initiate fertilisation. Pollinated pistils were then covered with a plastic film to prevent drying and ingress of unwanted pollen. The cross-pollinated pistils were then allowed to mature into fully developed siliques. Siliques were harvested when dry and the hybrid seeds recovered after threshing. Hybrid seeds were grown and DNA was extracted from a part of the leaf, to confirm the success of the cross via PCR (2.3.2) in the F1 generation. The same was done to select the different mutant combinations in the F2 generation.

2.3.22 microRNA Prediction

Prediction of miRNA targets was done using a web based programme called psRNATarget (Dai *et al*., 2018). FASTA sequences of target genes was first imported from the Arabidopsis Information Resource TAIR website (www.arabidopsis.org) using their ATG codes and then loaded into the paste sequences location on the “submit target candidates” tab. All other options were set at default and the sequences were submitted. Thereafter, the results downloaded in a .txt file showing predicted miRNAs and their supposed targets.

2.3.23 Measurement of Hydrogen Peroxide

100 mg of leaf material was harvested in liquid nitrogen and ground on ice with 500 µl of 100 mM Phosphate buffer (PB; pH 7). The samples were collected in a 1.5 ml Eppendorf tube and vortexed briefly for 10 seconds. The samples were then shaken continuously at RT for 30 minutes and then centrifuged at 12,000 x g for 5 minutes. The supernatant was

transferred into another 1.5 ml Eppendorf tube, re-centrifuged for 2 minutes with the supernatant transferred to a new Eppendorf tube without cell debris and stored on ice. A master mix of 485 µl of 100 mM PB buffer, 10 µl of 20 U/ml horseradish peroxidase and 5 µl of Amplex™ UltraRed Reagent (Invitrogen™ #A36006) was also made. Hydrogen Peroxide (H_2O_2) in the samples was measured by adding 5 µl of the extract to 45 µl of the master mix, incubated at 28°C for 30 minutes and measured at an excitation of 560 nm and an emission of 590 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany). The values obtained were compared against a standard curve of known concentrations of H_2O_2 to determine the concentration of H_2O_2 in the samples.

2.3.24 Biotic Stress Experiment: *Hyaloperonospora arabidopsidis* (*Hpa*)

Two week old plants grown under short day growth conditions were used for oomycete pathogen infection using the *Hyaloperonospora arabidopsidis* strain WAC09 (*Hpa*). To revive frozen oomycete spores, *Hpa* infected plant tissue was allowed to thaw on ice and spores were resuspended in 10ml of tap water by vortexing vigorously for 30-60 seconds. The solution was filtered through a layer of Miracloth to remove residual plant material and then placed into a sprayer bottle. A flat of ~100 two-week-old Arabidopsis Ws-0 seedlings was inoculated using a pressure sprayer. Plants were placed into a tray and allowed to dry at room temperature for 1hr. In order to maintain 100% humidity, water was placed in the bottom of the tray, a moistened lid was taped firmly to the tray and then placed into a 17°C ± 0.5°C growth cabinet for 24 hours. The lid was then opened and placed on the tray leaving a small gap for 24hrs. After 24hrs water was added to the bottom of the tray and the lid taped onto the tray to maintain humidity for 72 hours in order to encourage infection. To carry out *Hpa* infection, freshly infected wild type plant

tissue was recovered, resuspended as outlined previously and an initial inoculum level of ~ 5 x 10^4 spores/ml was calculated using a haemocytometer. After the infection cycle, spore counts were done for each mutant line by harvesting 50 mg of tissue into an Eppendorf tube and adding 1 ml of water and vortexing before counting using a neubauer improved haemocytometer (Neubauer Chamber).

2.4 Generating the CRISPR/Cas9 Vector

2.4.1 CRISPR/Cas9 Gateway Cloning Plasmid Set

The CRISPR/Cas9 gateway cloning plasmid set was kindly provided by Prof Holger Puchta (Fauser *et al.,* 2014) including information on how the plasmid set was made. The plasmid set include pDE-Cas9 (Nuclease), pDE-Cas9-10A (Nickase), pEn-Chimera (sgRNA for Nuclease) and pEn-C1.1 (sgRNA for paired Nickases). All plasmid vectors were transformed into *E.coli* by heat shock according to Section 2.3.5. pDE-Cas9 and pDE-Cas9-10A was transformed into DB3.1 and selected with Spectinomycin (100 µg/ml) while pEn-Chimera and pEn-C1.1 was transformed into TOP 10 and selected with Carbenicillin (100 µg/ml). All plasmids were digested (pDE-Cas9 and pDE-Cas9-10A: HindIII; pEn-Chimera and pEn-C1.1: EcoRI) and size-checked by running digested samples on agarose/TAE gel. Refer to Appendix 1A and B for plasmid maps.

2.4.2 sgRNA Guide Selection

To select sgRNA for both nuclease and nickase constructs, a number of different web tools were employed. The process of guide selection was done for both nuclease and nickase Cas9 from *Streptococcus pyogenes.* For the nuclease construct, 2-3 guides were first selected via CRISPR-P 2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR) and CRISPR DESIGN (http://crispr.mit.edu/) based on their on-target specificity and off-target score.

The guides were then checked with Cas-OFFinder (http://www.rgenome.net/cas-offinder/) a web tool that scans for off-targets when a guide RNA sequence is queried. The best of the 3 guides was then selected, synthesised and cloned. For the nickase construct, Desktop genetics (https://www.deskgen.com/landing/cloud.html) was used to search for suitable guides around the same location of the selected nuclease guide RNA. Based on their on-target specificity, off-target score, distance between both guide RNAs, a set of guides were selected and cloned.

2.4.3 Cloning sgRNA into pEn-Chimera and pEn-C1.1

Oligos for assembling both Nuclease and paired Nickases (Appendix 6A) were annealed by adding 1 µl of each oligo (100 µM) to 48 µl double distilled water in a 0.2 ml PCR tube and incubated for 5 min at 95°C in a thermocycler without a cooling step. The tubes were then removed to cooling at RT for 20 min. 1µg of pEn-Chimera and pEn-C1.1 was linearized with BbsI restriction enzyme (New England Biolabs) according to the manufacturer's instructions with the digested plasmid purified with a column according to Section 2.3.11 and DNA concentration adjusted to 5 ng/µl. 2 µl of digested pEn-Chimera and pEn-C1.1 and 3 µl of annealed oligos including 1 µl of T4 DNA Ligase (Thermo Scientific) and 1 µl of 10X T4 DNA Ligase Buffer (NEB) was added to a sterile PCR tube, made up to 10 µl with sterile water and incubated for 1 hour at room temperature for the ligation reaction. 5 µl of the ligation reaction was transformed into TOP10 competent cells (#C404010; Thermofisher Scientific) and selected on LB agar with Carbenicillin (100 µg/ml). Positive colonies were selected via colony PCR (Section 2.3.6), plasmid was extracted (Section 2.3.9) and sequenced (Section 2.3.13). The resulting plasmid with the sgRNAs was

therefore named pEn-C1.1:NI1 and pEn-C1.1:NI2 for paired Nickases and pEn-C:NU for the Nuclease construct.

2.4.4 Subcloning the sgRNA into pDE-Cas9 and pDE-Cas9-10A

The sgRNA together with the Arabidopsis U6-26 and sgRNA scaffold in pEn-C:NU was transferred into the expression vector pDE-Cas9 using Gateway® LR Clonase® II enzyme mix (Thermo Scientific). The reaction was carried out in a 10 µl volume consisting of 100 ng of pEn-C:NU, 150 ng of pDE-Cas9, 1 µl of LR clonase II enzyme mix and the rest made up to 10 µl using TE buffer pH 8. The reaction was incubated at room temperature for 3 hours and 1 µl of 2 µg/µl Proteinase K was added to the mix and incubated at 37°C for 10 minutes in a thermal cycler. 5 µl of the reaction mixture was then used to transform TOP10 competent cells and selected on LB agar with Spectinomycin (100 µg/ml). Positive colonies were selected via colony PCR (Section 2.3.6) and the plasmid extracted (Section 2.3.9) and stored as glycerol stock. The resulting expression vector was called pDE-Cas9:NU.

Assembling the sgRNA paired Nickases took 2 steps. Firstly, the sgRNA together with the Arabidopsis U6-26 and sgRNA scaffold in pEn-C1.1:NI1 was ligated to pDE-Cas9-10A and secondly, the resulting plasmid was used as the destination vector for the subcloning of pEn-C1.1:NI2 to give the final expression vector. For the first step, 1µg of pEn-C1.1:NI1 and pDE-Cas9-10A was digested with MluI (NEB) in 50µl according to the manufacturer's instructions. The DNA fragments of pEn-C1.1:NI1 was separated on an agarose/TAE gel and the sgRNA together with the Arabidopsis U6-26 and sgRNA scaffold of pEn-C1.1:NI1 (556bp band) was cut from the gel, DNA extracted and purified according to Section 2.3.8. 20ng of the purified DNA was then ligated to the linearized and purified pDE-Cas9-10A (12ng) with 1 µl of T4 DNA Ligase (Thermo Scientific) and 1 µl of 10X T4 DNA Ligase Buffer

(NEB) in a 10µl volume for 1 hour at room temperature. 5µl was then used to transform DB3.1 competent cells and selected on LB agar with Spectinomycin (100 µg/ml). Positive colonies were selected via colony PCR (Section 2.3.6), the plasmid extracted (Section 2.3.9) and stored as glycerol stock. The resulting expression vector was called pDE-Cas9-10A:NI1. The sgRNA, Arabidopsis U6-26 and sgRNA scaffold in pEn-C1.1:NI2 was then transferred into the new expression vector pDE-Cas9-10A:NI1 using Gateway® LR Clonase® II enzyme mix (Thermo Scientific) method described above. The expression vector for the paired nickases was called pDE-Cas9-10A:NI1&2. Refer to Appendix 1C for plasmid maps of pDE-Cas9:NU and pDE-Cas9-10A:NI1&2. Both vectors were transformed into GV3101 *Agrobacterium* strain.

2.4.5 Exchanging the Basta Cassette with Hygromycin

The pDE-Cas9:NU expression vector harboured a BAR cassette as the plant selection marker which was not suitable for the transformation therefore it had to be exchanged with a Hygromycin resistance gene (Hyg^R) cassette. The Hyg^R cassette was amplified with HindIII flanked oligos from pGWB40 with Phusion DNA polymerase according to the manufacturer's instructions and separated on an agarose/TAE gel. Hyg^R DNA band was extracted and purified according to Section 2.3.8 and digested with HindIII restriction enzyme. 1µg of pDE-Cas9:NU was also digested with HindIII restriction enzyme (Thermo Scientific) according to section 2.22 which removes the BAR cassette. 1µl of FastAP thermosensitive alkaline phosphatase (Thermo Scientific #EF0651) was added to the digested sample, incubated for 10 mins at 37°C and deactivated for 20 mins at 80°C. The plasmid vector was separated from the BAR cassette by loading the digested sample on agarose/TAE gel. The plasmid was then extracted and purified. 60ng of purified vector was

ligated to the digested Hygromycin cassette (100ng) with 1 µl of T4 DNA Ligase (Thermo Scientific) and 1 µl of 10X T4 DNA Ligase Buffer (NEB) in a 10µl volume for 1 hour at room temperature. 5µl was then used to transform TOP10 competent cells and selected on LB agar with Spectinomycin (100 µg/ml). Positive colonies were selected via colony PCR (Section 2.3.6), the plasmid extracted (Section 2.3.9) and stored as glycerol stock. The resulting vector was called pDE-Cas9:NU-HYG (Appendix 1D).

2.4.6 Inserting the mCHERRY Cassette

An mCHERRY cassette was added to pDE-Cas9:NU-HYG to enhance screening of positive transformants. The mCHERRY cassette driven by the strong AT2S3 seed coat promoter (Gao *et al.,* 2016) was double digested from 1µg of pHDE-35SCas9-mCherry (Addgene #78931; Hyg^R) with SpeI & XbaI (Thermo Scientific) in 10X tango buffer and separated on an agarose/TAE gel. The mCHERRY cassette (1434bp) was then purified from the gel according to Section 2.3.8. 1µg of pDE-Cas9:NU-HYG was linearized with SpeI and ligated to the purified mCHERRY cassette with 1 µl of T4 DNA Ligase (Thermo Scientific) and 1 µl of 10X T4 DNA Ligase Buffer (NEB) in a 10µl volume for 1 hour at room temperature. 5µl was then used to transform TOP10 competent cells and selected on LB agar with Spectinomycin (100 µg/ml). Positive colonies were selected via colony PCR (Section 2.3.6), the plasmid extracted (Section 2.3.9) and stored as glycerol stock. The resulting vector was called pDE-Cas9:NU-HYG-mCherry (Appendix 1D). The vector was then transformed into GV3101 *Agrobacterium* strain.

2.4.7 Screening CRISPR/Cas9 Positive Mutation Using T7 Endonuclease I (T7EI)

A 20µl PCR was run across the junction of the predicted mutation site with suitable primers (Appendix 6A) using Phusion high fidelity polymerase. 3.5 µl of the PCR product

was removed from each sample and replaced with 1.5 µl of Buffer (NEB Buffer 2). The samples were then incubated in a thermocycler at 95°C for 10 minutes and allowed to cool at room temperature. 10 units/ µl of T7EI was then diluted to 2U/ µl with NEB buffer 2 and added to the cooled sample to a final volume of 20 µl. The samples was then incubated at 37°C for 1 hour. The incubated samples was then run in a 1% agarose gel.

2.4.8 Hygromycin Spotting

100 mg/ml of Hygromycin was diluted to 25, 50, 75 and 100 µg/ml respectively in Triton X, a non-ionic surfactant. A drop of each diluted series including Triton X was spotted on an Arabidopsis leaves. The plant was then returned to the growth chamber for 5 days before visualising the presence or absence of leaf senescence or patches of cell death around the spotted droplet. Leaves that are susceptible to the Hygromycin senesce or have cell death patches and vice versa.

2.4.9 High Resolution Melting

2.4.9.1 PCR Protocol

PCR was performed in a Biorad CFX96 thermal cycler (Biorad Laboratories, Inc., USA) using SYBR Green chemistry. Each reactions were carried out in 20 µl volumes following a three-step program, 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds, 60°C for 15 seconds, and 72°C for 12 seconds. Refer to Appendix 6A for primers used.

2.4.9.2 Melting Curve Analysis

Melting analysis was performed in the Biorad CFX96 thermal cycler immediately after PCR amplification. A 170 bp DNA fragment was analysed with primers which spanned the predicted mutation site. For high-resolution melting, the sample was heated to 95 °C for

10 seconds, cooled to 65 °C for 5 seconds and then collected fluorescence data continuously at a ramping rate of 0.1 °C/s. Melting curve data was extracted to an Excel file and the Fluorescence-vs-temperature plots of all samples were plotted against wildtype (See chapter 4, Fig 4.6a).

CHAPTER 3

HSFA1b Regulation of Developmental Genes

3.1 INTRODUCTION

To survive a constantly changing environment, it is vital for plants to respond swiftly and efficiently or face detrimental costs on growth and reproduction. Consequently, plants have evolved different mechanisms to combat an array of abiotic stresses owing to their sessile nature. The Intergovernmental Panel on Climate Change (IPCC) predicts an increase in atmospheric temperature over the coming years leading to widespread impact on natural systems including a reduction in agricultural production (IPCC, 2014). Considering the effects of climate change, it has become increasingly important to focus on plant response mechanisms to rising temperatures. Different studies have shown the negative effects of high temperatures on crop yield, growth and productivity (Prasad *et al.,* 2011; Vile *et al.,* 2012; Suzuki *et al.,* 2014). Hence, plants suffering from extreme temperatures (heat stress) experience growth retardation and risk death (Bita and Gerats, 2013). However, the susceptibility to high temperatures in plants varies with heat intensity and duration, rate of temperature increase and plant developmental stage (Larkindale *et al.,* 2005; Wahid *et al.,* 2007).

In response to elevated temperatures, cells invoke a multitude of genes coding for protective proteins and chaperones that help mitigate the stress. This is termed the heat stress response (HSR) which is largely regulated by Heat shock transcription factors (HSF; Lindquist, 1986). HSFs are also central to the control of heat stress response in eukaryotes with a conserved basic structure (Baniwal *et al.,* 2004; Scharf *et al.,* 2012). During heat stress, HSFs are activated to initiate transcription leading to the transient accumulation of Heat Shock Proteins (HSPs; Schöffl *et al.,* 1998; Wu *et al.,* 1994) which assist in stabilizing partially folded proteins and also preventing aggregation of denatured ones

(Baniwal *et al.,* 2004). HSPs are also transcriptionally regulated by multiple HSFs that possess distinct and overlapping roles (Wang *et al.,* 2004). There are 21 HSFs in Arabidopsis which are divided into 3 classes; A, B and C (Nover *et al.,* 2001). The class A1 HSF family (HSFA1) in Arabidopsis consists of 4 Heat Shock transcription factors namely; *HSFA1a, HSFA1b, HSFA1d* and *HSFA1e* (Nover *et al.,* 2001; Liu *et al.,* 2011). Their role in Arabidopsis have been extensively studied in relation to heat stress as well as other stresses. Due to the high level of redundancy between the HSFA1s, studies were focused on knocking out all 4 *HSFA1* TFs (*hsfA1a-1/hsfA1b-1/hsfA1d-1/hsfA1e-1*; also called the QK mutant; Liu *et al.,* 2011), which resulted in a more sensitive plant to different abiotic stresses (Liu *et al.,* 2011; Yoshida *et al.,* 2011).

Developmentally, the QK mutant were smaller in comparison to wildtype at seedling stage, slow growth rate, significant increase in seed abortion as well as other altered morphologies (Liu *et al.,* 2011). Despite these altered morphologies, their viability and reproduction were not hampered under unstressed conditions but significantly reduced. This was the first time members of HSFA1s were collectively associated with a developmental function. Although it was suggested that the altered morphology of the QK was due to a significant drop in HSP90 levels under normal conditions, constitutive expression of *AtHSFA2* in the QK mutants only partially restored its phenotype despite increase in HSP90 levels (Liu and Charng, 2013). Subsequently, it was reported that overexpressing *AtHSFA1b* in Arabidopsis and *Brassica napus* enhanced water productivity, seed yield and harvest index (Bechtold *et al.,* 2013). At the time of this study, *HSFA1b* is the only member of the HSFA1s with a developmental effect when overexpressed.

AtHSFA1b, previously called HSF3 (Prändl *et al.,* 1998), is one of the most well studied HSFs in Arabidopsis (Albihlal *et al.,* 2018; Bechtold *et al.,* 2013; Panchuk *et al.,* 2002; Panikulangara *et al.,* 2004; Prändl *et al.,* 1998). It is constitutively expressed under non-stress conditions and is required for the regulation of early phase heat stress response (Lohmann *et al.,* 2004; Busch *et al.,* 2005). Overexpression of *HSFA1b* leads to the accumulation of HSPs under normal conditions which increases the plants tolerance to heat (Prändl *et al.,* 1998; Lohmann *et al.,* 2004) and knockout mutants do not show any marked difference in heat stress and development compared to wildtype (Lohmann *et al.,* 2004; Liu *et al.,* 2011; Persad, 2015). In a recent study, transcriptomic changes of *AtHSFA1b* overexpressing plants (*35S:HSFA1b*) compared to wild type plants were analysed under two conditions; no stress (NS) and heat stress (HS; Albihlal *et al.,* 2018). Results revealed that the expression profile of plants overexpressing *HSFA1b* under no stress partially resembles plants under a mild heat stress regime. This result gave a strong indication that overexpression of *HSFA1b* results in plants constitutively expressing stress responsive genes under no stress conditions which is consistent with previous studies (Prändl *et al*., 1998; Bechtold *et al*., 2013). Interestingly, Gene Ontology (GO) analysis in both plants under heat stress compared to no stress revealed enrichment for various biological processes including developmental processes. The biological functions of some of the differentially expressed genes in *35S:HSFA1b* were also enriched for growth and development associated functions. In the same study, a genome-wide mapping of the *HSFA1b* binding site under no stress and a short 30 min duration of heat stress using Chromatin Immunoprecipitation (ChIP)- combined with next generation sequencing (SEQ) was conducted and intersected with the RNA-SEQ data. Results revealed that *HSFA1b* was bound to 27 TFs which in parallel regulated a cohort of developmental as well as stress

associated genes under heat stress (Albihlal *et al.,* 2018). It also revealed an element of post-transcriptional control via long-non coding RNA when *HSFA1b* bound 817 long non-coding natural antisense transcript (lncNAT) genes and 79 long intergenic non-coding RNA (lincRNA) genes under both NS and/or HS conditions with 51% differentially expressed under HS and/or in *35S:HSFA1b* over-expressing plants (Albihlal *et al.,* 2018). This provided another level of organisation in which *HSFA1b* could regulate the expression of both stress and developmental genes during stress response.

This chapter aims to show experimentally how *HSFA1b* influences growth and development during normal and heat stress conditions, confirm its developmental targets and also shed light on its regulation of developmental genes.

3.2 RESULTS

3.2.1 Effect of Heat Stress on Arabidopsis Growth

To determine the effect of heat stress on Arabidopsis growth, 2 sets of wildtype (Col-0) seedlings were grown side by side. The first set was grown without stress (wildtype; Set 1) while the second set was grown in the same growth conditions as the first but heat stressed for 2 hours at 37°C on day 7 (wildtype + HS Day7; Set 2). Plant growth of both sets were identical until day 7 when heat stress was applied to set 2 (Fig 3.1A, black arrow). Thereafter, plants in set 2 appeared smaller relative to set 1 on subsequent days. A significant size difference was observed from day 10, 3 days after the initial stress ($p < 0.05$; Student *t*-Test; Fig 3.1A). Despite the size difference due to the application of heat stress on day 7 to set 2, it maintained exponential growth increase suggesting a slowing down rather than cessation of growth (Fig 3.1A).

AtHSFA1b overexpressing plants are suggested to be in an intermediate stage between normal and heat stress state based on its transcriptomic profile (Albihlal *et al.,* 2018). This would imply in theory, based on the results from wildtype + HS (Day 7), that *35S:HSFA1b* would be smaller in size compared to wildtype. Both wildtype and *35S:HSFA1b* were grown on soil according to section 2.2.1 while measuring daily the rosette area of the seedlings. A size difference could be seen from the onset of measuring both wildtype and *35S:HSFA1b* plants (Fig 3.1B). The overexpressing plant was significantly smaller in size than its wildtype with an average percentage decrease of 17%. This gives credence to the transcriptomic profile of *35S:HSFA1b*, at least to an extent, is in a mild state of heat stress due to its smaller size compared to wildtype under normal conditions confirming previous results.

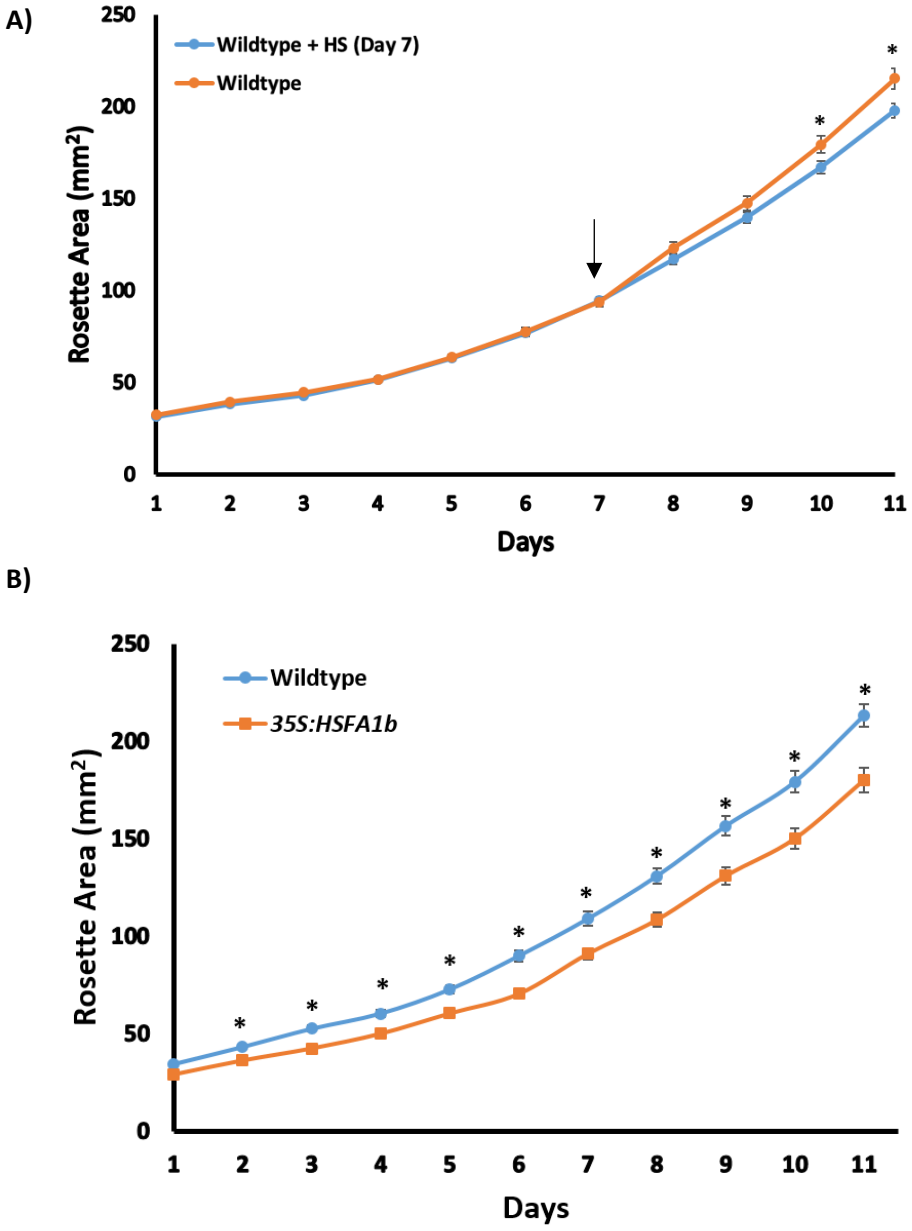


Figure 3. 1. Impact of heat stress on Arabidopsis seedlings growth. Rosette area of Arabidopsis seedlings grown in short day condition on soil-filled square plastic pots (3.5cmx3.5cmx5.5cm) for 2 weeks and measured for 11 days at 22°C. **A)** 2 sets of Col-0 plants grown without stress (**Wildtype**) and heat stress on day 7 for 2 hours (**Wildtype + HS (Day 7**). Black arrow indicates day of heat stress on set 2. **B)** Wildtype vs *35S:HSFA1b* under no stress. '*' means p value < 0.05, Student t-Test; HS; Heat stress; n = 50.

3.2.2 Phenotypic Effect of *35S:HSFA1b* on Growth and Development

Confirming the reduced size of *35S:HSFA1b* seedlings compared to wildtype (Col-0) led to an interrogation of observable phenotypic differences of *35S:HSFA1b* seedlings through to adult stage. The rosette area of 5-week-old soil-grown plants were measured and similar to the seedling stage, *35S:HSFA1b* plants were significantly smaller compared to wildtype with a 17% decrease in size (Fig 3.2A). The reduced growth phenotype observed in *35S:HSFA1b* plants also suggested that *HSFA1b* might regulate cell expansion. Flowering time was also affected in the transgenic plants compared to the wildtype. This was measured when the emerging flower bolt was ≥ 1 cm. In long day conditions (16h light/ 8h dark), *35S:HSFA1b* plants bolted and flowered earlier than wildtype (Fig 3.2B, C). The flowering time difference between *35S:HSFA1b* and wildtype was estimated to 8 hours since increase in cell size occurs during the dark period. In the same vein, the inflorescence height was measured when half the number of samples had open flowers. Once more, the inflorescence height of *35S:HSFA1b* was almost 3 times longer than that of the wildtype (Fig 3.2B). Note that while inflorescence height was measured in plants grown in SD for 4 weeks and moved to LD for an additional 2 weeks when half the number of plants had open flowers, flowering time was measured in plants exclusively grown in LD. In addition to these phenotypic changes observed in *35S:HSFA1b* plants, improvement in seed yield and harvest index compared to wildtype have already been reported (Bechtold *et al.,* 2013).

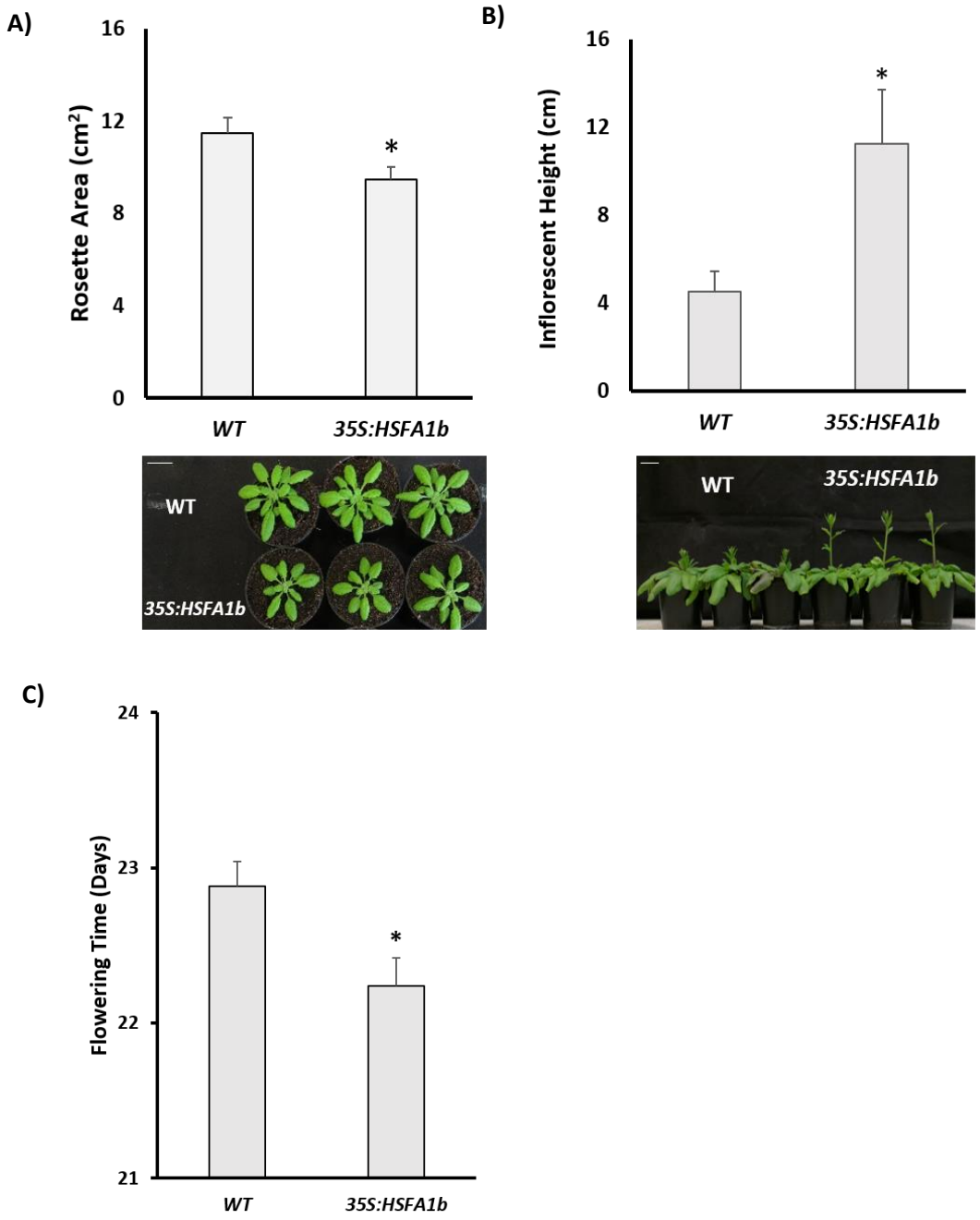


Figure 3. 2. Phenotypic changes of *35S:HSFA1b* plants compared to wildtype (Col-0). **A)** Rosette area of 5-week-old plants grown in short day conditions (n=12). **B)** Inflorescence height between WT and *35S:HSFA1b*. Plants were grown for 4 weeks on soil in SD and transferred to LD to induce flowering at 22°C. Inflorescence was measured when half the number of plants (n = 20) had open flowers. **C)** Flowering time (i.e. number of days flowering bolt ≥ 1cm) of plants growing in long day conditions at 22°C (n=50). '*' means *p value* < 0.05; Student *t*-Test. Scale bar = 2m

3.2.3 *35S:HSFA1b* Plants are under a Mild Heat Stress State

Results from genome wide transcriptomics profile of unstressed *35S:HSFA1b* plants suggested that the plants are in an intermediate state between unstressed wildtype and heat stressed wildtype plants as heat responsive genes were significantly upregulated compared to the wildtype, Col-0 (Albihlal *et al.,* 2018). The upregulation of several HSPs in unstressed *35S:HSFA1b* plants compared to unstressed and heat stressed wildtype can be summarized with the heat map below (Fig 3.3A). Majority of the HSPs were upregulated compared to the unstressed wildtype albeit lower than that of the heat stress. A couple of the upregulated HSPs were confirmed quantitatively by RT-PCR (Fig 3.3B). The transcript levels of 2 HSPs known to interact with HSFA1s, HSP90.1 and HSP70, were higher by more than 10-fold in *35S:HSFA1b* than the unstressed wildtype and 10-fold lower than the heat stress wildtype (Fig 3.3B). MBF1c, also an HSR gene (Suzuki *et al.,* 2008), had a similar pattern of expression in *35S:HSFA1b* compared with the unstressed and stressed wildtype with more than 10-fold difference respectively (Fig 3.3B). To confirm that the upregulation of HSR genes is due to *HSFA1b* overexpression, the changes in transcript level of these genes were observed in an *hsfa1a/hsfa1b* double knockout mutant compared to its wildtype, Ws-0 (Fig 3.3C). *HSP90.1*, *MBF1c*, *HSFA2* and *HSFB2b* all had an overall delayed response highlighted by the decrease in transcript level in the *hsfa1a/hsfa1b* mutant compared to the Ws-0 during heat stress. Except for *HSP90.1,* the other HSR genes were delayed by about 30 mins in the *hsfa1a/hsfa1b* mutant compared to the Ws-0 (Fig 3.3C). These results, in combination, agrees with the transcriptomic profile of *35S:HSFA1b* plants which suggests that overexpressing *HSFA1b* in Arabidopsis*,* puts the plant in an intermediate state between unstressed and heat stress wildtype plants.

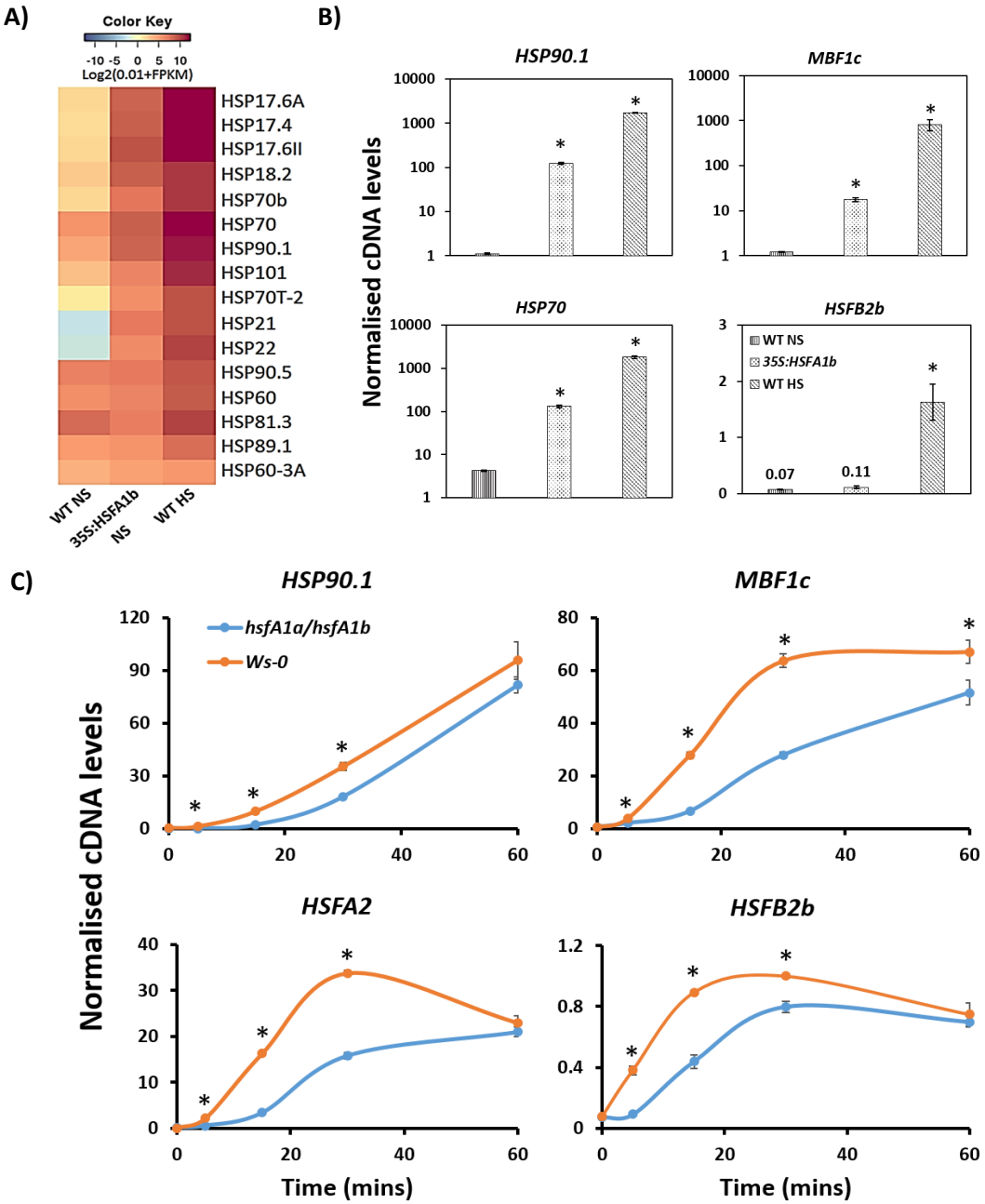


Figure 3. 3. Expression of Heat stress responsive (HSR) genes in different conditions. A) Heat map showing the upregulation of different HSPs in *35S:HSFA1b* no stress compared to wildtype (Col-0) in both Heat and no stress condition. **B)** Normalised cDNA levels (Log10) of some HSR genes in *35S:HSFA1b* plants compared to wildtype in both conditions. Plants were grown for 5 weeks on soil in SD condition at 22°C (NS) before applying heat stress (37°C) for 30 mins (HS). HS; Heat stress, NS; No stress. '*' means significant difference; $p < 0.05$; ANOVA with Tukey's HSD post hoc test. **C)** Time series of some heat responsive genes in *hsfa1a/hsfa1b* plants compared to Ws-0. Plants were grown for 5 weeks on soil in SD condition at 22°C before applying heat stress (37°C) at different time points. Time points 0 (No stress), 5, 15, 30 and 60 mins. 3 biological replicates were used to determine transcript level in B & C, normalised against *PP2AA3*. Heat map from Albihlal *et al.,* (2018).

3.2.4 *HSFA1b* Directly Regulates Developmental Genes

Albihlal *et al.,* (2018) reported that intersecting the ChIP-SEQ binding and transcriptomics data of *HSFA1b* overexpressing plants revealed 27 transcription factors which were bound and directly regulated by *HSFA1b*, significantly altering their expression under no stress conditions (Fig 3.4A). Eight of these TFs have been experimentally confirmed with published papers (See Albihlal *et al.,* (2018) for reference list) to have developmental roles (Fig 3.4A, Asterisk TFs.). To test the hypothesis that these TFs are directly regulated by *HSFA1b* and/or other members of HSFA1*,* the transcript expression of 7 TFs (randomly selected from Fig 3.4A) were investigated in the *HSFA1* quadruple knockout (QK) mutant due to the functional redundancy of *HSFA1s* (Fig3.4B). Since the QK was in the Col-0 and Ws-0 background, both ecotypes were used as controls (Fig 3.4C). The 7 TFs which include *BASIC LEUCINE ZIPPER28 (BZIP28), REVIELLE7 (RVE7), REVIELLE1 (RVE1), SALT INDUCED ZINC FINGER1 (SZF1), HEAT SHOCK TRANSCRIPTION FACTOR B2b (HSFB2a), G-BOX BINDING FACTOR3 (GBF3)* and *HEAT SHOCK TRANSCRIPTION FACTOR B2a (HSFB2a)* were tested in both NS and HS conditions due to their response to heat. The expression of all the TFs tested in both conditions were perturbed in the QK mutant compared to the controls. However, significant difference could only be attained by some TFs depending on which of the controls was compared with the QK.

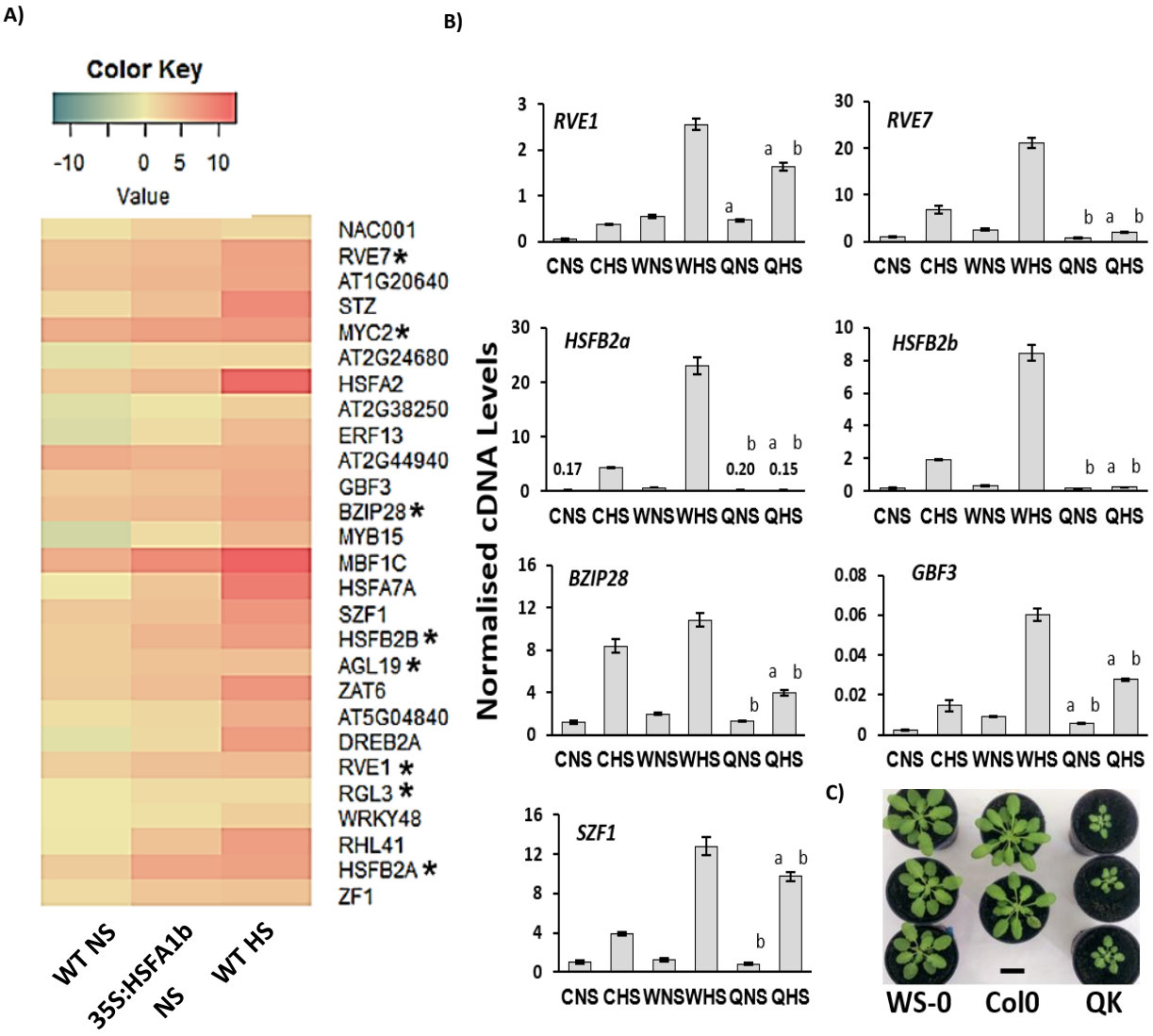


Figure 3. 4. Transcription factors directly regulated by *HSA1b*. A) Heat map showing the expression of 27 *HSFA1b*-directly regulated TFs in *35S:HSFA1b* compared to WT under both heat stress (HS) and no stress (NS). '*' signifies developmental TFs (Albihlal *et al.,* 2018). **B)** Expression of selected TFs in QK and its wildtypes (Col-0 & Ws-0) under both heat and no stress. Plants were grown for 5 weeks on soil in SD condition at 22°C (NS) before applying heat stress (37°C) for 30 mins (HS). 3 biological replicates were used to determine transcript level, normalised against *PP2AA3*. CNS, Col-0 No stress; CHS, Col-0 Heat stress; WNS, Ws-0 No stress; WHS, Ws-0 Heat stress; QNS, QK No stress; QHS, QK Heat stress. 'a' implies significant difference when QK expression is compared with Col-0; 'b' implies significant difference when QK expression is compared with Ws-0. *P value* < 0.05; ANOVA with Tukey's post hoc test. **C)** Image showing 5-week old QK plants compared to its wildtype genotypes. Scale bar = 2cm.

For example, *RVE1* was significantly altered in QK when compared with Col-0 but not Ws-0 under NS. *RVE7*, *SZF1, BZIP28, HSFB2a, HSFB2b* were significantly altered in QK when compared with Ws-0 but not Col-0 under NS. Under HS however, all TFs tested were significantly altered in the QK compared with both controls. Of the TFs tested, all except *SZF1* and *GBF3* have developmental roles in Arabidopsis (Kuno *et al.,* 2003; Kolmos *et al.,* 2014; Wunderlich *et al.,* 2014; Xu *et al.,* 2015; Zhang *et al.,* 2017). These results highlight the possibilities of *HSFA1b* function by binding and regulating not only stress related TFs but also developmentally associated TFs during NS but especially during HS. The use of the QK in this instance however, introduces an element of complication as the transcript levels differ when comparing the QK with the controls. For example, the transcript level of *GBF3* in the QK is upregulated when compared with Col-0 but downregulated when compared with Ws-0 under both conditions. The same applies to *RVE1, RVE7, BZIP28 and SZF1* in the QK compared to both controls under HS. To reiterate, at the time of this experiment, the QK, was the only mutant available without the functional *HSFA1*s albeit in 2 parental genotypes.

3.2.5 *35S:HSFA1b* Indirectly Regulates Of Some Developmental Genes via Long Non-Coding RNA

Albihlal *et al.,* (2018) suggested that the indirect regulation of developmental genes could be achieved via *HSFA1b's* interaction with Natural Antisense Transcripts (NAT), a type of long non-coding RNA. They identified 413 NATs transcripts that were differentially expressed in WT HS plants and/or *35S:HSFA1b* NS plants which corresponded to 357

putative sense targets. Of these sense targets, 39 were transcription factors of which most were enriched for stress associated functions. However, 8 were developmentally-associated. They include, *CYCLING DOF FACTOR1* (*CDF1*), *LIGHT REGULATED ZINC FINGER PROTEIN 1 (LZF1), MYB DOMAIN CONTAINING PROTEIN 16 (MYB16), HOMOBOX PROTEIN 2 (HB2)* and *ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 56, 78, 83 (ANAC056, ANAC078, ANAC083)*. The sense and NAT antisense transcript expression of these TFs were observed in WT and QK genotypes in both NS and HS conditions via qRT-PCR (Fig 3.5). Results showed that both the sense and the NAT antisense transcript expression are perturbed in the QK mutant compared to at least one of its parental genotypes. This suggests that both sense and NAT transcripts in some way interact with *HSFA1b* and/or other members of the *HSFA1* clade either directly or indirectly.

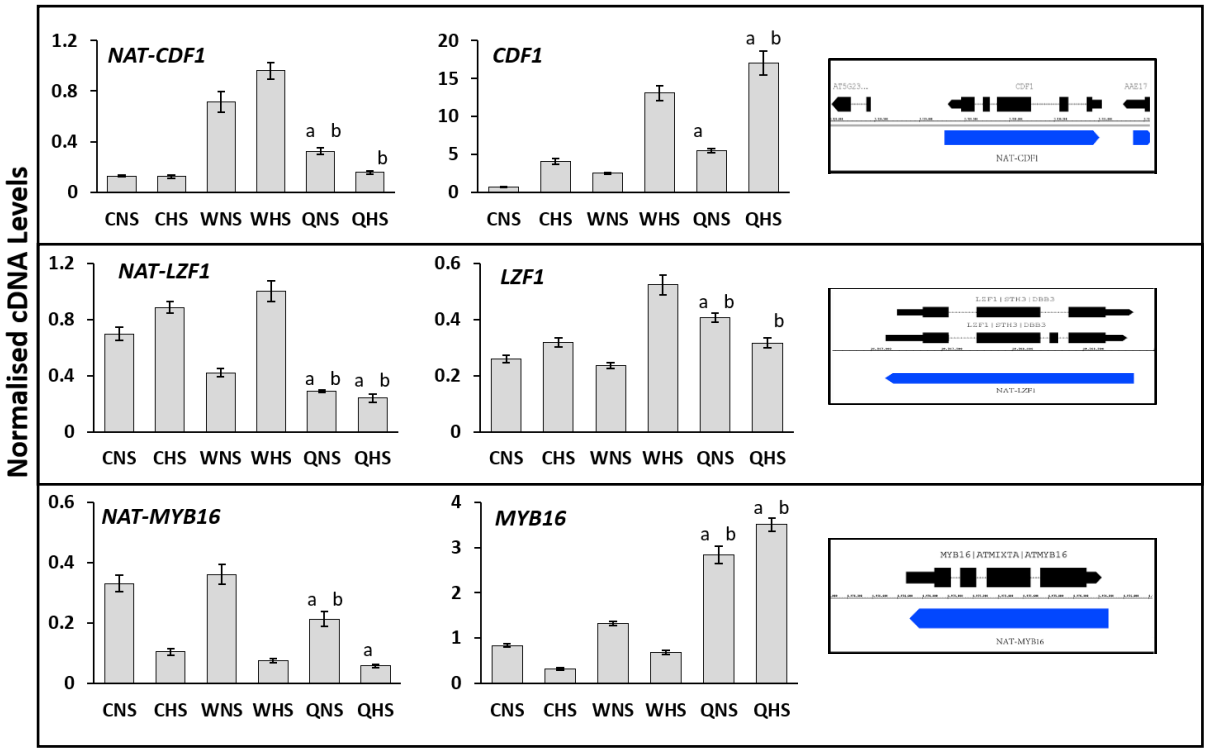


Figure 3. 5. Normalised transcript level of selected lncNATs and their sense targets. Expression of selected lncNATs in QK and its wildtypes (Col-0 & Ws-0) under both heat and no stress. Plants were grown for 5 weeks on soil in SD condition at 22°C (NS) before applying heat stress (37°C) for 30 mins (HS). 3 biological replicates were used to determine transcript levels, normalised against *PP2AA3.* Images (far right) represent the corresponding transcription factor (Black) and its corresponding lncNAT (Blue). Pointed tip signifies direction of transcription. Numbers represents position on the genome. CNS, Col-0 No stress; CHS, Col-0 Heat stress; WNS, Ws-0 No stress; WHS, Ws-0 Heat stress; QNS, QK No stress; QHS, QK Heat stress. 'a' implies significant difference when QK expression is compared with Col-0; 'b' implies significant difference when QK expression is compared with Ws-0. *P value* < 0.05, ANOVA with Tukey's post hoc test.

3.3 DISCUSSION

3.3.1 *35S:HSFA1b* NS Plants Phenocopy WT Plants under a Mild Heat Stress Regime Due To Over-Accumulating Heat Stress Genes

Temperature is an important factor in the life cycle of a plant especially that of growth and development. Extreme temperatures can cause drastic developmental changes in the growing plant significantly affecting crop yield. While high temperatures are detrimental to plant development, a mild change in ambient temperature can be tolerated and, in some cases, beneficial. Wildtype plants under a slight warming regime are characterised by early flowering and improved seed yield at the expense of leaf biomass. Accordingly, Jin *et al.,* (2011a) examined the effects of warming on Arabidopsis leaves and found that a slight elevation in growth temperature from 23°C to 25.5°C increased the total weight of seeds by 37%, albeit with a reduced life span of 7%. However, a 5°C growth increase from 23°C to 27°C decreased seed weight, biomass and life span by 14%, 37%, 21% respectively (Jin *et al.,* 2011a). In addition, it was also reported that a mild increase in growth temperature from 23°C to 25°C and/or 27°C induces early flowering in Arabidopsis accessions (Balasubramanian *et al.,* 2006). These pleotropic developmental effects of wildtype plants in mild temperature changes are comparable to that of *35S:HSFA1b* plants under unstressed conditions characterised by early flowering and reduced rosette area (Fig 3.2) validating observations made by Bechtold *et al.,* (2013) including an improved seed yield and harvest index.

In contrast, high temperatures (>10°C) can be detrimental to plant growth and development which often leads to a reduction in plant size amongst other developmental effects. Compared to unstressed WT plants, *35S:HSFA1b* plants have a reduced rosette

area which could be as a result of constitutively upregulating heat stress responsive genes under normal conditions (Fig 3.3B; 3.2B). These do not seem to impede the photosynthetic performance as *35S:HSFA1b* plants do not show deleterious effects of plants growing under extreme heat stress regime (Bechtold *et al*., 2013). Therefore, it can be concluded that *35S:HSFA1b* plants are in a state equivalent to a mild heat stress regime. While the mild heat stress state of *35S:HSFA1b* plants promotes and influences growth and development, it also puts the plant in a poised state, priming the plant for an imminent heat stress. This is as a result of the increase in heat inducible HSF and HSP transcripts, a consequence of *HSFA1b* overexpression, however lower than those of heat stressed WT (Fig 3.3A). The upregulation of heat inducible stress genes is also observed when HSF or heat stress inducible genes themselves are overexpressed. For example, overexpression of *AtHSFA2* resulted in the upregulation of heat stress genes (Ogawa *et al.,* 2007). Although, *HSFA2* knockout mutants do not show any obvious morphological abnormalities, its constitutive overexpression led to growth retardation as well as abiotic stress tolerance (Ogawa *et al.,* 2007). The growth retardation phenotype of *AtHSFA2* overexpressing plants can explain that observed by *35S:HSFA1b* plants.

These reports highlight the possibility of an interconnected pathway between stress response and development involving HSFA1s and HSPs. It thus suggests that the temperature sensing pathway(s) can intersect with that of growth and development with HSFs at the hub (Fig 3.8). This in turn supports the narrative that Arabidopsis plants over-expressing *HSFA1b* is in an intermediate state between heat stressed and non-stressed wildtype plants, constitutively expressing heat stress and other abiotic stress responsive genes without adversely affecting growth and development (Bechtold *et al.,* 2013; Albihlal *et al.,* 2018).

3.3.2 *HSFA1b* Modulates Plant Growth and Development by Regulating Different Developmental TFs

Animals have between 1-4 HSFs that are not only involved in stress response but also in development, cell proliferation and differentiation (Pirkkala *et al.,* 2001). The multiplicity of HSFs in plants mean that several different HSFs can have collective as well as distinct roles in the regulation of abiotic stress and development (Liu and Charng, 2013). The interaction between the master *HSFA1*s, other HSFs and HSPs appears to be crucial for normal development and abiotic stress tolerance in maintaining plant homeostasis. The overexpression of one of the master HSFs, *HSFA1b*, led to minor pleiotropic developmental effects indicating its role in development (Fig 3.2) although, *hsfa1b* mutants have no obvious morphological differences due to its redundancy with other *HSFA1* members (Liu *et al.,* 2011). Its developmental function was also highlighted when *AtHSFA1b* was overexpressed in *Brassica napus* leading to an improved seed yield and harvest index (Bechtold *et al.,* 2013). It showed that Arabidopsis HSFs could still perform its function in other related species (Li *et al.,* 2003). Furthermore, in addition to its role in stress tolerance, *HSFA1b* directly regulates the expression of 27 TFs especially during heat stress (Fig 3.4). These TFs not only respond to heat stress but are also involved in other developmental aspects. These TFs are also important because they can in turn regulate the expression of a wide variety of target genes, thus providing a link to indirect regulation. For example*, MYC2*, a basic-helix-loop-helix (bHLH) TF, is the regulator of most aspects of the jasmonate signalling pathway in Arabidopsis which plays an essential role in defence and development (Wasternack, 2007). A notable function of *MYC2* involves mediating the crosstalk between JA and other phytohormones such as abscisic acid (ABA), salicylic acid (SA), gibberellins (GAs), and auxin (IAA) as it acts as both an activator and

repressor of target gene expression (Kazan and Manners, 2013). It is also involved in JA-regulated plant development, lateral and adventitious root formation, oxidative stress tolerance and phytochrome signalling amongst other functions (Kazan and Manners, 2013). In order to successfully be involved in a myriad of functions, it is suggested that *MYC2* regulates directly or indirectly other TFs, which in turn regulate downstream JA-response genes involved in diverse JA-dependent plant processes (Dombrecht *et al.,* 2007). Interestingly, it has been shown that *MYC2* interacts with all members of the DELLA proteins (Hong *et al.,* 2012) of which one of their genes, *RGL3* (Wild *et al.,* 2012), is also directly regulated by *HSFA1b* (Albihlal *et al.,* 2018). Furthermore, at least 21 genes from the differentially expressed indirectly regulated set of genes are positively or negatively regulated by *MYC2* during JA signalling (Fig 3.6; Dombrecht *et al.,* 2007). These include (*AT1G66100, DEHYDROASCORBATE REDUCTASE (DHAR1), 5'ADENYLYLPHOSPHOSULFATE REDUCTASE 2 (APR2), ANTHOCYANIN 5-AROMATIC ACYLTRANSFERASE 1 (AACT1), APS REDUCTASE 3 (APR3), ARGININE DECARBOXYLASE 2 (ADC2), AVRRPT2-INDUCED GENE 2 (AIG2), BASIC CHITINASE (HCHIB), CYTOCHROME P450, FAMILY 71, SUBFAMILY A, ETHYLENE RESPONSE FACTOR 1 (ERF1), ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6 (ERF6), GLUTAMATE DECARBOXYLASE (GAD), JACALIN-RELATED LECTIN 23 (JAL23), OSMOTIN 34 (OSM34), PEROXIDASE 71 (PRX71), PHYTOALEXIN DEFICIENT 3 (PAD3), REDUCED SUGAR RESPONSE 4 (RSR4), SENESCENCE-ASSOCIATED GENE 13 (SAG13), UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE (UF3GT), VEGETATIVE STORAGE PROTEIN 1 (VSP1), VITAMIN C DEFECTIVE 2 (VTC2)* and *PLANT DEFENSIN 1.2 (PDF1.2a). PDF1.2a/b* was shown to be significantly upregulated in *hsfB1/B2b* double knockout plants (Kumar *et al.,* 2009). The negative effect of *HSFB1/B2b* on *PDF1.2a/b* expression was suggested to be indirect. Interestingly, *HSFB2b* and *MYC2* (which regulates

PDF1.2a/b) are directly regulated by *HSFA1b* which means *HSFA1b* could interact with other TFs (*HSFB2b* and/or *MYC2* in this case) to regulate a large variety of genes involved with either stress defence or development. Similar to MYC2, 29 genes which are differentially expressed in *35S:HSFA1b* plants are direct targets of *BZIP28* (Albihlal *et al.,* 2018). *BZIP28* is one of 2 TFs important for thermotolerance during vegetative and reproductive stages of plant growth to maintain fertility during heat stress (Zhang *et al.,* 2017). These examples highlighting a method by which *HSFA1b* could indirectly regulate 281 developmental genes (Appendix 2A) as well as other non-developmental genes.

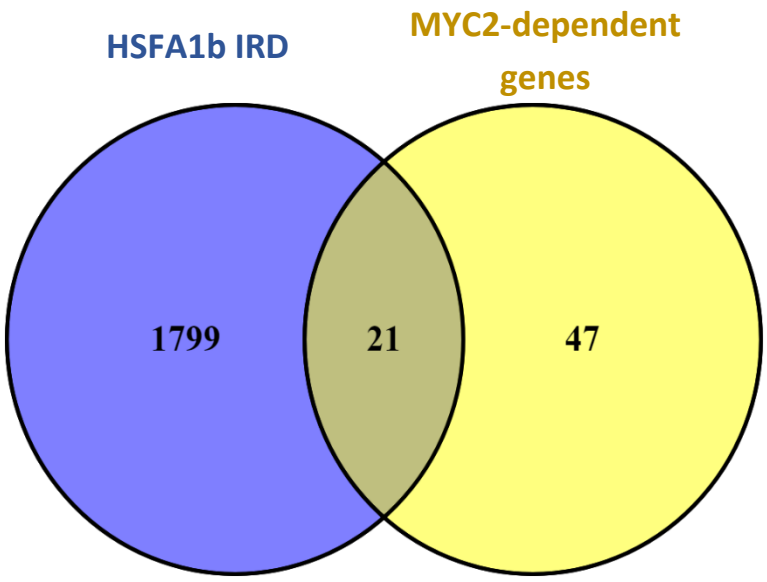


Figure 3. 6. Venn diagram showing overlap between *HSFA1b* indirectly regulated developmental/non-developmental genes and *MYC2*-dependent genes. Overlap between *HSFA1b* indirectly regulated developmental genes (IRD) and MYC2-dependent genes. IRD, Indirectly regulated developmental; IR, Indirectly regulated non-developmental. *p* = 5e-07*;* Hypergeometric test.

HSFA1b along with *HSFA1a* can also indirectly regulate genes especially during heat shock response by forming heteromeric complexes with other HSFs (Schramm *et al.,* 2006). It was shown that tomato *HSFB1* (ortholog of *AtHSFB1*) can interact with *HSFA1a* and/or *HSFA2* and also with other general TFs specifying a co-activator function (Baniwal *et al.,* 2004; Röth *et al.,* 2017). In a similar way, it was also proposed that the *AtHSFB1/B2b* could interact with other HSFs and/or other general TFs in exerting a HSF-dependent repressive role on target gene expression. The interaction was shown to be involved in the repression of 2 diseases resistance genes despite being devoid of a perfect HSE (Kumar *et al.,* 2009). Consequently, the indirectly regulated developmental (IRD) genes were scanned for the presence of either a perfect or imperfect HSE. Of the 281 IRD genes, 40 possessed at least one imperfect HSE (one base mismatch) located within approximately 1Kb upstream of the initiation codon while 2 had a perfect HSE. Amongst the set of 42 IRD genes with perfect/imperfect HSEs, the transcript levels of 33 were upregulated (Appendix 2B) while 9 were downregulated in the *35S:HSFA1b* compared to wildtype under no stress (Fig 3.7). Some of the non-developmental indirectly regulated genes also possessed both perfect and imperfect HSEs also possibly extending their regulation to the interaction between A- and B-class HSF. Of the 1540 non-developmental genes identified, 196 possessed at least one imperfect HSE (one base mismatch) located within approximately 1Kb upstream of the initiation codon while 32 had a perfect HSE. These indicate a possibility that the interactions between A- and B-class HSFs and/or other general TFs could mediate the DE of *HSFA1b* indirect developmental targets although more experiments are needed to confirm their involvement.

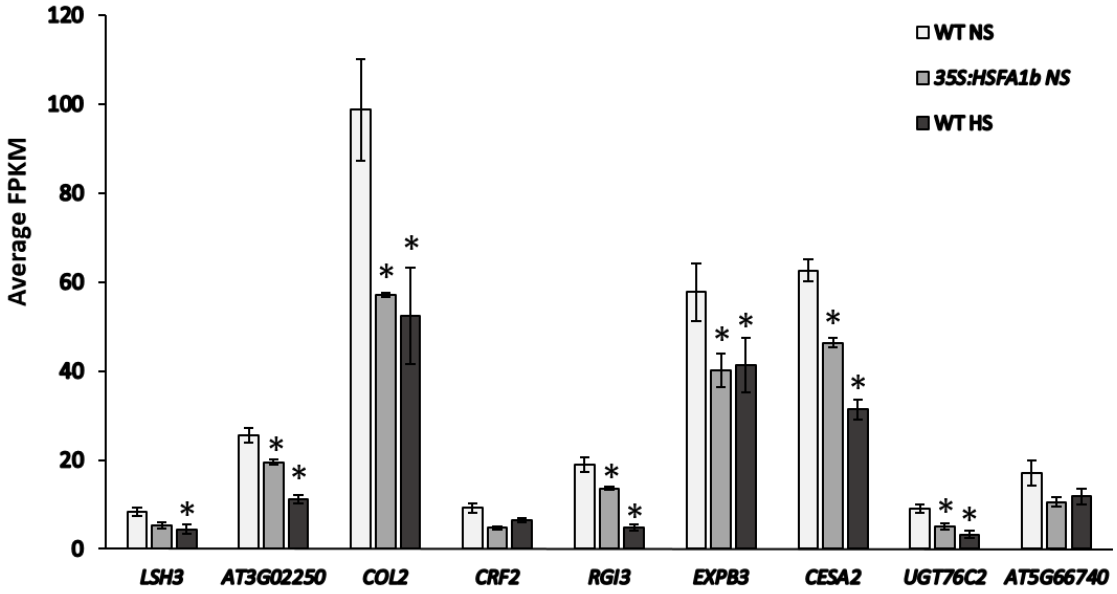


Figure 3. 7. *HSFA1b* indirectly regulates expression of developmental genes. Downregulated IRD genes with imperfect HSE in *35:HSFA1b* plants in no stress compared with Wildtype in both conditions (Heat and no stress). Data from RNA-seq from Albhilal *et al.,* 2018. *p <0.05*; ANOVA with Tukey's HSD post hoc test. IRD, indirectly regulated developmental genes.

Furthermore, *HSFA1b* could indirectly regulate developmental genes by long non-coding RNAs (lncRNAs). These RNAs are typically longer than 200 nts and are termed antisense lncRNAs (lncNATs), intronic lncRNAs or intergenic lncRNAs (lincRNAs) due to their genomic position against coding genes (Rinn and Chang, 2012; Bonasio and Shiekhattar, 2014). With the advent of high resolution genome wide screening of plant transcriptomics in recent times, lncRNAs have been shown to be active in plants, regulating aspects of stress and development (Zhang and Chen, 2013) although it is estimated that 70% of annotated mRNAs are associated with lncNATs in Arabidopsis (Wang *et al.,* 2014a). In respect to heat

stress, several lncRNAs have been characterised in different plant species. Di *et al.,* (2014) identified and confirmed via qRT-PCR 15 lncRNAs that were differentially expressed under heat stress in Arabidopsis. In wheat, 125 putative long non-protein-coding RNAs were characterized during powdery mildew infection and heat stress (Xin *et al.,* 2011), 34 specifically expressed lncRNA were identified during heat stress which in turn regulated some heat responsive target genes in Chinese cabbage. (Song *et al.,* 2016b). These show a clear relationship between heat stress and expression of lncRNAs. Developmentally, the "ying-yang" interaction between *HSFB2a* and its antisense lncNAT, *asHSFB2a*, has been described; both of which are heat inducible and involved in gametophyte development in Arabidopsis (Wunderlich *et al.,* 2014). Interestingly, *HSFA1b* binds to both *HSFB2a* and its antisense lncRNA *asHSFB2a* (Albhilal *et al*, 2018). Transcript of *asHSFB2a* is only expressed after heat stress and is dependent on the activity of *HSFA1a/HSFA1b*. This goes to show that *HSFA1b* can indirectly regulate developmental genes by directly altering the expression of their lncNAT during heat stress (Fig 3.5) which adds another layer of complexity in the regulation of genes during response to high temperatures. Chromatin modification, DNA methylation and post-transcriptional histone modifications leading to transcription repression have also been shown to be regulated by lncRNAs (Ariel *et al.,* 2015). Therefore, *HSFA1b* can exert indirect regulation on growth, development and stress defence by directly regulating lncRNAs affecting epigenetic markers leading to changes gene expression.

3.3.3 HSFA1s Could Mediate the Switch between Abiotic Stress and Development by Their Interaction with *HSFA2*, *HSFB1/B2b* and HSPs

The role of HSFs and HSPs have been extensively studied in relation to abiotic stress response, particularly during heat stress in conferring plant thermotolerance. The response to mild temperature increase results in the promotion of growth while the opposite occurs during heat shock (Mittler *et al.,* 2012). At the heart of both responses (ambient temperature increase and heat shock) involve the interaction between *HSFA1*s and HSPs which suggests a possibility for both to be involved in growth processes at normal temperatures in the absence of stress or during a mild temperature increase (Mittler *et al.,* 2012). For example, temperature-dependent seedling growth has been shown to be regulated by the stabilization of the auxin co-receptor F-box protein, *TIR1*, by *HSP90* (Wang *et al.,* 2016b). Inhibition of HSP90 activity with geldanamycin led a range of auxin-mediated growth processes at higher and normal temperatures including inhibiting root and hypocotyl growth due to the rapid degradation of *TIR1*. In the same vein, knockout of 4 *HSFA1*s (QK) brought about a significant drop in *HSP90* transcript level resulting in a hypersensitive plant with severe developmental defects under normal conditions (Liu *et al.,* 2011). Reduction in *HSP90* transcripts have also been previously linked with altered plant morphologies (Queitsch *et al.,* 2002; Sangster *et al.,* 2007). Aside from *HSP90*, overexpression and/or knockout of different homologues of *HSP70* led to thermotolerance and developmental effects respectively including plant lethality. Knockout mutants of cytosolic *HSP70-15* have been characterised with severe growth retardation including increased virus resistance and accelerated wilting due to constantly open stomata (Jungkunz *et al.,* 2011). Heat treatment of *HSP70-15* deficient mutants also resulted in a higher mortality rate compared to wild type plants, however, these mutants

accumulated various HSPs including *HSFA2* whose expression have both been shown to be regulated by HSFA1s (Liu *et al.,* 2011).

The interaction between heat stress response and development is not only associated with the A class HSFs. Heat inducible HSFs and small HSPs have also been shown to be regulated by B class HSFs which are known transcription repressors. Ikeda *et al.,* (2011) showed that knocking out 2 members of the HSF B class (*HSFB1* and *HSFB2b)* lead to a plant overexpressing heat inducible HSFs (*HSFA2* and *HSFA7a*) under normal conditions, a characteristic also shared with *35S:HSFA1b* plants. It is estimated that under normal conditions these 2 members of the B class repress the activity of heat inducible HSFs and HSPs of which they (B class) are in turn regulated by *HSFA1*s on the onset of stress. The *hsfb1/b2b* knockout also possessed longer hypocotyls while maintaining a higher thermotolerance compared to wildtype genotypes (Ikeda *et al.,* 2011). Overexpressing *HSFB1* resulted in plants with smaller rosettes compared to wildtypes (Ikeda *et al.,* 2011), while knockout of *HSFB2a* affected gametophyte development leading to embryo lethality (Wunderlich *et al.,* 2014). Finally, while *HSFB4* has been shown to be involved in root development in Arabidopsis (Begum *et al.,* 2013), *HSFB3* has no known function. Since all members of the B Class are heat inducible, there exist a possibility for them being regulated by HSFA1s during heat stress, case in point *HSFB1* and -*B2b* (Fig 3.4b). However, only *HSFB1* and -*B2b* have repressive functions (Zhu *et al.,* 2012).

Accordingly, *HSFA2* also exerts its function in growth and stress response. It was shown that in the absence of HSFA1s, *HSFA2* can to some extent promote growth and development (Liu and Charng, 2013). It suggests that the function of *HSFA2*, when overexpressed can occupy the same loci as the HSFA1s and perform their function in

relation to development. The possibility of *HSFA2* promoting growth in the absence of HSFA1s was strengthened when one splice variant (*AtHSFA2.1*) complemented the deleted yeast HSF1 strain and promoted growth while the other splice variant did not (*AtHSFA2.2*; Albihlal *et al.,* 2018, unpublished data). In contrast, overexpressing *HSFA2* resulted in a dwarf phenotype compared to the wildtype and its severity was dose dependent (Ogawa *et al.,* 2007). Interestingly, *HSFB1* and *HSFB2a* were also up-regulated in the *AtHSFA2* overexpressing plant. Ogawa *et al.,* (2007) also showed that overexpressing *AtHSFA2* enhanced callus growth in Arabidopsis root explant compared to wildtype but no obvious difference in *hsfA2* mutant suggesting that other HSFs might play a role in cell proliferation. This inadvertently implicates the *HSFA1*s as they are known to regulate the expression of *HSFA2* during stress and the knockout mutant (QK) resulting in a dwarf plant. Although the inhibition of several heat response pathways were observed in *hsfA2* plants, deficiency in *HSFA2* did not negatively affect heat tolerance in plants (Kataoka *et al.,* 2017).

In summary, these reports suggest a tight interaction between B class HSFs (*HSFB1* and *HSFB2*b) and Heat inducible HSFs and HSPs (HSFA2, HSP90, HSP70 and sHSPs) in regulating growth during heat stress strictly coordinated by the master regulators HSFA1s (Fig 3.8A). A simplified working model (Fig 3.8B) is proposed to summarize the points discussed above. AtHSFA1s are required for normal growth and development at normal growth temperatures (22-23°C) prioritising growth over stress defence with the B class HSFs (*HSFB1/B2b*) repressing the activity of heat inducible HSFs and HSPs (Fig 3.8B (i)); but when stress is encountered which is often gradually in nature, *HSFB1/B2b* is deregulated while the *HSFA1*s primes the plant for an imminent stress by upregulating heat inducible HSFs and HSPs to an optimum level although maintaining growth during the increase in

ambient temperature (25-28°C; Fig 3.8B (ii)). At this point growth slows but still maintained as resources divert from growth to stress response. If the temperature returns to normal levels (22-23°C), *HSFB1/B2b* orchestrates the attenuation of HSPs and *HSFA2*, supressing stress response and returning the balance to favour growth responses. However, if the heat stress persists and/or reaches threshold of heat shock (>37°C), heat stress inducible HSFs take over from *HSFA1*s in establishing thermotolerance. As a result, *HSFA2* surpasses its threshold becoming the dominant HSF thereby affecting development by slowing down growth while HSPs reach their maximum level carrying out their chaperone duties to cope with the damaging effects of the stress (Fig 3.8B (iii)). When the temperature becomes extreme and/or persistent, important proteins begin to break down triggering cell death responses. The fine-tuning interaction between HSFs and HSPs is important due to the gradual and dynamic temperature changes in nature helping the plant maintain a balance between growth and stress response.

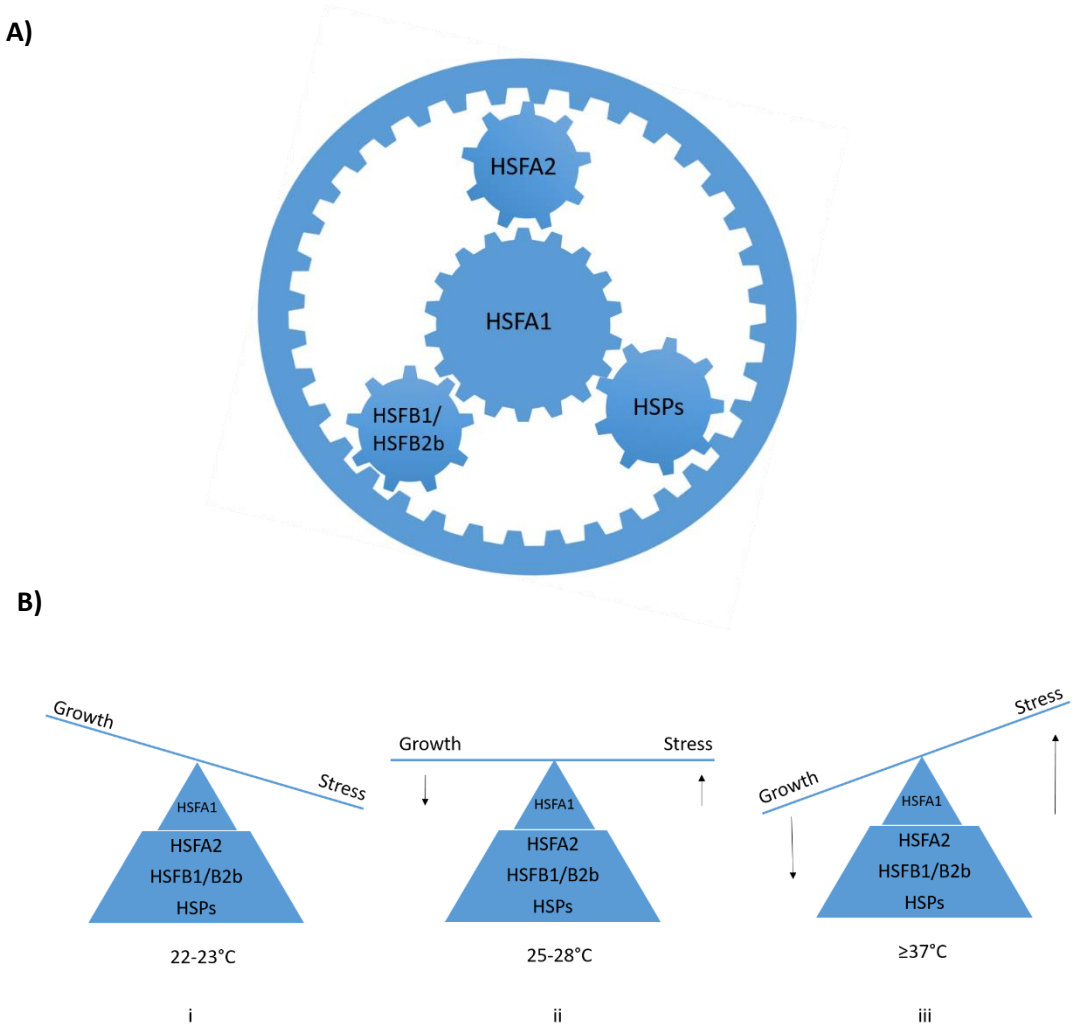


Figure 3. 8. Working model of the interaction between HSFs and HSPs in the regulation of growth (i.e. rosette area) and stress defence. **A)** Illustration of the tight-knit interaction between HSFA1s, *HSFB1/B2b* and *HSFA2.* The proper functioning of *HSFA2*, *HSFB1/B2b* and HSPs depend on the master regulators HSFA1s. The big gear encompassing the TFs represents physiological aspects of growth and stress defence. **B)** Diagram showing the physiological aspects prioritised during normal growth conditions (i), mild increase in temperature (ii) and heat stress (iii).

CHAPTER 4

Generating an *Arabidopsis* Clade A1 Quadruple Knockout Mutant

4.1 INTRODUCTION

The Arabidopsis clade A1 HSFs, which functions as the master regulators of heat shock response, have been shown to be involved in other abiotic stress response along with growth and development when all four highly redundant homologous transcription factors *HSFA1*a/b/d/e were knocked out (Liu *et al.,* 2011; Yoshida *et al.,* 2011). The resulting mutant dubbed QK was generated when *hsfa1*a/b double knockout in a Ws-0 background was crossed with *hsfa1d/e* double knockout in a Col-0 background (Liu *et al.,* 2011). This at the time was interesting as it was the first time all four members of the clade were attributed with something other than stress response; a developmental role, however it was only observed when all four TFs were knocked out and not in any of the triple knockout mutants (Liu *et al.,* 2011). Despite the discovery, Liu and colleagues did not follow-up on how these TFs affected development or their effect on developmental genes genome-wide. This could be due in part that the QK is a hybrid of 2 naturally occurring accessions; Col-0 and Ws-0. It is important to note there exists a natural variation between both accessions leading to differences in morphological and physiological traits making them very different (Koornneef *et al.,* 2004). For example, Ws-0 differs in size and flowering time compared to other accessions (Passardi *et al.,* 2007). Due to these natural occurring morphological differences in Arabidopsis accessions, a cross between ecotypes can lead to developmental differences between the hybrid progeny making it difficult and challenging to score developmental changes or effects of functional genes on developmental traits because of a random mix of Col-0 and Ws-0 alleles in different QK hybrid individuals. For example, in the research by Persad (2015), a number of flowering time characteristics was observed in a number of *HSFA1* single overexpressing mutants, single, double, triple and quadruple triple knockout mutants

compared to their wildtypes (Ws-0 and Col-0). The single overexpressing mutants (*35S:HSFA1a, -b, -d, -e)* as well as single and double knockout mutants in the Col-0 background (*b-, d-, e-, bd-, be-, de KO*) had between 8-10 leaves while *abKO* in Ws-0 background had 4-6 leaves. On the other hand, the triple knockout mutants (*abdKO, bdeKO, adeKO, abeKO*) which is a Col-0/Ws-0 hybrid had between 6-8 leaves except for *bdeKO* which had between 4-6 leaves. This was also reflected in the number of days till bolting, days to first flower open and days to anthesis (Fig. 4.1). Therefore, it can be argued that the triple knockout mutants have acquired the early flowering trait from its Ws-0 parent compared to their double knockout counterparts in a Col-0 background or it could be the knockoff effect of the functional *HSFA1* gene knockout. Whichever the case, it is undeniable that the QK presents a myriad of problems due to inherent differences between its parents therefore it was imperative a QK in a single background be generated to avoid ambiguous and convoluted conclusions.

Different methods of gene editing have been used to knockout functional genes in Arabidopsis. Transcription Activator-like Effector Nucleases (TALENS; Boch *et al.,* 2009; Moscou and Bogdanove, 2009) and Zinc Finger Nucleases (ZFN; Kim, Cha and Chandrasegaran, 1996) have been used successfully in generating double stranded breaks in desired regions but in recent times the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) + CRISPR associated protein 9 (Cas9) or CRISPR/Cas9 has been shown to not only be successful but also easy and inexpensive to use (Cong *et al.,* 2013; Jinek *et al.,* 2013; Fauser *et al.,* 2014). The CRISPR/Cas9 system was employed to knockout the last functioning member of the clade A1, *HSFA1*a, from a homozygous *bdeKO* mutant in Col-0 background isolated from crossing individual knockouts (*bKO, dKO*

and *eKO*) due to unavailability of a T-DNA insertional mutant after a long and extensive search (Persad, 2015).

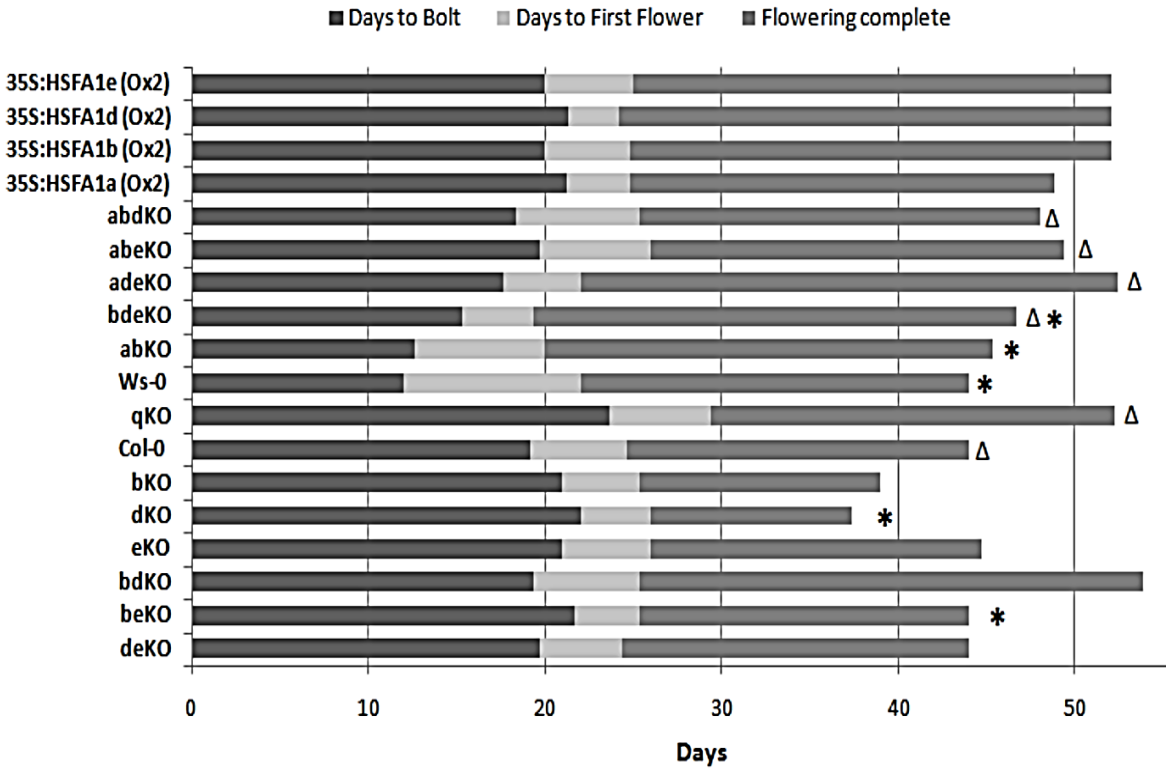


Figure 4. 1. Comparison of flowering characteristics between different Clade A1 mutants and their wildtypes. Flowering time determined by the number of days till bolting, days to first flower open and days to anthesis (flowering complete) of different *HSFA1* knock outs and overexpressors. Single and double knockouts: *bKO, dKO*, *eKO, bdKO, beKO* and *deKO*, and all over-expressing lines are compared to Col-0; *abKO* is compared to Ws-0. Triple and quadruple knockouts: *bdeKO, adeKO, abeKO* and *abdKO* are compared to both Col-0 and Ws-0. Plants were grown in long day conditions. Significant difference is indicated by Δ when compared to Ws-0; * when compared to Col-0. $p < 0.05$; Two-sample Kolmogorov-Smirnov Test) Figure from Persad (2015).

4.1.1 CRISPR/Cas9 System

CRISPR/Cas9 is a type II RNA-guided genome editing tool adapted from the bacteria immune system from *Streptococcus pyogenes* (Jinek *et al.,* 2012; Cong *et al.,* 2013). Cas9 is a DNA-endonuclease that cleaves DNA at specific sites causing double stranded breaks which invokes the cells error-prone non-homologous end-joining repair mechanism thereby introducing mutations (Jinek *et al.,* 2013) The Cas9 gene was also found to be closely associated with short homologous repeats interspaced with non-homologous spacer sequences (CRISPR sequences; Jansen *et al.,* 2002). Cas9 has two conserved nuclease domains, HNH and RuvC-like nuclease domain with each cleaving one strand of the DNA creating a double stranded break (Jinek *et al.,* 2012). The Cas9 nuclease is directed to its target site by two non-coding RNAs; CRISPR-RNA (crRNA) and trans-activating crRNA (tracrRNA; Fig 4.2a; Jinek *et al.,* 2012). The crRNA directs target specificity by binding directly to a 20 nucleotide (nt) sequence on the DNA called the protospacer by complimentary base paring. This protospacer/target recognition is dependent on the presence of a protospacer adjacent motif (PAM), a 3nt sequence with a NGG consensus present on the DNA strand downstream of the cleavage site (Jinek *et al.,* 2012). In order to cause a double stranded break (DSB), crRNA and tracrRNA form a heteroduplex which in turn forms a complex with Cas9. The crRNA base pairs with the complimentary strand of the DNA with its 3' end adjacent to the PAM sequence (Figure 4.2A; Jinek *et al.,* 2012). The Cas9 nuclease domains then cleaves both DNA strands 3 bp upstream of the PAM recognition site causing a DSB. The DSB is repaired by the cells error-prone non-homologous end joining (NHEJ) creating indels. Furthermore, both crRNA and tracrRNA were fused together with a GAAA tetraloop to form a single chimeric RNA called single guide RNA (sgRNA) which was shown to be effective in driving the Cas9 nuclease to its

target site and cause a double stranded break making the CRISPR/Cas9 system simpler (Fig 4.2B; Jinek *et al.,* 2012). This system from *Streptococcus pyogenes* is typical for all type II CRISPR systems to ward off invasion of foreign DNA (Barrangou *et al.,* 2007; Wiedenheft *et al.,* 2012) and was easily adapted for genome editing. This system has been used for gene knockouts, gene knock-ins, gene tagging, gene activation, gene repression in different cell types and organisms (Dicarlo *et al.,* 2013; Gratz *et al.,* 2013; Wang *et al.,* 2013). Unlike other gene editing tools, Zinc finger Nuclease and TAL effector nuclease (Gaj *et al.,* 2013), CRISPR/Cas9 is easy to design and engineer, allows multiplexing, easy to deliver into cells and has a wide range of target sites with the PAM motif (Cong *et al.,* 2013).

To achieve a DSB in plant genome editing, a plant codon optimised version of Cas9 from S. pyogenes have been used (Miao *et al.,* 2013; Jiang *et al.,* 2013a) as well as a human-codon optimised version (Nekrasov *et al.,* 2013; Xie and Yang, 2013). To ensure delivery of Cas9 into the nuclei, the cas9 protein is flanked by a nuclear localisation signal (NLS; Jiang *et al.,* 2013a; Xie and Yang, 2013). The first 20 sequences from the 5' end of the sgRNA confers DNA target specificity (Hsu *et al.,* 2013). Therefore, to target any site on the DNA, only the first 20 sequences of the sgRNA needs to be modified as long as it is adjacent to the PAM sequence 'NGG' on the DNA strand with the consensus sequence (N)20NGG (Jinek *et al.,* 2013). sgRNA is expressed using the plant RNA polymerase III promoters U6 or U3 depending on the starting nucleotide. If a "G" starts the sgRNA, U6 promoter is used and U3 promoter if an "A" is the starting nucleotide (Nekrasov *et al.,* 2013; Xie and Yang, 2013).

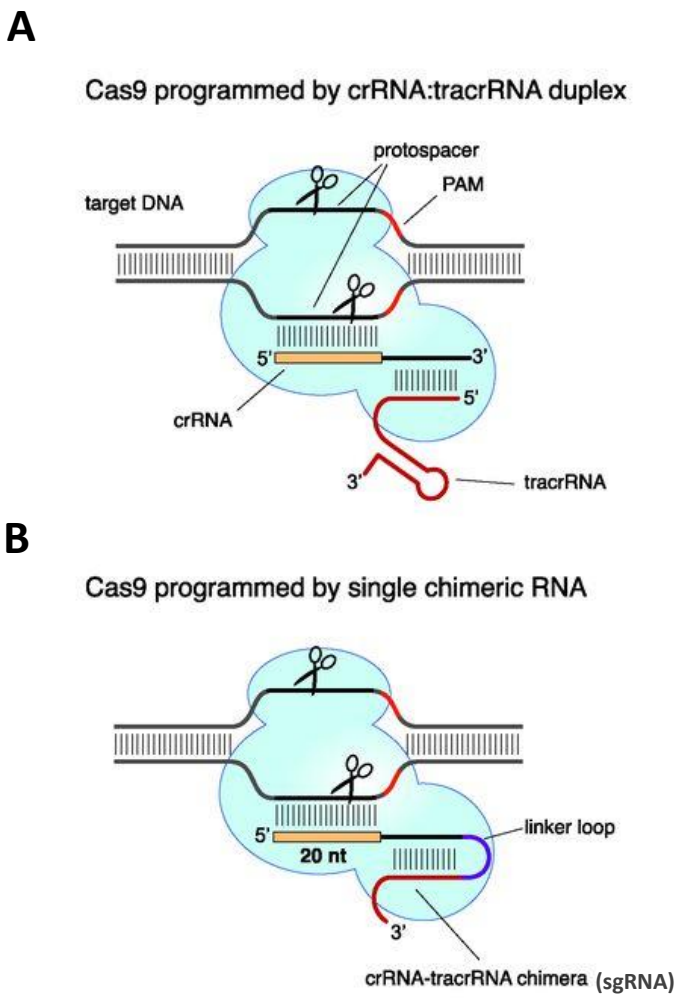


Figure 4. 2. RNA programmed Cas9 typical of type II CRISPR/Cas System. A) Cas9 nuclease is guided to its target site by the heteroduplex formation with crRNA:tracrRNA duplex. The first 20nt of the crRNA binds to the target sequence/protospacer via complimentary base pairing adjacent to its PAM motif. A complete match of the crRNA and protospacer triggers the nuclease domains to cut the DNA 3 bp upstream of the PAM motif. **B)** Cas9 is guided by the chimeric fusion of crRNA:tracrRNA duplex by the GAAA tetraloop (linker loop; Jinek *et al.,* 2012)

A major challenge of using this genome editing tool is the issue of specificity and off-target activity (Hsu *et al.,* 2013; Pattanayak *et al.,* 2013). Specificity of the sgRNA is determined by the complementarity of the guide sequence and the target DNA. A perfect match between the last 8-12 bases of the guide sequence and its complimentary DNA directly before the PAM is essential (Hsu *et al.,* 2013; Pattanayak *et al.,* 2013). While off target activity has been reported in animal systems it is suggested to be less prevalent or rare in plant system as the mutational efficiency of Cas9 is much lower in plant cells (Peterson *et al.,* 2016; Tang *et al.,* 2018). Nevertheless the use of bioinformatics tools is required to search for unique target sites within the plant genome to choose the best sgRNAs (also called guide RNAs) and to eliminate off-target activity. In addition, Cas9 nuclease was also engineered to work as a nickase which produces single-stranded breaks. This was achieved by introducing point mutations in one of the two nuclease domains disabling its nuclease activity (Jinek *et al.,* 2012). This is also useful in avoiding unwanted mutagenesis caused by off-target effects.

4.1.2 Design and Selection of Guide RNA

One of the crucial steps in achieving successful gene editing using CRISPR/Cas9 is the design of the guide RNA. The mutagenic efficiency of the CRISPR/Cas9 system to a large extent depends on the guide RNA (Hsu *et al.,* 2013; Fu *et al.,* 2014). Different guide RNAs produce different editing results as some perform better than others. Due to this slight drawback a set of “rules”/ guidelines have been suggested to get the best editing results. These rules were elucidated with animal cells but can be applied to plant cells with similar results. Firstly, sgRNA that are guanine-rich and adenine-depleted are more stable and more mutagenic (Doench *et al.,* 2014; Moreno-Mateos *et al.,* 2015). It was suggested that guanine-rich sgRNA, especially at the 5’ end, results in the formation of G-quadruplexes

which may protect the guide RNA from 5' exonuclease degradation, stabilizing the sgRNA-CRISPR/Cas9 complex to its target site increasing its activity (Moreno-Mateos *et al.,* 2015). Secondly, G/C content of sgRNAs between 40-80% have been shown to give the best results because they influence binding efficiency (Gagnon *et al.,* 2014; Montague *et al.,* 2014; Wang *et al.,* 2014b). Thirdly, the position of the nucleotides within the guide RNA play a significant effect on its activity. Having a G in position 20 or immediately upstream of the PAM sequence greatly increases its activity (Doench *et al.,* 2014; Gagnon *et al.,* 2014; Moreno-Mateos *et al.,* 2015). Furthermore, position 3 in the guide RNA favours a G (Moreno-Mateos *et al.,* 2015) and greatly disfavours a C (Doench *et al.,* 2014; Kuscu *et al.,* 2014; Moreno-Mateos *et al.,* 2015). Many bioinformatics tools use these guidelines in predicting the best guides for optimum cas9 activity. Different bioinformatics tools were used in generating the sgRNA used in this study for both Cas9 variants (Nuclease and Nickase). Refer to section 2.4.2.

4.2 RESULTS

4.2.1 Using CRISPR/Cas9 to Generate a Quadruple KO Mutant in a Single Background

So far using the QK mutant has been challenging posing different biological problems most especially during data analysis and reproducibility (Fig 3.4; 3.5). To circumvent this problem a QK mutant in a single background was paramount. Therefore, the CRISPR/Cas9 genome editing tool was employed to knockout *HSFA1*a from an existing triple knockout *hsfa1*b/d/e (TKO) in a Col-0 background. To achieve this, 3 sgRNAs were cloned into 2 variant CRISPR/Cas9 expression plasmids and used to transform both TKO and wildtype Col-0 via agrobacterium mediated transformation of Arabidopsis flower buds described in Chapter 2.3.16. Both constructs were designed to target the first exon of the *HSFA1*a gene for effective knockout of the TF (Fig 4.3A).

The first CRISPR/Cas9 construct pDE-Cas9:NU-HYG was made with Hygromycin as the plant selection because the TKO possesses other plant antibiotic resistance genes namely Sulfadiazine, Basta™ and Kanamycin. The second CRISPR/Cas9 construct, pDE-Cas9-10A:NI1&2, was made to address a possible off-target effect that could be observed using pDE-Cas9:NU-HYG. Kanamycin plant selection was adopted using this construct as it turned out that the kanamycin resistance in the triple knockout had been silenced (Appendix 3A). The TKO and Col-0 plants were transformed with both constructs and screened by growing on MS media with their respective selection; Kanamycin and Hygromycin (Chapter 2.3.16 – 2.1.18). Positive transformants were transferred to soil and screened for the presence of mutations at the target region. Genomic DNA was extracted and PCR products (1280bp) were amplified using primers that spans across the predicted mutation site. Distance of predicted mutation site to the left and right primer was 400bp and 880bp respectively (Fig 4.3a). The PCR products was briefly denatured, annealed and

treated with T7 Endonuclease I enzyme (T7EI) which cleaves DNA heteroduplexes caused by indel mutations as described in Chapter 2.4.7. The resulting samples were run in 1.5% agarose gel detecting positive bands of both predicted sizes; 880bp and 400bp including the wildtype band of 1280bp (Fig 4.3B). Lines 4 and 6 from the TKO mutant was selected from the Nickase and Nuclease construct respectively following T7EI treatment (Fig 4.3C) for further screening in the following generation for plants which have lost the CRISPR/Cas9 T-DNA. This is to ensure that the mutation is stable and inheritable in the following generations. Due to the difficulty in extracting and purifying the smaller of the two DNA band, new primers that spans across the predicted mutation site were designed to give a PCR product of 811bp. Treating the PCR product with T7 Endonuclease I enzyme gave a single band of ~411bp which was easier to extract and purify. The purified band was then cloned into pJET2.1 blunt cloning vector and sequenced. The resulting sequence confirmed that both pDE-Cas9:NU-HYG and pDE-Cas9-10A:NI1&2 was successful in creating mutations at the target region.

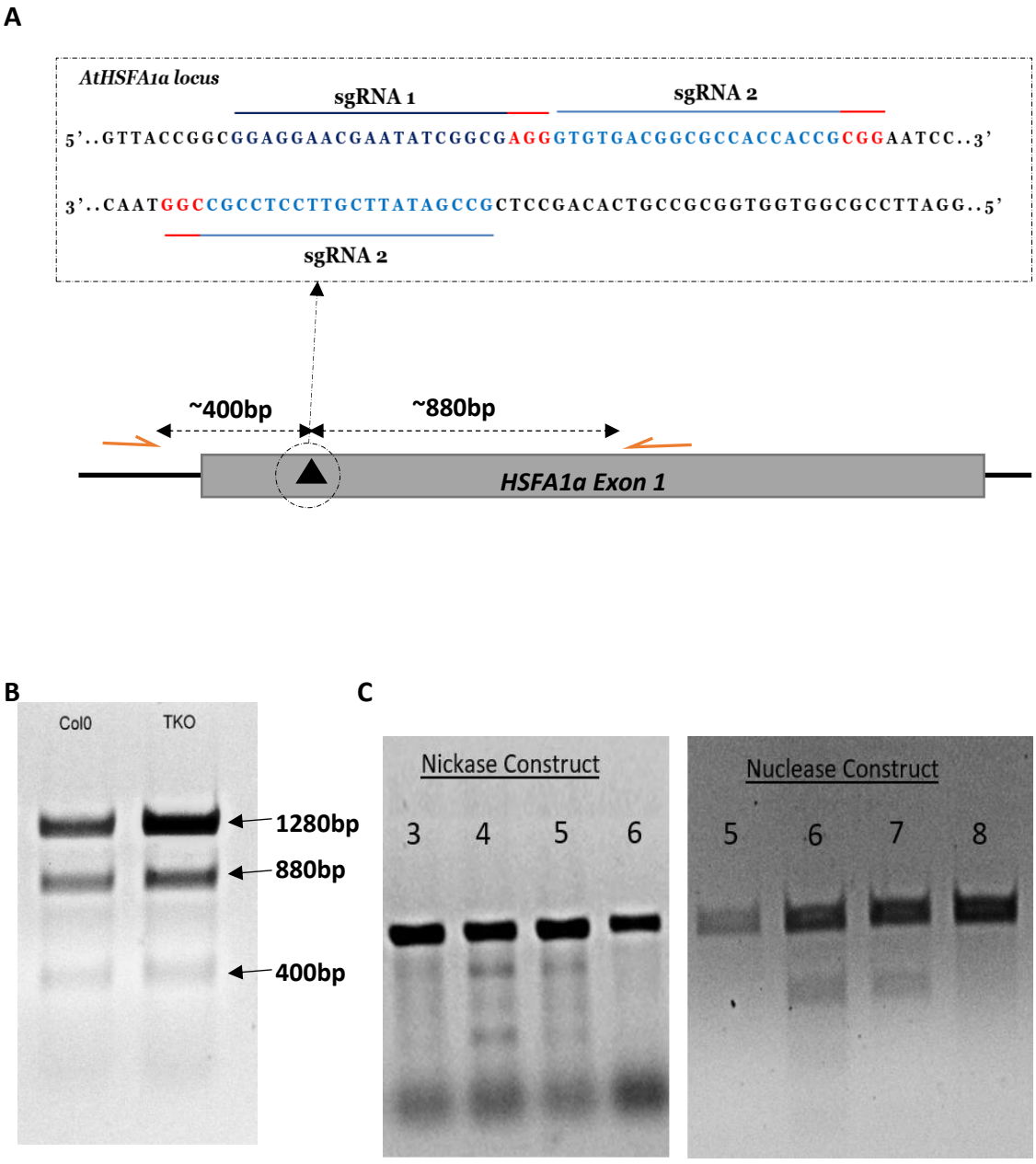


Figure 4. 3. CRISPR/Cas9-mediated gene editing of At*HSFA1*a. A) Schematic diagram of *HSFA1*a exon 1 showing mutation site (Black triangle). Above it is the *HSFA1*a sequence showing the location of the sgRNAs used for pDE-Cas9:NU-HYG (sgRNA1) and pDE-Cas9-10A:NI1&2 (sgRNA 2). Orange arrows depict primers used to amplify mutation site for T7EI analysis. **B)** Image showing pooled samples from Col-0 and TKO (lane 1 and 2) after T7EI analysis. The 880bp and 400bp band indicate successful mutation by the Cas9. The 1280bp band represents the non-mutated WT sequence. **C)** Gel image showing positive T7EI screening of Line 4 (Nickase construct) and Line 6 (Nuclease construct).

4.2.2 Screening of Positive CRISPR/Cas9 Transformants

Seeds from Line 4 were grown on soil with 100 plants checked for the absence of the CRISPR/Cas9 T-DNA via PCR. It is important to segregate out the CRISPR/Cas9 T-DNA in the following generations in order to avoid the Cas9 from re-editing target site and off-target sites. Unfortunately, all 100 plants still harboured the CRISPR/Cas9 T-DNA. Therefore a new method was developed to screen for the absence of the CRISPR/Cas9 T-DNA. This method involved spotting different concentrations of Hygromycin diluted in a detergent, Triton X, and used to spot Arabidopsis leaves as described in Chapter 2.4.8. Depending on the leave size and position of the Hygromycin droplet, cell death was induced on the leaf at the site of droplet (Fig 4.4A). The Hygromycin spot method was used to screen 260 plants from line 6 and 18 plants were selected for further screening. Genomic DNA was extracted and the presence of the CRISPR/Cas9 T-DNA was checked via PCR using Phusion DNA polymerase with primers specific to the Cas9. Of the 18 selected plants 3 had big bright bands indicating the presence of Cas9 while the others had faint bands of varying intensity (Fig 4.4B). No plants survived when seeds from the plant in lane 3-8 were grown on Hygromycin plate indicating a possible loss of the CRISPR/Cas9 T-DNA. The Hygromycin spot test could not be replicated with Kanamycin for screening plants from Line 4 because Kanamycin in the pDE-Cas9-10A:NI1&2 T-DNA can be silenced.

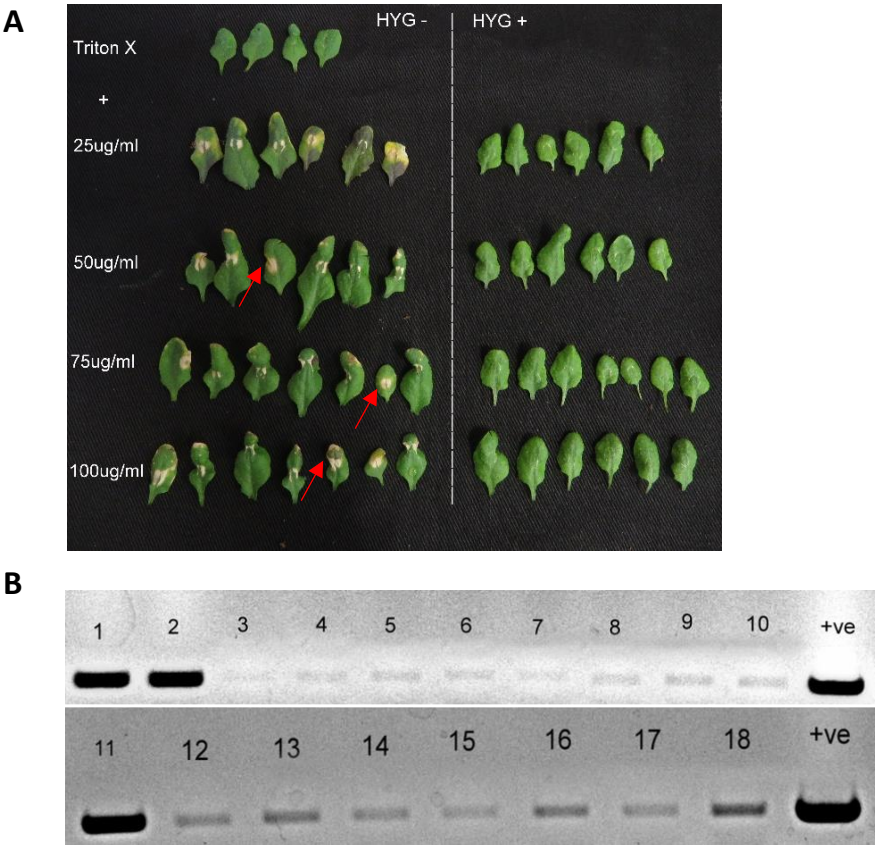


Figure 4. 4. Screening for Cas9-free Mutants. A) Hygromycin spot test using different concentrations of Hygromycin diluted with Triton X. Leaves on the left are Hygromycin sensitive while leaves on the right are Hygromycin resistant. Red arrows shows induced cell death on Hygromycin sensitive leaves. **B)** Presence of Cas9 protein on 18 plants selected with the Hygromycin spot test using phusion DNA polymerase for 35 cycles. +ve indicates positive control.

Despite the Hygromycin test being time effective, it wasn't robust hence the need for another type of screening method. This came in the form of adding a mCherry cassette driven by the strong AT2S3 seed promoter to pDE-Cas9:NU-HYG as described in Chapter 2.4.6. The new construct pDE-Cas9:NU-HYG-mCherry was then used to transform TKO and Col-0 and the T1 seeds screened by growing on MS media with Hygromycin. Positive transformants were transferred to soil and screened for mutations with T7EI (Chapter 2.2.7). Lines 22 & 25 from TKO and Lines 32 & 33 from Col-0 were selected for further analysis due to the increased band intensity of the digested products (Fig. 4.5A; Red rectangles). The higher the band intensity of digested products, the higher the chance of the mutation being inheritable in the following generations. Seeds from these lines were collected and checked for the loss of the CRISPR/Cas9-mCherry T-DNA. This was done by checking seeds for loss of fluorescence when excited with a higher wavelength. Seeds which fluoresce still harboured the CRISPR/Cas9-mCherry construct and those which didn't had potentially lost the Cas9 nuclease (Fig. 4.5B). Non-fluorescent seed from each line were selected, grown and checked for the presence of mutations by High Resolution Melting described in Chapter 2.4.9. Line 22-51 had a similar melting curve to the WT which was rejected. The others had a divergent melting curve with respect to the wild type were selected for further analysis (Fig. 4.6A). Genomic DNA from a subset of these samples were cloned into pJET1.2, Sanger sequenced to confirm HRM analysis and number of indels (Fig. 4.6B). Of the samples selected L22-54 had a 10 bp deletion while L22-15 had a 1 bp insertion which was enough to change the amino acid sequence disrupting the function of the *HSFA1*a protein by introducing several stop codons. Table 4.1 shows data from selected lines, mutation event via HRM and mutation frequency.

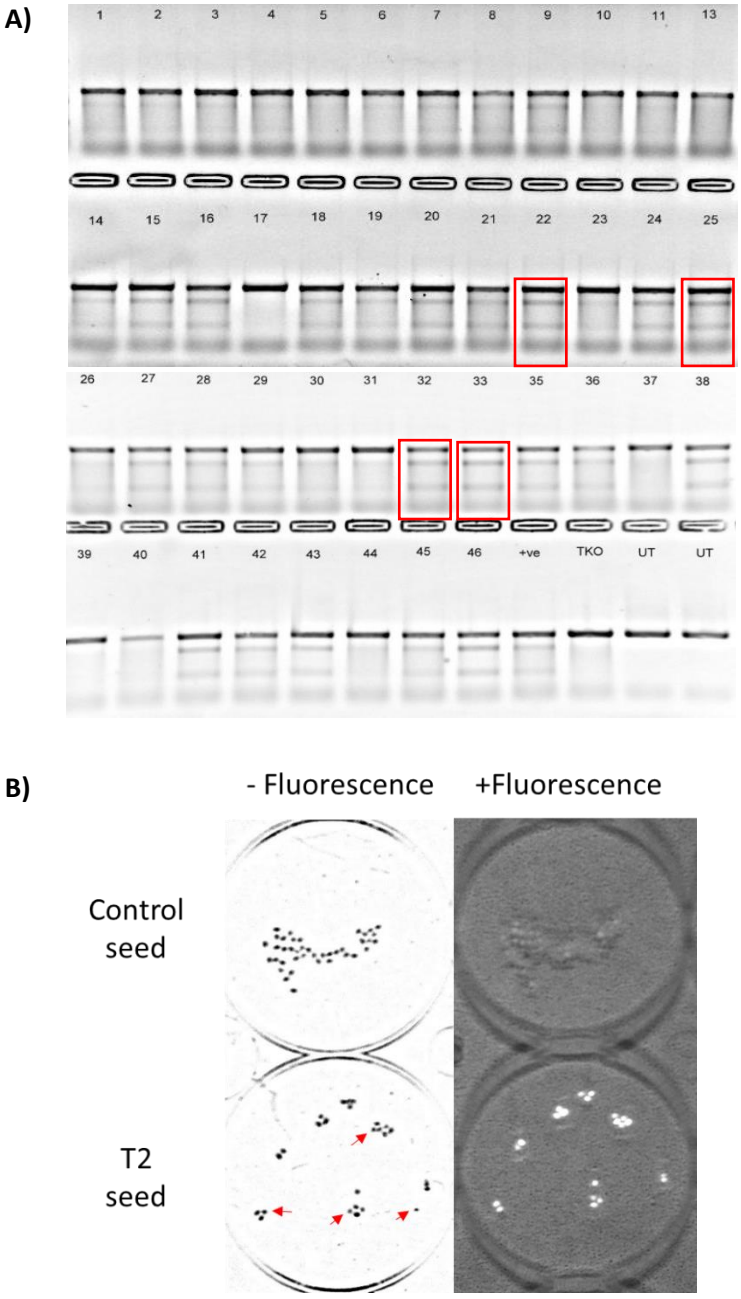


Figure 4. 5. Screening of positive T2 mutants. A) Gel image showing positive T7EI screening of T2 plants. DNA from leaves of T2 plants were extracted and a PCR was run across the junction of the predicted mutation site followed by sample incubation in a thermocycler at 95°C for 10 minutes. 2U/ µl of T7EI enzyme with buffer was added to the PCR sample and incubated again at 37°C for 1 hour. The incubated samples was then run in a 1% agarose gel. Top band belong to unedited WT allele (1280bp), middle band and bottom bands (880 and 400 bp) indicate DBS due to edited allele. Lines 22, 25, 32 and 33 (Red rectangles) were selected due to smaller size band intensity. +ve - positive control; TKO – triple knockout band untreated with T7EI; UT – untreated Col-0 band. **B)** Loss of Cas9 T-DNA by screening seeds without fluorescence after the addition of mCherry cassette. Red arrows indicate seeds which are not fluorescent hence cas9-free.

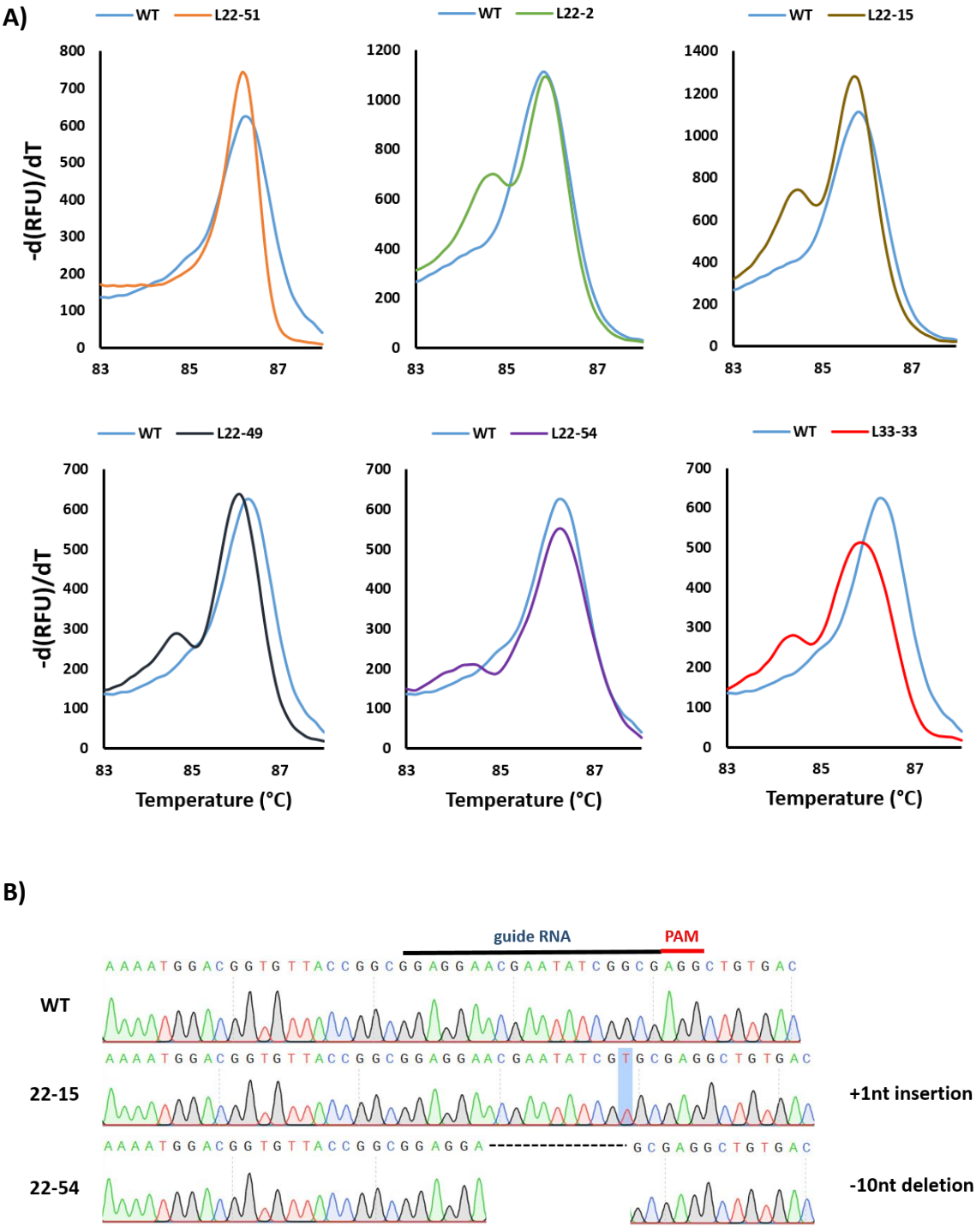


Figure 4. 6. Screening for heritable mutation in Cas9-free T2 mutants using High Resolution Melting (HRM). A) HRM analysis showing altered melting profile of different lines relative to WT sequence. **B)** Sanger sequencing results of lines 22-15 and 22-54 compared with WT sequence showing mutations. Numbers beside sequence represents number of indels. Line 22-15 has an extra base (blue highlight) 3 bp upstream of PAM site while L22-54 has a 10 bp deletion 3 bp upstream of PAM site.

Table 4. 1. Mutation Frequency of CRISPR/Cas9 Positive Lines.

Genotype	Plant Line	T2 Seeds checked	Seeds (-ve mCherry)	Plants Tested	Heritable Events (HRM)	Mutation Frequency (%)
TKO	L22	193	54	40	7	17.5
	L25	150	35	32	2	6.3
Col0	L32	179	46	34	8	23.5
	L33	287	44	35	2	5.7
Total				141	19	13.5

Four selected CRISPR/Cas9 positive lines showing number of T2 seeds checked for the loss of Cas9T-DNA by checking seed fluorescence, number of plants from each line tested for heritable mutations via HRM and calculated mutation frequency for each line.

4.2.3 Quadruple Knockout Showed Altered Developmental Morphologies

Line L22-54, which possessed a 10 bp deletion, was selected for further experimental analysis with three homozygous lines isolated. The transcript level of *HSFA1* and the Cas9 transgene in the 3 homozygous plants from Line L22-54 (*QK2*-2, *QK2*-5, *QK2*-7) could not be detected compared to their controls confirming the mutant as a *HSFA1* quadruple knockout (hereby called *QK2*; Fig 4.7A). *QK2* mutant possessed smaller sized seeds on average (Fig 4.7C) with about 20-40% of seeds falling in the < 200 µm range in the 3 homozygous lines compared to wildtype (Col-0) with 10%. Col-0 also had 20% of its total seed in the >297 µm range compared to the *QK2* homozygous lines with only 6%, 3% and 4% respectively. Similarly, despite the majority of the seeds in both genotypes fall within the 200-297 µm range, wildtype had significantly more seeds in that range compared with 2 homozygous lines (Fig 4.7B). Consequently, the variation in seed distribution in both wildtype and *QK2* is unequivocally manifested in the 1000-seed weight with wildtype weighing significantly more than the *QK2* homozygous lines (Fig 4.7D). Generally, seeds of the QK2 mutant were lighter in colour compared to wildtype with varied shapes (Fig 4.7E).

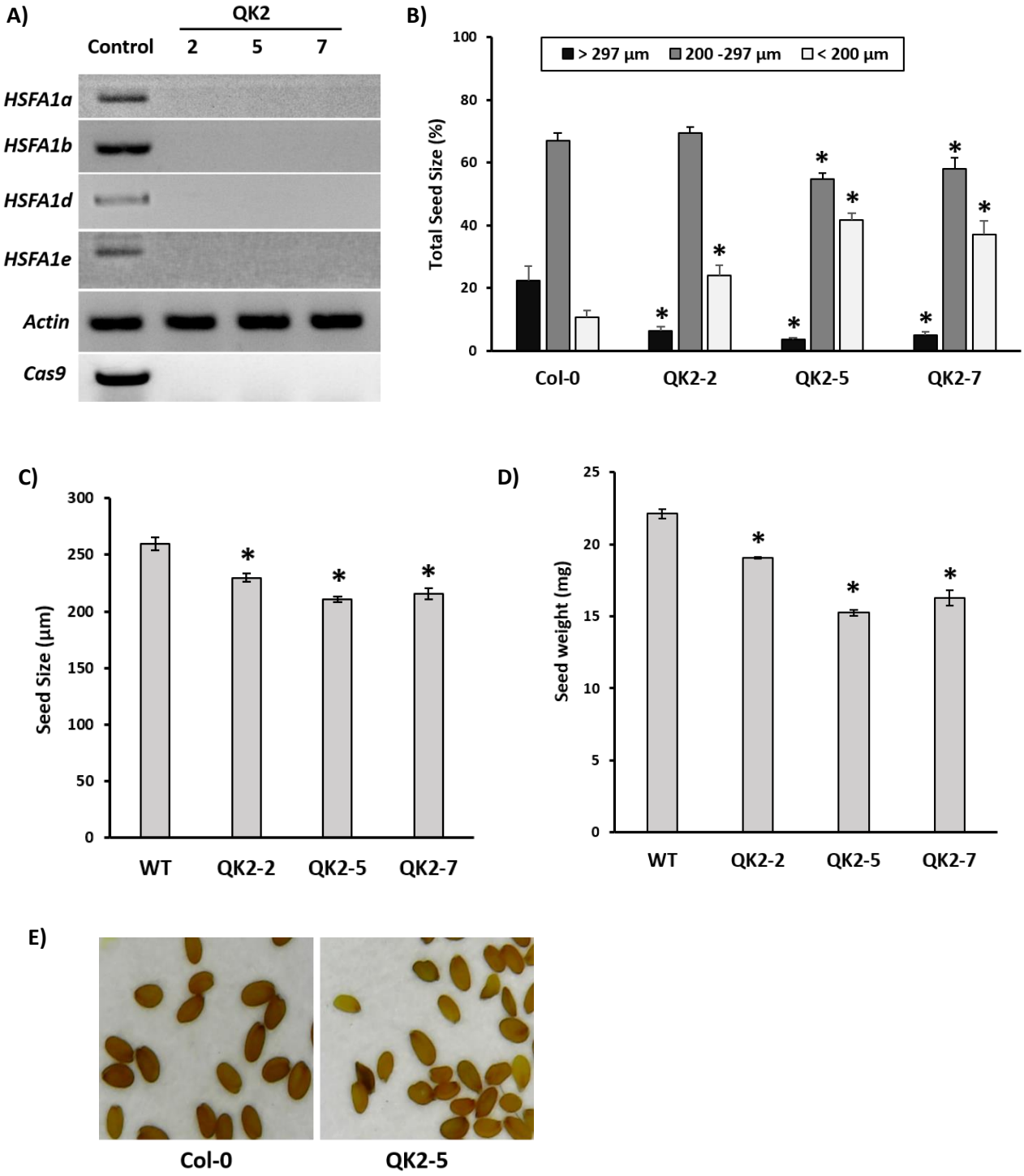


Figure 4. 7. Transcript levels and seed size of Col-0 and QK2. A) RT-PCR analysis of transcript levels of HSFA1 genes between Col-0 and 3 homozygous plants from Line L22-54 (QK2-2, QK2-5, and QK2-7). **B)** Distribution of seed sizes. **C)** Average seed size. **D)** 1000 Seed weight. Results are mean values of 3 replicates (n >900 each). *p value < 0.05*; Student *t*-Test. **E)** Seeds of Col-0 and QK2-5.

Similarly, the timing to reach certain developmental stages (Boyes *et al.,* 2001) in the *QK2* (or *QK2*-5) was longer than that of wildtype. When grown on solid medium plates, *QK2* was delayed by 0.5 days in reaching growth stage 0.5 (radicle emergence) and another 2.2 days in reaching growth stage 1.0 (cotyledons fully open) from stage 0.7 (radicle and cotyledon emergence) compared to wildtype (Fig 4.8A). Irrespective of delay in reaching certain developmental stages, the *QK2* was smaller in size to wildtype. When the cotyledons were fully open in both genotypes, the rosette area was smaller by 47% in the *QK2* than in wildtype in long day conditions when grown on solid medium supplemented with 1% sucrose (Fig 4.8B). Similar rosette size was also observed in 17-day old soil-grown plants in the *QK2* mutant compared to wild type in short day conditions (Fig 4.8C). Additionally, the *QK2* mutant possessed pale green semi-translucent cotyledons with a pointed tip compared to the greenish oval-shaped cotyledons of the wildtype with a rounded tip (Fig 4.8B). The primary root length in the *QK2* mutant was also shorter than wildtype (Fig 4.8D). Despite the clear developmental differences between both genotypes at seedling stage, these differences become less obvious as they got older especially the timing to reach certain developmental stages. For example, 3-week old *QK2* plants grown in short day conditions have the same number of leaves as wildtype albeit smaller in size (Fig 4.8E, G). The leaf number of both QK2 and wildtype remained the same at bolting as well as fresh weight (Fig 4.8H, I). However, despite the QK2 mutant catching up to the wildtype in terms of rosette size, the cotyledons remained smaller at 5 weeks (Fig 4.F).

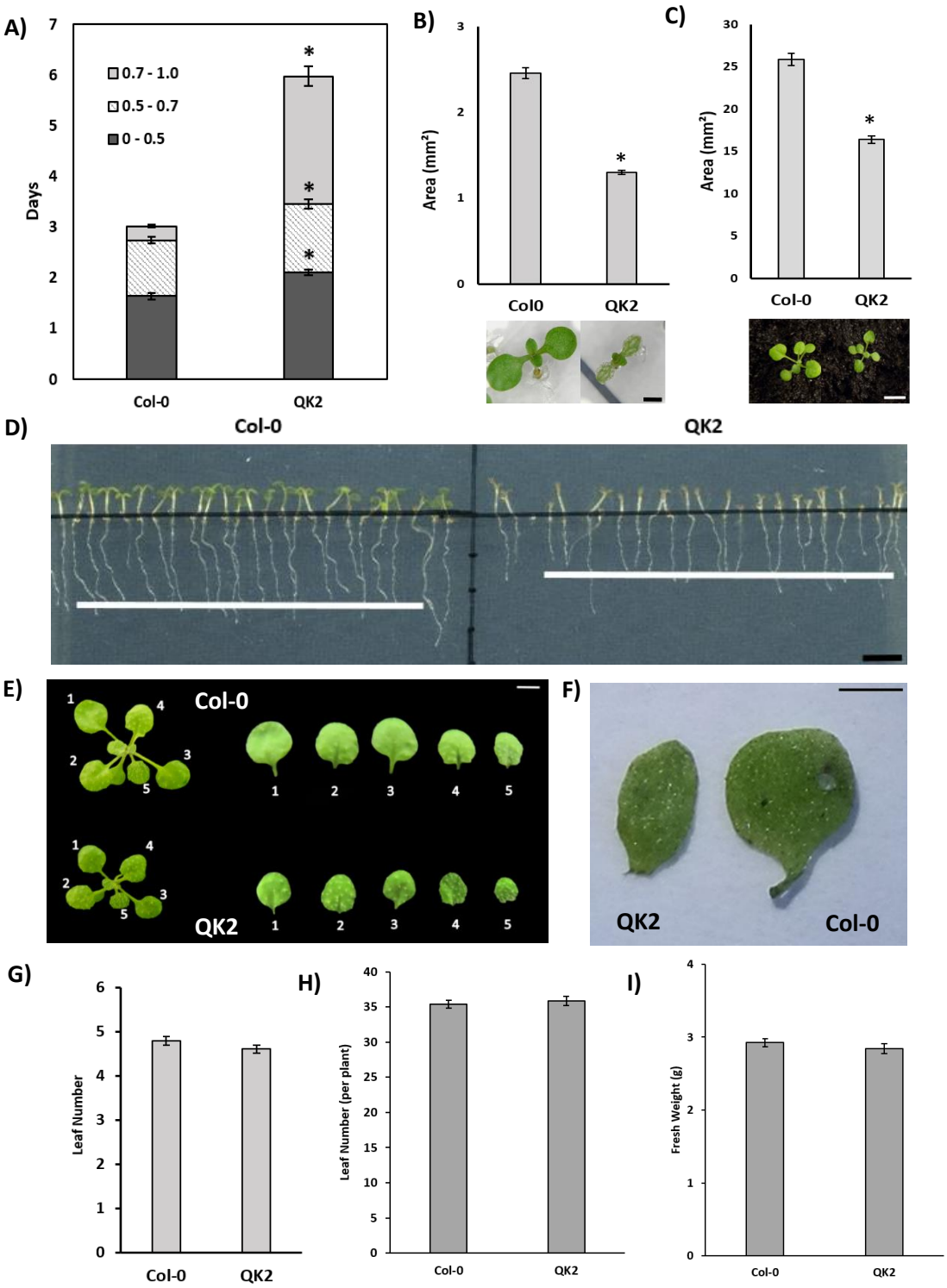


Figure 4. 8. Morphological differences between Col-0 and QK2. A) Growth rate of Col-0 and QK2: 0.5, radicle emergence; 0.7, hypocotyl and cotyledon emergence; 1.0, cotyledons fully opened. Results are a mean of 6 replicates. n = 50. **B)** Rosette area at Day7; Scale 1mm. **C)** Rosette area at Day17; Scale 5mm. **D)** Primary root length at Day5; Scale 5mm. **E)** Leaf arrangement at Day22; Scale 2mm. **F)** Cotyledon size and shape at 5 weeks; Scale 1mm. **G)** Leaf number at Day 22. n = 40. **H, I**) Leaf number and fresh weight at bolting. n = 60. Plants were grown on soil at 22°C in SD except for A, B and D which were grown in LD on ½ MS media plates supplemented with 1% sucrose. *p value* < 0.01; Student *t*-Test.

4.2.4 QK2 Mutant Is Sensitive To High Temperatures

To further confirm the *QK2* mutant, its ability to withstand abiotic stress needed to be compromised. This was done by subjecting the mutant under heat stress (HS) at different developmental stages. Firstly, the imbibed seed was incubated for 2 hours at 45°C and returned to normal growing conditions in long day conditions (16h light) to test for seed thermotolerance. The germination rate of the *QK2* mutants was significantly reduced after the stress treatment compared to the control. It took the *QK2* mutant seeds 5 more days to attain > 95% seed germination compared to wildtype (Fig 4.9A). The delay in germination in the *QK2* mutant is also exemplified in the developmental stage after HS (Fig 4.9B). Majority of the *QK2* HS seeds were between growth stage 0.5 (radicle emergence) and 0.7 (radicle and cotyledon emergence) while wildtype (no stress/HS) and *QK2* no stress had reached growth stage 1.0 (cotyledons fully open; Fig 4.9B). Secondly, the thermotolerance of seedlings was tested after subjecting 7-day old plants to different HS regime to test for thermotolerance i.e. Acquired Thermotolerance after a Short Recovery (ATSR) and Basal Thermotolerance (BT). *QK2* mutant were non-viable 7 days after heat treatment while the wildtype showed no visible signs of leaf damage for ATSR (Fig. 4.9C). Similar effects was observed when tested for BT (Fig. 4.9C)

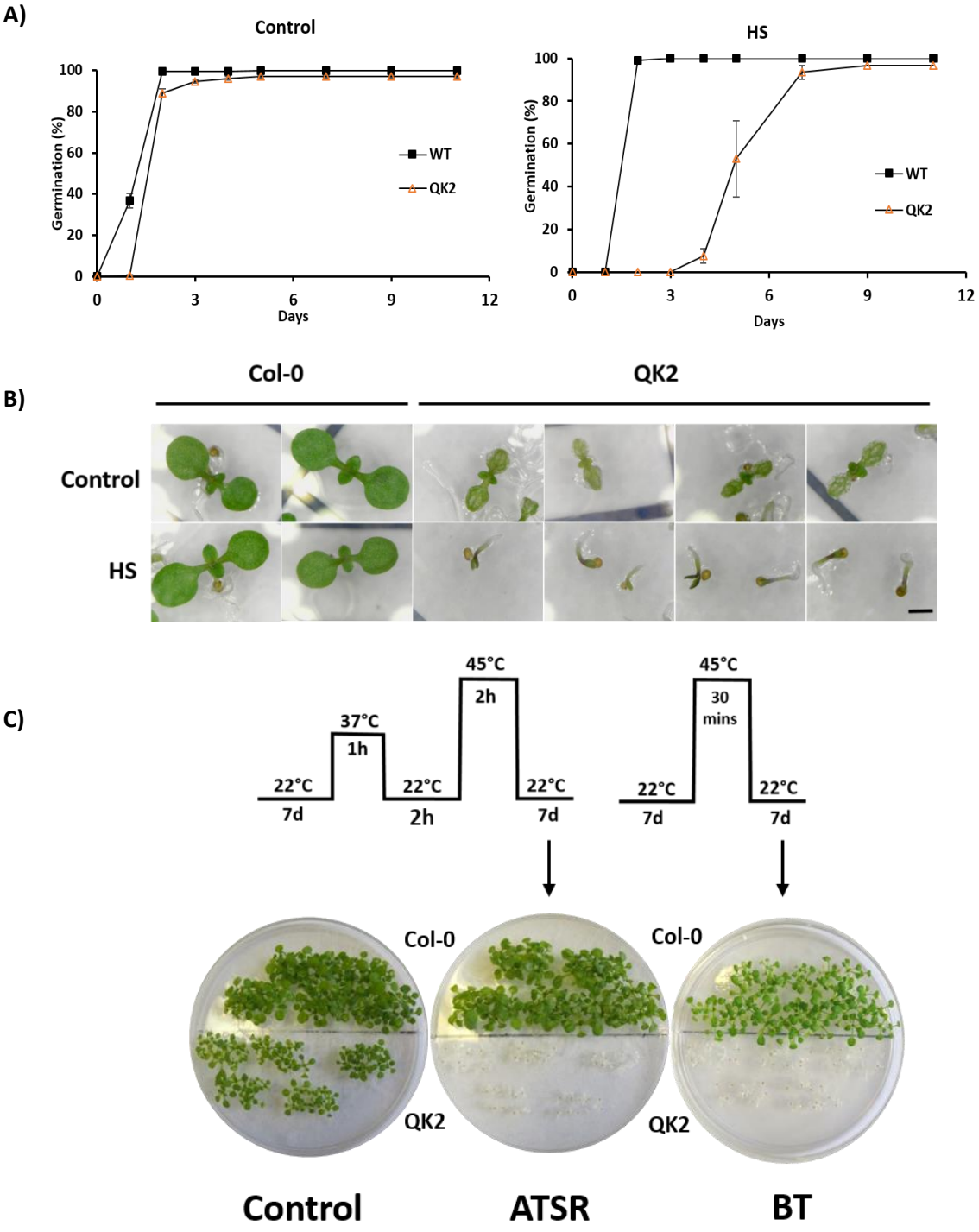


Figure 4. 9. QK2 sensitivity to heat treatment. A) Seed germination rate of Col-0 and QK2 after heat treatment. Imbibed seed were sown on ½ MS plates with 1% sucrose in LD followed by 45°C (HS) for 2 hours. Controls were kept at a constant 22°C. Result represent mean value of 5 replicates. **B)** 7 day old seedlings of plants from (A). Scale 1mm. **C)** Survival rates of seedlings following acquired thermotolerance after a short recovery (ATSR) and Basal thermotolerance (BT) grown in LD on ½ MS media plates supplemented with 1% sucrose. Controls were kept at a constant 22°C. Image is representative of 2 replicates. n = 50.

4.2.5 QK2 Mutant Exhibits Varied Phenotype in Ambient Temperature

Following failure to survive exposure to different heat stress regimes, the QK2 mutants were subjected to a temperature increase of +5°C (from 22 to 27°C). At control temperatures (22°C), hypocotyl length of QK2 was slightly reduced compared to wildtype. However at 27°C, QK2 plants failed to increase hypocotyl length which was significantly increased in the wildtype (Fig 4.10A). Furthermore, QK2 seedling at this temperature increase had greener cotyledons compared to those grown in control temperatures for 9 days, although there was a slight increase in cotyledon size (Fig 4.10B). In order to confirm that QK2 mutant were not responding developmentally to changes in temperature, 5-week old plants were grown in soil at control temperatures and then moved to a 27°C growth chamber for an additional 2 weeks. At the end of the experiment, the QK2 mutant had a smaller rosette area with smaller leaves at 27°C compared to wildtype (Fig 4.10C) despite having an almost identical phenotype at control temperatures (Fig 4.10C, insert image). In addition, the petiole length of the QK2 mutant was shorter than wildtype at 27°C (Fig 4.10D).

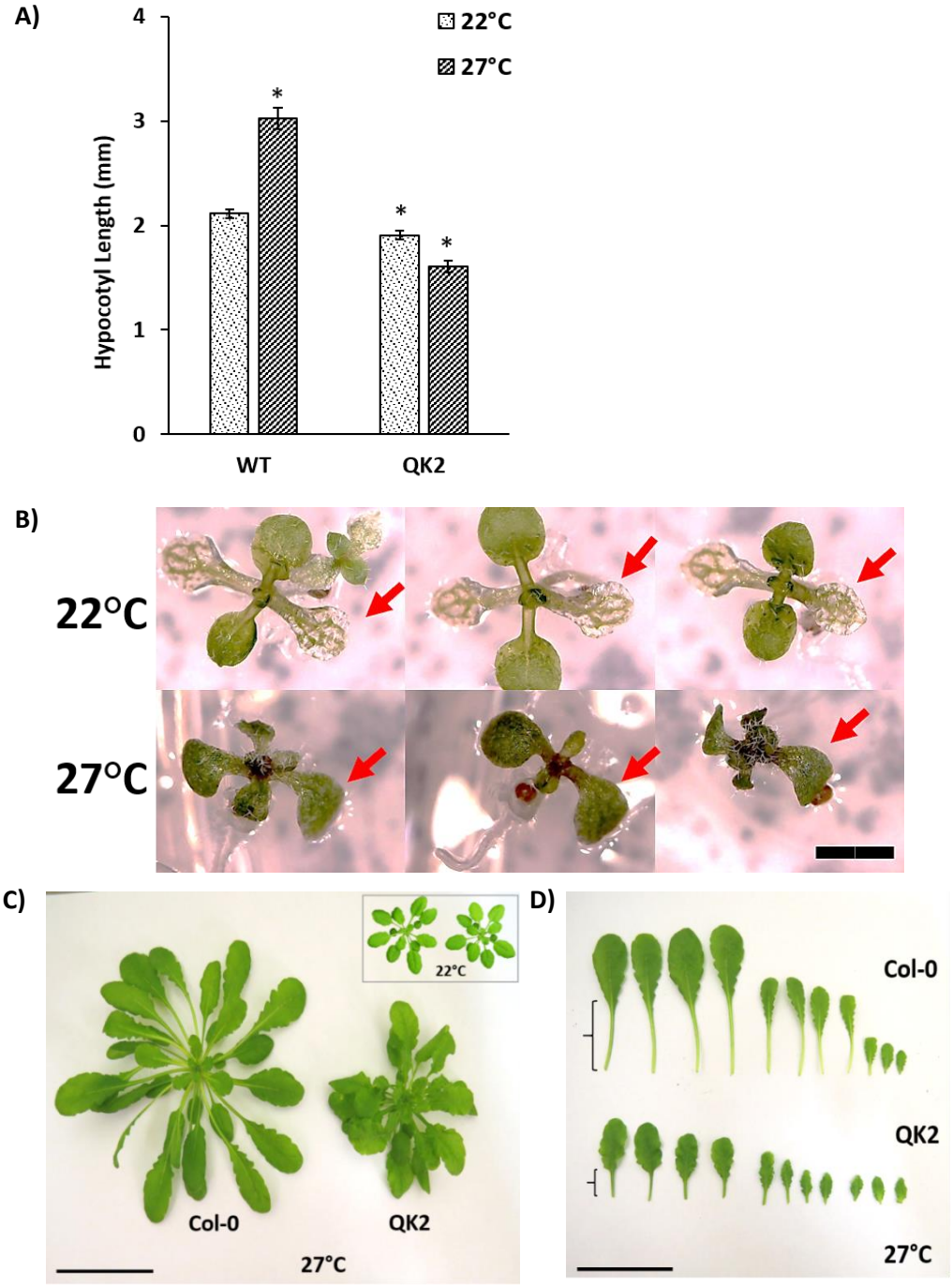


Figure 4. 10. Pleiotropic effect of QK2 in mild temperature change. A) Hypocotyl length of 8-day old QK2 seedlings compared to wildtype at 22°C and 27°C. Seedlings were grown in LD on ½ MS media plates supplemented with 1% sucrose. n = 40. **B)** 9-day old QK2 seedling showing differences in cotyledon development (red arrows) in control (22°C) and mild temperature increase (27°C). Seedlings were grown in LD on ½ MS media plates supplemented with 1% sucrose. Scale = 2mm **C)** Wildtype and QK2 mutants grown in soil at 22°C for 5 weeks (insert image) and then moved to 27°C for additional 2 weeks. Scale = 5cm **D)** Leaves of QK2 and wildtype showing differences in petiole length from (C). Scale = 5cm. *p value* <0.05; Student *t*-Test.

4.2.6 HSFA1s Regulate Several Developmental Genes

Owing that HSFA1s regulate the heat shock response, a few heat stress responsive genes were tested in the wildtype and QK2 under normal and heat stress (30 mins) on 3-week old seedlings grown on solid media. Results show a significant upregulation on all genes tested in the wildtype heat stressed plants compared to control. The same could not be observed however for the QK2 heat stress plants as the expression of these genes were not significantly upregulated compared to QK2 control plants with the exception of *HSFA2* (Fig 4.11A). Furthermore, in normal conditions, the expression of *HSP90, HSP70* and *HSFA2* was significantly downregulated compare to QK2 in the same condition. Since the expression of these heat responsive genes were expected in the QK2 mutants efforts was shifted to determine the expression of the same *HSFA1b*-directly regulated developmental TFs tested with the original QK (Fig 3.4). Result showed difference in expression pattern between some of the TFs tested (4.11B). For example, *bZIP28* was significantly upregulated in both controls and QK after heat stress but not in QK2. A similar pattern was also observed in *GBF3, RVE1* and *SZF1* but not in *RVE7* and *HSFB2a* which showed a similar pattern of expression between QK and QK2. The difference in expression pattern observed between QK and QK2 compared to their controls especially after heat stress highlights the need for generating a HSFA1 quadruple knockout in a single accession.

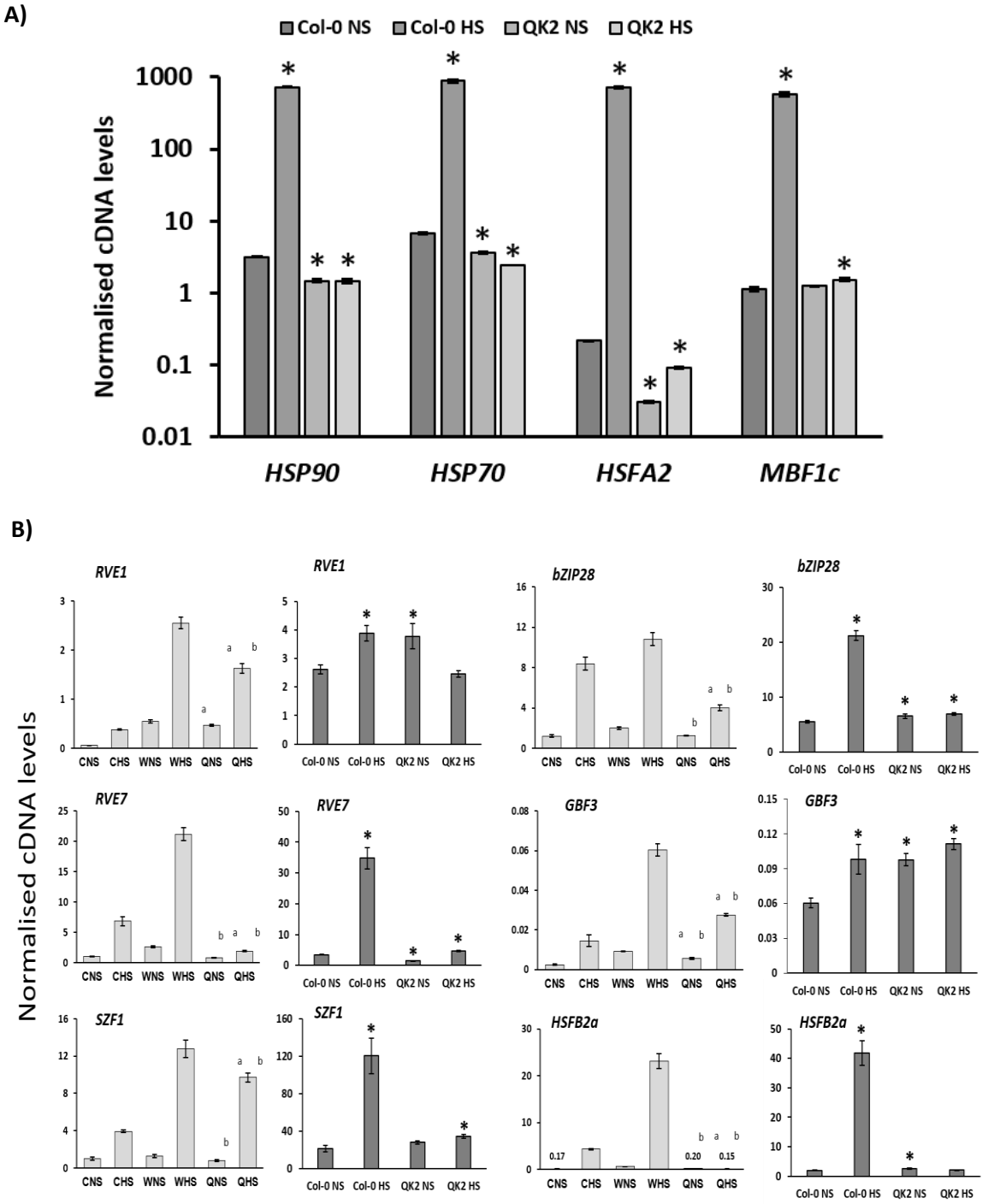


Figure 4. 11. Real-time RT-PCR transcripts of selected HSR and developmental genes. **A)** Graph showing transcript levels of 3 week old plants between Col-0 and QK2 grown in SD at 22°C (No stress; NS) and at 37°C for 30 mins (Heat stress; HS), normalised against *PP2AA3*. Result represents log 10 mean value of 3 replicates. **B)** Side by side comparison of *HSFA1b* directly regulated TFs in the original QK (Light bars) and QK2 (Dark bars) of 5 week old soil grown plants in SD at 22°C (No stress; NS) and at 37°C for 30 mins (Heat stress; HS). 'a', significant difference between QK compared to Col-0 and; 'b', significant difference between Ws and QK; '*', significant difference compared to Col-0 NS. *p value* <0.05.

4.3 DISCUSSION

Genetic redundancy of protein coding genes is a common feature of higher organisms (Nowak *et al.,* 1997) and the Arabidopsis clade A1 HSFs is no exception. Despite the 4 members of this clade been suggested to have specialised roles (Liu *et al.,* 2011), they still share a high homology and similar function in regulating heat stress responses protecting plants from elevated temperatures (Liu *et al.,* 2011; Yoshida *et al.,* 2011). The implication of functional genes performing similar roles is that inactivation of one of its members may have little or no effect on the biological phenotype or plant fitness (Rutter *et al.,* 2017). This is a feature of the clade A1s as the different combinations of triple knockouts shows no observable effect on the mutant phenotype compared with the wildtype plant in normal conditions. Only when all 4 are inactivated do they exhibit defects affecting growth and development (Liu *et al.,* 2011; Yoshida *et al.,* 2011). On the flipside, this phenotype has only been shown in a QK mutant, a hybrid of 2 naturally occurring accessions. Aside from the mere fact that these Arabidopsis accessions are physiologically, phenotypically and developmentally different, a cross can result in heterotic vigour which contributes to the development in the hybrid progeny (Birchler *et al.,* 2010; Groszmann *et al.,* 2014). However, hybrid vigour, maintained only in the F1 progeny, is reduced in subsequent generations due to segregation which varies from plant to plant (Greaves *et al.,* 2014; Groszmann *et al.,* 2014) leading to hybrid progeny variation. It is also suggested that siRNA populations and DNA methylation patterns change in Arabidopsis hybrids altering their transcriptome which in effect contributes to the changes in mRNA levels observed in hybrids compared to their parental equivalents (Chen, 2013; Groszmann *et al.,* 2013; Greaves *et al.,* 2014). This singular characteristic of hybrids together with hybrid progeny variation poses a huge problem as transcriptomic experiments conducted with hybrid

progenies produce data that can be complex to interpret and sometimes irreproducible. With the complexity of an altered transcriptomic profile in a hybrid compared to its parents, a knockout of all clade A1s in a Col-0 /Ws-0 hybrid, in this case QK, might further affect/change its transcriptomic profile making concrete conclusions difficult and challenging which has been highlighted (Chapter 3.2.4; Fig. 3.4b, 4.11b; Cortijo *et al.,* 2017). Therefore generating a QK mutant in a single background was imperative which was achieved using engineered nucleases to introduce disruptive mutations on the remaining functional member of the clade A1 in a triple knockout mutant in a Col-0.

4.3.1 CRISPR/Cas9 Was Effective In Knocking Out *HSFA1a*

In recent years, the CRISPR/Cas9 gene editing system has been used with success to introduce mutations of various kinds to different crop species including Arabidopsis (Miao *et al.,* 2013; Nekrasov *et al.,* 2013; Brooks *et al.,* 2014; Fauser *et al.,* 2014; Li *et al.,* 2014; Zhang *et al.,* 2014; Wang *et al.,* 2015; Svitashev *et al.,* 2016; Andersson *et al.,* 2017). The CRISPR/Cas9 construct used in this study had been previously shown to induce heritable mutations at target site with about 15.3% mutation efficiency (Fauser *et al.,* 2014). Although using this construct was successful in inducing mutation at the target region (Fig 4.3b, c), it had to be optimised by adding a fluorescent protein to the CRISPR/Cas9 T-DNA driven by a seed promoter to ease the screening process in the T2 generation (Fig 4.5b). Adding fluorescent proteins is a current feature of Arabidopsis CRISPR/Cas9 plasmids as it makes selection of positive transformants easy eliminating the use of antibiotics and harsh herbicides (Shimada *et al.,* 2010; Gao *et al.,* 2016). However, different types of phenotype-based methods of selection have been proposed; by size, colour and resistance to pathogen or abiotic stress (Feng *et al*., 2013; Čermák *et al*., 2015; Svitashev *et al*., 2016). It is important to screen for cas9-free plants in T2 generation in order to identify heritable

mutations because the CRISPR/Cas9 system generates a high frequency of somatic mutation depending on the activity of the cas9 promoter albeit it can be vastly reduced with specific germline promoters (Mao *et al.,* 2016). As reported, Cas9-free T2 plants could not be isolated for Line 4 which was transformed with the CRISPR/Cas9 T-DNA without mCherry using the PCR assay. This could be due to cross-contamination from extracting genomic DNA from a large sample size. Despite using the Hygromycin spot test to identify Cas9-free T2 plants in Line 6, no plant with a mutation was isolated which could only mean that the mutation was somatic and not heritable.

Accordingly including mCherry to the T-DNA eased the identification of Cas9-free plants in T2 by screening seeds negative for mCherry which is indicative of the loss of the T-DNA. An overall mutation efficiency of 13.5% (Table 4.1) was achieved which is similar to the 15.3% reported from the creators of the construct (Fauser *et al.,* 2014). Highly efficient CRISPR/Cas9 construct has also been reported in recent years which involves using the *RIBOSOMAL PROTEIN S5 A* (RPS5A) promoter to drive the expression of the Cas9 increasing mutation efficiency albeit with suitable terminators (Tsutsui and Higashiyama, 2017). The RPS5A promoter is constitutively expressed at all developmental stages from egg cell to meristematic cells. It is 30x more efficient than PcUbi4-2 promoter used in this study (*personal communication*; Ordon *et al.,* 2017). It is important to note herewith that increasing the mutagenic efficiency of CRISPR/Cas9 increases the chances of mutations at unwanted sites especially if the sgRNA, which also influences efficiency, isn't designed correctly.

The common method for selecting T2 plants with heritable mutation is by Restriction enzyme-loss assay (Nekrasov *et al.,* 2013) which involves having a restriction site within

the sgRNA preferably close to the PAM site. After transformation with CRISPR/Cas9, genomic DNA is amplified across the target site, the product is digested with the specific enzyme and the sample is run on DNA separating gel. DNA band resistant to the digestion is indicative of a mutation as the Cas9 had destroyed the restriction enzyme recognition site. This method could not be applied with Cas9-free T2 plants as there were no available restriction enzyme site located within the sgRNA hence the use of High Resolution Melting analysis. This method was successful in identifying mutations in the target region as it works with the principle that the melting temperature (TM) of a DNA duplex of a certain size and nucleotide composition will differ from another with different size and/or nucleotide composition. Thus the TM of a CRISPR/Cas9 edited sequence will have a different melting curve to its WT sequence (Fig 4.6a). This method was used to successfully identify T2 plants with heritable mutations and confirmed via Sanger sequencing (Fig 4.6b). The sequenced plants harboured mutations ranging from 10bp deletions to 1 bp insertions at the predicted site, 3bp upstream of the PAM. It shows that using HRM is sufficient in screening for heritable mutations with mutation frequency in the different lines ranging from 5.7% to 23.5% (Table 4.1). Using HRM to screen for mutations in a large sample size eliminates the cost of buying the expensive T7EI enzyme. Other methods for screening mutations include Restriction Fragment Length Polymorphism (RFLP; Feng *et al.,* 2013) and High-Resolution Fragment Analysis (HRFA; Andersson *et al.,* 2017). How they compare to HRM is not known at this time.

4.3.2 QK2 Is Sensitive To Mild and High Temperatures

Temperature is an important environmental factor in growth and development because plants react differently when it changes. While mild temperature increase (from 22 to 28°C) promotes growth, high (30 - 38°C) and extremely high temperatures (45°C) are detrimental. Sensitivity to high temperatures in the QK mutant can be attributed to the inability of the mutant to induce heat stress responsive (HSR) genes including chaperones like HSP90 and HSP70 which are direct targets of *HSFA1*s. This was corroborated with the transcript expression of a select number of HSR genes in the wildtype and QK2 under heat stress. *HSP90, HSP70, HSFA2* and *MBF1c* failed to induce under heat stress in the QK2 mutant compared to wildtype (Fig. 4.11a). Similarly, a reduction in transcript expression was observed with *HSP90, HSP70* and *HSFA2* under no stress which is in line with previously published data (Liu *et al.,* 2011). This explains why the *QK2* mutant is deficient in basal and acquired thermotolerance as the seedling are lacking important chaperones involved in the protecting against heat stress (Fig. 4.9c).

Mild temperature increase on the other hand have been shown to alter development in Arabidopsis especially hypocotyl elongation without significant induction of stress responses suggesting that the players involved in this temperature-dependent pathway are developmentally-associated (Wigge, 2013). At the core of this temperature-dependent pathway is the basic Helix loop Helix transcription factor, *PIF4* and together with its direct target *BRASSINAZOLE-RESISTANT1* (*BZR1*), a transcription factor involved in Brassinosteroid hormone signalling, have been shown to regulate a diverse number of genes involved in growth and development especially hypocotyl elongation (Oh *et al.,* 2012; Ibañez *et al.,* 2018). Auxins have also been implicated in the regulation of temperature-dependent hypocotyl growth which is also dependent on the presence of

PIF4 (Gray *et al.,* 1998; Koini *et al.,* 2009; Franklin *et al.,* 2011; Ibañez *et al.,* 2018). Furthermore, it was revealed that PIF4-regulation of hypocotyl elongation during high temperatures is regulated by the circadian clock TF *EARLY FLOWERING 3* (*ELF3;* Box *et al.,* 2015; Nieto *et al.,* 2015). Although these reports do not show a link between HSFs and temperature-dependent growth, Wang *et al.,* (2016) provided evidence of how HSP90 regulated temperature-dependent hypocotyl elongation by stabilizing the auxin co-receptor protein TIR1. They also showed that inhibition of HSP90 activity with geldanamycin (GDA) resulted in the degradation of TIR1 preventing hypocotyl elongation at a higher temperature. Furthermore, it has been shown that HSP90 activity is important for Brassinosteroid signalling (Samakovli *et al.,* 2014). Since *HSP90* expression was downregulated in the QK2 in normal and heat stress condition compared to wildtype, phenotypic changes in the QK2 compared to wildtype was examined at 27°C (Fig 4.10). The failure of QK2 to increase hypocotyl length and petiole elongation, typical characteristics of plants grown in mild temperature increase, suggests that HSFA1s are involved in the temperature-dependent growth pathway. It is also possible that HSFA1s are not directly involved in this pathway like *PIF4* and *BZR1* but indirectly by their ability to regulate key protein chaperones like HSP90 important for client protein stabilization. However a counter argument to the statement above; it has been suggested that *HSFA1b*, one of the HSFA1 members, binds and regulates similar genes to *PIF4* (Albihlal *et al.,* 2018), therefore, *PIF4* and *HSFA1b* (and/or other HSFA1s) could be co-regulators of the temperature-dependent growth pathway. Furthermore, the interaction between HSP90 and hormone signalling especially in the stabilization of proteins suggest that HSFA1s are indirectly involved in hormone homeostasis and important for temperature-dependent development.

4.3.3 HSFA1s Affects Genes Involved In Growth and Development

Majority of the morphological and physiological differences between *QK2* and wildtype is not unique as they have been previously reported in the original QK mutant (Liu *et al.,* 2011). However, the justification in generating the *QK2* mutant lies not only in the knocking out of the *HSFA1* TFs in a single background but being able to plan and execute unambiguous experiments (due to using a single control) and to make concise conclusions in relation to transcriptomics data. This is most important as this study aims to identify developmental targets of *HSFA1* TFs under normal and heat stress conditions. Looking at the morphology of the *QK2* mutant, there are differences in seedling size within individual plants and to that of wildtype. This could be due to seed size (Fig 4.7c), distribution (Fig 4.7b) or a reduction in cell expansion in the *QK2* compared to wildtype.

Seed size is determined primarily by the co-ordinated growth between the embryo, endosperm and seed coat (Chaudhury *et al.,* 2001; Becker *et al.,* 2014). In Arabidopsis, development of embryo and endosperm begins after double fertilization followed by a rapid expansion of the endosperm increasing the seed volume (Chaudhury *et al.,* 2001; Nowack *et al.,* 2010). Later in the developing seed, the embryo expands and fills the cavity of the endosperm and is restricted by the outer seed coat which has been proposed to determine the final size of the developing seed (Ohto *et al.,* 2009). Hence, the embryo, consisting mainly of the both shoot and root meristems, cotyledons and hypocotyl, occupies the majority of the mature seed (Chaudhury *et al.,* 2001). QK2 consists of 83% less, 18% less and 74% more seed in the >297 µm, 200-297 µm and <200 µm range respectively compared to wildtype (Fig 4.7b). On average QK2 is 11% smaller than wildtype (Fig 4.7c). This generally implies that the *QK2* mutant due to its smaller seed size compared to wildtype starts its lifecycle at least from germination smaller than wildtype

(Fig 4.7c). Aside from environmental cues, determination of the final seed size is pre-determined by the genetic information from parental gametes which influences the rate of growth the embryo, endosperm and the seed coat. Several mechanisms that affect the development of the embryo, endosperm and seed coat, inadvertently influencing seed size, have been elucidated including the effects of hormone signalling (Schruff *et al.,* 2006; Bartrina *et al.,* 2011; Jiang *et al.,* 2013b), ubiquitin pathway (Li *et al.,* 2008; Xia *et al.,* 2013) and certain transcription factors (Ohto *et al.,* 2009; Prasad *et al.,* 2010; Cheng *et al.,* 2014). To identify the precise mechanism affecting seed size in *QK2*, a complete transcriptional profile is necessary to identify key players in the development of the mutant regulated by *HSFA1*s under normal growth conditions.

Aside from the seed size, *QK2* is smaller than wildtype although the biggest size difference occurs in seedlings. Despite this size difference, the *QK2* maintains the same developmental stage in adult plants suggesting that the effects of plant size may be due to cell elongation/expansion. Cell expansion is also implicated in determination of seed size (Johnson *et al.,* 2002; Yu *et al.,* 2014). Cell elongation/expansion is primary mediated by the plant hormone brassinosteroid (BR) as well as other phytohormones. BRs are involved in other aspects of plant development including cell division, stress response and vascular system differentiation (Azpiroz *et al.,* 1998; Krishna, 2003; Yamamoto *et al.,* 2007). Plants deficient or insensitive to BR show abnormal developmental phenotypes including dwarfism and reduced fertility. For example, *dwf5* mutants, defective in BR biogenesis, have a dwarf phenotype, small round dark-green leaves, short stems, pedicels and petioles, small round seeds with poor germination (Choe *et al.,* 2000); Brz-*insensitive-long hypocotyl 4* (*BIL4*), a positive regulator of BR signalling, is expressed early in development especially in young elongating cells. Plants with a reduced expression of *BIL4*

have smaller leaves and hypocotyl compared to wildtype and its severity is dose dependent (Yamagami *et al.,* 2017). ARGOS-LIKE (*ARL*), another BR signalling-regulated gene, plays a role in cell expansion during organ growth. Altered expression of this genes resulted in the changes in leaf and cotyledon size as well as flower organs (Hu *et al.,* 2006). *ARL* is mainly expressed in cotyledons and expanding leaves as well as in the roots. *TCP1*, a member of the TEOSINTE BRANCHED1, CYCLOIDEA and PCF TFs, has also been identified to positively regulate BR biosynthesis and the longitudinal elongation of leaves and petioles (Koyama *et al.,* 2010). These 4 genes amongst others involved in seed germination and post-germination growth are indirectly regulated by *HSFA1b,* one of the 4 TF knocked out in the *QK2* mutant. Therefore, it is plausible that the size difference between *QK2* and WT is because of cell elongation mediated by BR or other phytohormones partly involved in cell elongation. Furthermore, unlike other phytohormones, BRs cannot be transported over a long distance (Symons *et al.,* 2008) meaning they are synthesised in the same tissues in which they function although most actively synthesised in young actively developing organs (Shimada *et al.,* 2003; Symons *et al.,* 2008). This supports the idea the *QK2* mutant, especially during germination, might not accumulate the same level of endogenous BR compared to wildtype resulting in 47% decrease in cotyledon size.

Additional to cotyledon size, cotyledon development was also affected in the *QK2* mutant. The cotyledons were pointed, pale-green and translucent (Fig 4.9B). Nevertheless, this did not translate to the whole plant as true leaves developed just as wildtype. This is not uncommon as chloroplast biogenesis in cotyledons is different from that in true leaves (Albrecht *et al.,* 2008). It is unknown if the poor cotyledon development is a direct result of the aberrant seed size/development. However, looking at the *35:HSFA1b* dataset of

bound developmental but not differentially expressed set of genes, 3 chloroplast and embryogenesis genes stood out amongst the rest namely; HEAT SHOCK PROTEIN 90.5 (HSP90.5)/ EMBRYO DEFECTIVE 1956 (emb1956); SCHLEPPERLESS (SLP) and RIBOSOME RECYCLING FACTOR (RRF). HSP90.5 is a chloroplast localized molecular chaperone in Arabidopsis involved in chloroplast function and biogenesis necessary for proper growth and development. Knockout mutants of HSP90.5 are embryonically lethal but co-suppression mutants are defective in thylakoid formation resulting in a small semi-albino looking plant with white flower petals (Feng *et al.,* 2014; Oh *et al.,* 2014b). Chloroplastic RRFs (cpRRF) are essential for the proper functioning of chloroplasts and viability of Arabidopsis. Null mutants of this gene conveniently called *high chlorophyll fluorescence and pale green mutant 108-1 (hfp 108-1)* is embryonically lethal but plants with reduced transcript were small, pale and possessed few internal thylakoid membranes with severely reduced accumulation of chloroplast-encoded proteins (Wang *et al.,* 2010). Unlike the 2 previous genes where knockout mutants were embryonically lethal, *spl* mutants causes retardation of embryo development before the heart stage. After this stage of development, *spl* embryos morphologically remains normal albeit with highly reduced cotyledons. Furthermore, embryos dissected from the seed and rescued by tissue culture were completely white indicating an inability of plastids to form proper functioning chloroplasts (Apuya *et al.,* 2001). These embryogenesis and chloroplast biogenesis mutants however do not phenocopy the QK2 but can, to an extent, suggest that the small-sized pale-green translucent nature of its cotyledon could to be related to its seed/embryo/chloroplast development. Other *HSFA1b*-bound genes that were identified to regulate seed size or embryo development include but not limited to *SHORT HYPOCOTYL UNDER BLUE1 (SHB1), MICRORNA172A (MIR172A), EMBRYO SAC*

DEVELOPMENT ARREST 25 (EDA25), AUXIN RESPONSE FACTOR 2 (ARF2), POLLEN DEFECTIVE IN GUIDANCE 1 (POD1), and HEAT SHOCK TRANSCRIPTION FACTOR B2a (HSFB2a). Other chloroplast biogenesis and embryogenesis genes not found in the *35S:HSFA1b* bound developmental genes dataset like *EMBRYO-DEFECTIVE DEVELOPMENT (EDD1;* Uwer, Willmitzer and Altmann, 1998) and RASPBERRY3 (RSY3; Apuya *et al.,* 2002) have mutants that are impaired in chloroplast development and embryo lethal however, plants heterozygous for RSY3 are small, have pale-green cotyledons and leaves till they reached adult stage (Apuya *et al.,* 2002). Further search of literature uncovered 3 chloroplast deficient mutants restricted only to the cotyledon. *SIGMA FACTOR 2 (SIG2)* anti-sense plants have a pale-green cotyledon and normal true leaves as opposed to knockout mutants pale-green rosettes though chlorophyll deficiency was more severe in the cotyledon of anti-sense plants than knockout. It was suggested that the difference in chlorophyll deficiency was as a result of a reduced *psbA* expression in the anti-sense plants than in the knockout (Privat *et al.,* 2003). *WHITE COTYLEDONS (WCO)* mutants on the other hand results in small plants with albino (white) cotyledons but require the addition of sucrose to survive until true leaves emerge. *wco* mutants accumulate low levels chloroplast mRNAs with abnormal chloroplasts but nuclear-encoded genes for photosynthetic-related proteins were expressed similar to wildtype levels (Yamamoto *et al.,* 2000). Finally, *SNOWY COTYLEDON (SCO)* mutants *sco1* have an amino acid substitution in the gene coding for chloroplast elongation factor G in the predicted 70S ribosome-binding domain (Albrecht *et al.,* 2006). The mutation causes white cotyledons early in development but start to green much later except at the tips. The *sco1* mutants have similar chloroplast mRNA transcript levels but a significant reduction in photosynthetic proteins in 3-day old seedlings. Chloroplast development was also greatly

impaired in the cotyledons but not in the true leaves which was reflected in the transcript levels of some nuclear genes like *LHCP* and *POR B* (Albrecht *et al.,* 2006). So far, the *sco1* mutants is the only mutant that phenocopies the QK2 mutant in that they have a delayed germination, slower growth rate, smaller rosettes and a reduced seed weight compared to wildtype. Although the mutation in the *sco1* mutant did not cause a reduction in the transcript level of the gene, chloroplast mRNA translation was hampered in the cotyledon leading to reduced chlorophyll accumulation and other developmental processes. It would be interesting to see if any of the chloroplast biogenesis and embryogenesis genes highlighted are affected in the QK2 mutant which could potentially explain its characteristic phenotype.

In addition, 9-day old QK2 plants grown at 27°C had green cotyledons compared those grown in normal conditions (Fig 4.10b) suggesting that the genes responsible for cotyledon greening in QK2 is temperature regulated and possibly by HSF? A literature search revealed 3 Arabidopsis HSPs implicated in cotyledon greening and regulated by heat and HSF namely; Chloroplastic *HSP90.5*, already discussed above and 2 stromal HSP70s (*cpHSC70-1* and *cpHSC70-2*) important for chloroplast development, protein translocation into chloroplasts as well as thermotolerance of germinating seed (Su and Li, 2008, 2010; Latijnhouwers *et al.,* 2010; Feng *et al.,* 2014). While knockout mutants of *cpHSC70-1* exhibit variegated cotyledons and growth retardation, *cpHSC70-2* knockout plants had no phenotype (Su and Li, 2008). It was found that both knockout mutants were affected in photosynthetic and non-photosynthetic protein import into the chloroplasts during early stages of plant development including significantly reduced import efficiencies as they grew older (Su and Li, 2010). Furthermore, *cpHSC70-1 cpHSC70-2* double knockouts are embryo lethal but co-suppression and/or RNAi of both genes

revealed a white plant with stunted growth which suggest an important role in plant and chloroplast development (Latijnhouwers *et al.,* 2010). Interestingly, *cpHSC70-1*, *cpHSC70-2* and *HSP90.5* are significantly upregulated in both controls (Col-0 and Ws-0) compared to QK after heat stress (Liu *et al.,* 2011; Yoshida *et al.,* 2011). Similarly, *cpHSC70-2* and *HSP90.5* transcripts are upregulated in *hsfb1/hsfb2b* double knockout under both normal and heat stress condition suggesting that these chloroplast genes are regulated by HSFs (Ikeda *et al.,* 2011). Additionally *HSP90.5* and *cpHSC70-1* are both bound and differentially expressed in *35S:HSFA1b* plants compared to wildtype under both NS and HS conditions. Scanning the promoter regions of all 3 chloroplastic genes revealed the presence of HSE elements further strengthening the argument. *HSP93-V* and *Tic40* are also involved in chloroplast protein import but do not possess an HSE element on their promoters nor where they detected in the QK microarray or *35S:HSFA1b* dataset (Liu *et al.,* 2011; Yoshida *et al.,* 2011; Albihlal *et al.,* 2018). Therefore, it can be theorised that knocking out the HSFA1s resulted in the downregulation of one or all 3 chloroplastic genes under normal conditions affecting cotyledon chloroplast development and greening. Meanwhile, subjecting the QK2 mutant to a mild temperature increase (27°C) increased the transcript levels of one or all three chloroplastic genes to wildtype levels thereby increasing cotyledon greening (Fig 4.10b). This hypothesis however, is yet to be confirmed.

4.4 CONCLUSION

A quadruple knockout (*QK2)* mutant in a Col-0 background was successfully generated using the engineered nuclease CRISPR/Cas9 by introducing mutations in the functional *HSFA1*a gene in an existing TKO triple mutant disrupting its function (Fig 4.6B; 4.7A). Morphologically, the biggest observable difference between the QK2 and wildtype is in the small pale-green cotyledons (Fig. 4.8). Under normal growth conditions however, the small QK2 mutant catches up, in terms of growth and development, to wildtype despite a delayed germination (Fig 4.8). This is only possible as long as the growing conditions remain constant. Any slight alteration to growing conditions (e.g. higher temperature) derails proper development of the QK2 mutant (Fig. 4.9; 4.10).

The mutant was sensitive to different heat regimes as it lost its ability to regulate heat responsive genes affecting basal and acquired thermotolerance (Fig.4.9C). Furthermore, the QK2 mutant failed to show typical characteristic of plants growing under a mild temperature increase (27°C) i.e. petiole and hypocotyl elongation (Fig. 4.10A, C) which implicates the hormone signalling pathway. Aside from being involved in promoting growth and development, Brassinosteroids have been implicated in protecting the translational machinery and heat shock protein synthesis after heat stress as well as tolerance to a range of abiotic stresses in different plant species (Dhaubhadel *et al.,* 2002; Mü *et al.,* 2002; Anuradha and Rao, 2003; Kagale *et al.,* 2007; Kurepin *et al.,* 2008; Bajguz and Hayat, 2009). Growth promotion via BR has also been linked to the proper functioning of HSP90 (Samakovli *et al.,* 2014). Therefore, there is a possibility that the loss of HSFA1s affects BR accumulation directly via TFs involved in BR signalling and biogenesis or indirectly by affecting the expression of HSP90 in normal and heat stress condition.

Loss of HSFA1s also affected the expression of other heat stress responsive genes as well as developmental genes identified to be directly regulated by *HSFA1b* (one member of the HSFA1s; Fig. 4.11A). The difficulty in making concise conclusions when the expression a few developmental TFs were tested in the original QK (Col-0 and Ws parental background) fuelled the decision to generate the QK2 mutant in a single parental background (Col-0; Fig.3.4B, 3.6). A side by side comparison showing difference in expression patterns of those developmental TFs between QK and QK2 justified the importance of generating a mutant in a single accession (Fig. 4.11B). It removes the complexity of relating results from the mutant to 2 controls that are morphologically and physiologically different.

CHAPTER 5

Regulation of microRNA Expression by HSFA1s

5.1 INTRODUCTION

Plants process and accumulate 21-24nt small RNAs (sRNAs) at different stages of growth and development which interfere with gene expression either by degradation or translation inhibition of target mRNAs that display near perfect complementarity to them. Small RNAs are classified into microRNAs (miRNAs), trans-acting siRNAs (ta-siRNAs), natural antisense siRNAs (nat-siRNAs), repeat associated siRNAs (rasiRNAs) and long siRNAs (lsiRNAs) which are 30-40nt in size (Bartel, 2004; Katiyar-Agarwal *et al.,* 2007). miRNAs are predominantly 21nt that regulate gene expression post-transcriptionally (Bartel, 2004). In Arabidopsis, the miRNA pathway begins when miRNA precursors (pri-miRNA) are transcribed by RNA polymerase II (Pol II) into pre-miRNAs that forms a stem-loop structure (Bologna and Voinnet, 2014). The step-loop structure is then further processed by *DICER-LIKE (DCL)* RNaseIII proteins to form a miRNA:miRNA* duplex with a 2nt 3' overhang in each strand. There are 4 DCL proteins in Arabidopsis; DCL1 mainly processes miRNAs while DCL2, DCL3 and DCL4 processes long double stranded RNAs (dsRNAs) into 22-24nt nat-siRNAs, ta-siRNAs and other siRNAs (Henderson *et al.,* 2006). After processing by DCL1, the miRNAs duplexes are then 2'-O-methylated by *HUA ENHANCER 1 (HEN1)* to protect against exonuclease degradation (Li *et al.,* 2005b). The miRNA strand of the duplex is then loaded into the RNA-induced silencing complex containing an ARGONAUTE (AGO) protein which acts as a RNA slicer. The miRNA strand acts as a guide for AGO-mediated cleavage or translation repression with near perfect complementarity to its target gene resulting in its downregulation (Xie *et al.,* 2012).

There are 10 AGO proteins in Arabidopsis divided into 3 phylogenetic clades based on protein similarity: AGO1, AGO5 and AGO10; AGO2, AGO3 and AGO7; and AGO4, AGO6,

AGO8 and AGO9 (Vaucheret, 2008; Fig. 5.1A). Developmentally, *ago1* mutants have a severe phenotype compared to other members that either exhibit limited (*ago7* and *ago10*) or no obvious defects (*ago2, ago3, ago4, ago5, ago6 and ago9*; Vaucheret, 2008). The different *ago1* knockout mutants are either lethal (null) or with severe developmental defects (hypomorphes) but deficient in PTGS (Fig. 5.1B, C; Morel *et al.,* 2002). For example, the hypomorphic *ago1-27* has a reduced growth, highly serrated leaves, delayed flowering and deficient in PTGS, nonetheless still viable (Fig. 5.1C). This is as a result of a single nucleotide substitution (C -> T) at the 3' end of the coding sequence changing the amino acid from Alanine to Valine (Morel *et al.,* 2002). Due to the varying developmental effect of *ago1* hypomorphes, it is suggested that PTGS is more sensitive to AGO1 perturbation than is development (Morel *et al.,* 2002). On the other hand, while *ago2* null mutants have no developmental effects, *ago10* mutants have defective shoot apical meristem (Lobbes *et al.,* 2006; Zhu *et al.,* 2011).

AGO1 is the main ARGONAUTE protein involved in the miRNA pathway because most miRNAs have a 5'-terminal uridine which AGO1 preferentially associates with (Mi *et al.,* 2008; Takeda *et al.,* 2008), although *miR165/166*, which has a 5'-uridine, associates almost exclusively with AGO10 (Zhu *et al.,* 2011). Other miRNAs with 5'-adenosine or 5'-cytosine associates preferentially with AGO2, -4, -6 and 9 and AGO5 respectively (Mi *et al.,* 2008; Takeda *et al.,* 2008). Of the 10 AGO proteins, only *AGO1* and *AGO2* are post-transcriptionally regulated by *miR168* (Vaucheret *et al.,* 2004) and *miR403* (Allen *et al.,* 2005). In addition, both AGO1 and AGO2 are also known to play antiviral roles (Morel *et al.,* 2002; Garcia-Ruiz *et al.,* 2015).

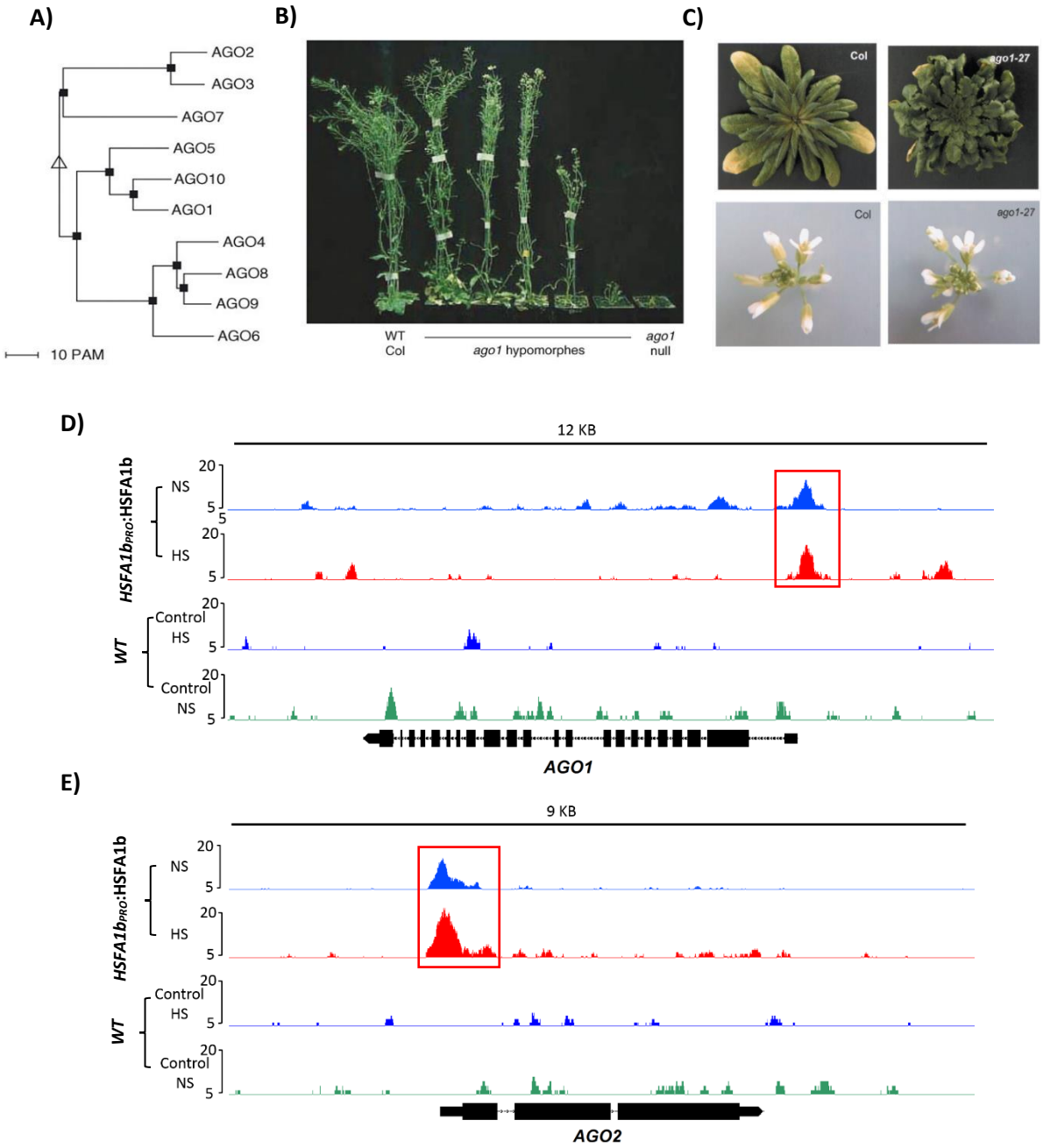


Figure 5. 1. Arabidopsis ARGONAUTE proteins, mutant phenotypes, binding and expression data. A) Phylogenetic classification of AGO proteins divided into 3 clades. PAM indicates the point accepted mutation, the minimal distance between sequences. **B)** Phenotypes of *ago1* mutants grown in long-day conditions. Null mutants are dwarf and sterile (right). Hypomorphic alleles exhibit amino acid substitutions within various domains of the protein, and developmental defects that vary between null mutants and wild type (WT) plants. **C)** *ago1-27* hypomorph showing developmental severity to Col-0. **D)** Binding of *HSFA1b* to AGO1 and AGO2 **(E)** during no stress (NS, Blue) and heat stress (HS, Red) in native promoter *HSFA1b* plants (*HSFA1b*$_{PRO}$:HSFA1b) compared to Control plants (WT Col-0). Red rectangles indicate binding intensity. Images in pane A and B are from Vaucheret, H. (2008) while C is from (Vaucheret *et al.,* 2004).

It was recently established after surveying *HSFA1b* genome-wide binding and *35S:HSFA1b* transcriptomics data that *HSFA1b* binds to the promoters of *AGO1* and *AGO2* under both normal and heat stress conditions altering their expression (Fig 5.1D, E; Albihlal *et al.,* 2018). This was the first time AGO proteins have been linked with HSFs. Interestingly, a survey of other proteins involved in the sRNA biogenesis pathway showed different expression patterns but were not directly bound by *HSFA1b* under any condition (Appendix 4A; Albihlal *et al.,* 2018). The binding of *HSFA1b* to AGO1/2 highlights a possible mechanism of how *HSFA1b* and other members of the clade might regulate some aspects of development via miRNAs since both genes play important roles in growth and stress defence (Sunkar, 2012; Bechtold *et al.,* 2013; Albihlal *et al.,* 2018). Therefore, understanding the interplay between *HSFA1b*, AGO proteins (AGO1 and 2) and miRNAs during stress might uncover a new pathway by which the switch from growth to stress defence and vice versa is attained. To this effect, this chapter aims to investigate how the binding of *HSFA1b* (or HSFA1s) to AGO1/2 during stress might regulates Arabidopsis development and/or developmental genes via the action of miRNAs.

5.2 RESULTS

5.2.1 *AGO1* and *2* Expression is Perturbed by Heat and *HSFA1b* Overexpression

As mentioned previously *AGO1* and 2 was identified to be bound and regulated by *HSFA1b* in both normal and heat stress condition following results from ChIP- and RNA-SEQ data (Fig. 5.1D; Appendix 4A; Albihlal *et al.,* 2018). To confirm, *AGO1* and 2 transcript levels were checked in heat stress wildtype plants and unstressed transgenic *35S:HSFA1b* plants. In agreement with transcriptomics data, *AGO*1 and 2 expression was significantly upregulated in wildtype heats stress plants compared to unstressed wildtype plants. In unstressed *35S:HSFA1b* plants however, it was not significantly altered (Fig 5.2A, top row). Since both AGO genes were bound by *HSFA1b* under heat stress, attention was shifted to test if other members of clade A1 could also regulate their expression. Firstly, promoter regions of both AGO genes was examined for the presence of the canonical HSE elements which is required for HSF binding. Results showed that both *AGO1* and 2 possessed an intact HSE element on their promoters located 369 bp and 197 bp upstream of the translation initiation site (ATG) respectively in agreement with the ChIP-SEQ data (Fig. 5.2B; Albihlal *et al.,* 2018). To then investigate the possibility of their expression being regulated by HSFA1s, transcript expression of both genes were tested in wildtype and QK2 plants in normal and heat stress conditions. Results indicate the failure of both *AGO1* and *AGO2* transcripts to accumulate in the QK2 mutant under heat stress conditions compared to wildtype (Fig 5.2A, bottom row) suggesting HSFA1s are needed for the accumulation of *AGO1* and *AGO2* transcripts under heat stress. Furthermore, under normal conditions, their expression remained unchanged in the QK2 plants compared to wildtype.

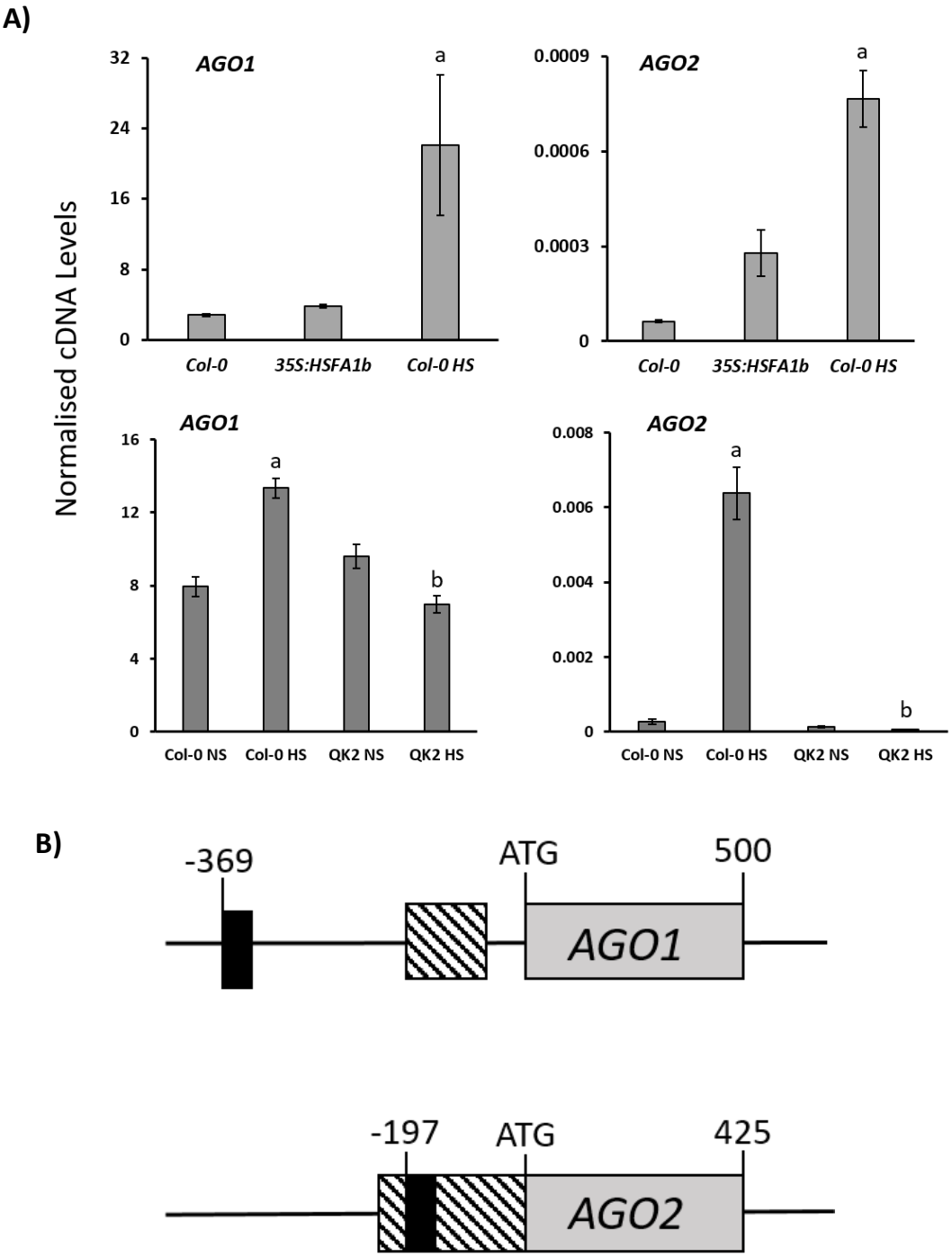


Figure 5. 2. Expression of ARGONAUTE genes in different plant genotypes. A) Expression of *AGO1* and *AGO2* in 5 week old plants grown in soil at control temperatures (22°C) or heat stress (37°C for 30 mins), in SD conditions. Expression was normalised against 2 reference genes *PP2AA3* and *TIP41-like*. 3 biological replicates were used to determine transcript level. **B)** Schematic diagram of *AGO1* and *AGO2* transcript. Black box represents HSE element, diagonal striped box represents 5' UTR, and grey box represents coding region. ATG; translation initiation site; numbers is the sequence distance from TSS. NS, No stress; HS, Heat stress. 'a' means significant difference to Col-0 NS; 'b' means significant difference to Col-0 HS (*p value* <0.05; ANOVA and Tukey's HSD post hoc test).

5.2.2 Generating the *35S:HSFA1b-ago1/2* Mutant

To determine if the developmental effect of *35S:HSFA1b* was as a result of altered *AGO1* and 2 expression, *ago1/2* double knockout (dko) plants (♀, Garcia-Ruiz *et al.,* 2015) was crossed with *35S:HSFA1b* (♂; Bechtold *et al.,* 2013). The resulting F1 seed was screened for the presence of the monomeric Red Fluorescent Protein (mRFP) by RT-PCR which is fused to *35S:HSFA1b* (Fig 5.3A). The F1 mutant with positive mRFP band (Fig 5.3A, lane 3) was taken to the next generation and screened for the different mutant permutations. To screen for the *ago1* mutation, 2 reverse primers with either 1 or 2 mismatches to the wildtype allele were designed. Both were tested and the reverse primer with 2 mismatches was sufficient to differentiate the wildtype allele from the *ago1* mutant allele (See Appendix 4B). *ago2* was screened using SALK LBb 1.3 and *ago2* reverse primer while mRFP forward and reverse primers were used for *35S:HSFA1b* (See Appendix 6A for primer list). Different mutant combinations were generated including the desired *35S:HSFA1b-ago1/2* mutant (Lane 3), 35SHSFA1b (Lane 1), ago1/2 dko (Lane 2) and wildtype (Lane 8; Fig. 5.3B). Phenotypic differences between the different mutants compared to wildtype plants were then investigated.

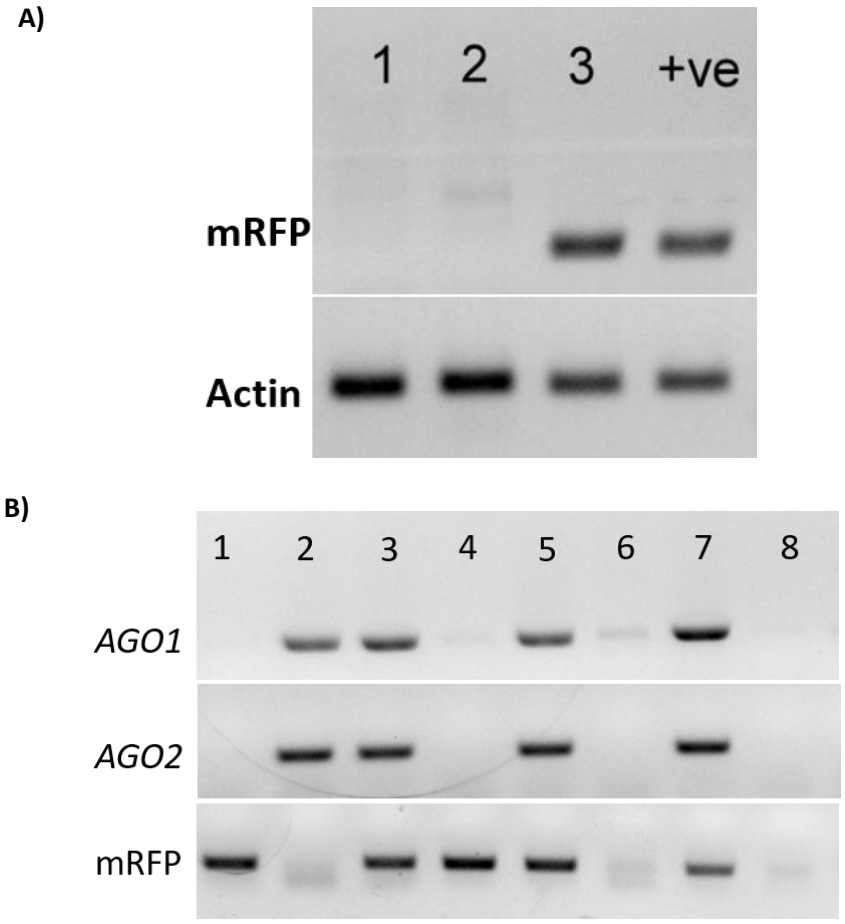


Figure 5. 3. Gel images showing PCR and RT-PCR screening of the different mutants. **A)** RT-PCR Gel image of 3 F1 plants after crossing. Primers were against mRFP. **B)** Gel image confirming different mutants isolated. Lane 1- *35S:HSFA1b*; 2- *ago1/2 dko*; 3- *35S:HSFA1b*-- *ago1/2 dko*; 4- *35S:HSFA1b*; 5- *35S:HSFA1b-ago1/2 ko*, 6- WT; 7- *35S:HSFA1b-ago1/2 ko*, 8- WT. *ago 1 KO* primers span the amino acid substitution; *ago 2 KO* spans the T-DNA; *35S:HSFA1b* spans mRFP.

5.2.3 Phenotypic Differences between Generated Mutants

Developmental differences observed in *35S:HSFA1b* plants (Chapter 3.2.2) were determined in the following mutants; *35S:HSFA1b-ago1/2 dko, ago1/2 dko* and *35S:HSFA1b* compared to wildtype to identify if the phenotypic difference observed in *35S:HSFA1b* was due to changes in *AGO 1* and 2 expression. Rosette area of 5-week-old soil-grown plants was measured in all mutants compared to wildtype. While there was a significant size reduction in *35S:HSFA1b* plants compared to wildtype as previously reported (Chapter 3.2.2), there was no size difference observed in the *35S:HSFA1b-ago1/2 dko* mutants compared to *ago1/2 dko* (Fig 5.4A). Furthermore, the flowering time in all mutants was also determined. Flowering time was determined by counting the number of days until the flowering bolt was >2cm (Boyes *et al.,* 2001). Results showed that *35S:HSFA1b* flowered a day earlier than wildtype as well as the *35S:HSFA1b-ago1/2 dko* compared to the *ago1/2 dko* in long day conditions (Fig 5.4B; 16h light/ 8h dark). Similarly, inflorescence height was determined when more than half the number of plants had open flowers (Boyes *et al.,* 2001). Once more, longer inflorescence was observed in *35S:HSFA1b* compared to wildtype and *35S:HSFA1b-ago1/2 dko* when compared to *ago1/2 dko* (Fig 5.4C). Taking together, the phenotypic results observed in all mutants suggests that *HSFA1b* might be in the same pathways *AGO1* and 2 during plant growth since the observed phenotype in *35S:HSFA1b-ago1/2 dko* were not additive compared to *35S:HSFA1b* and *ago1/2 dko* respectively.

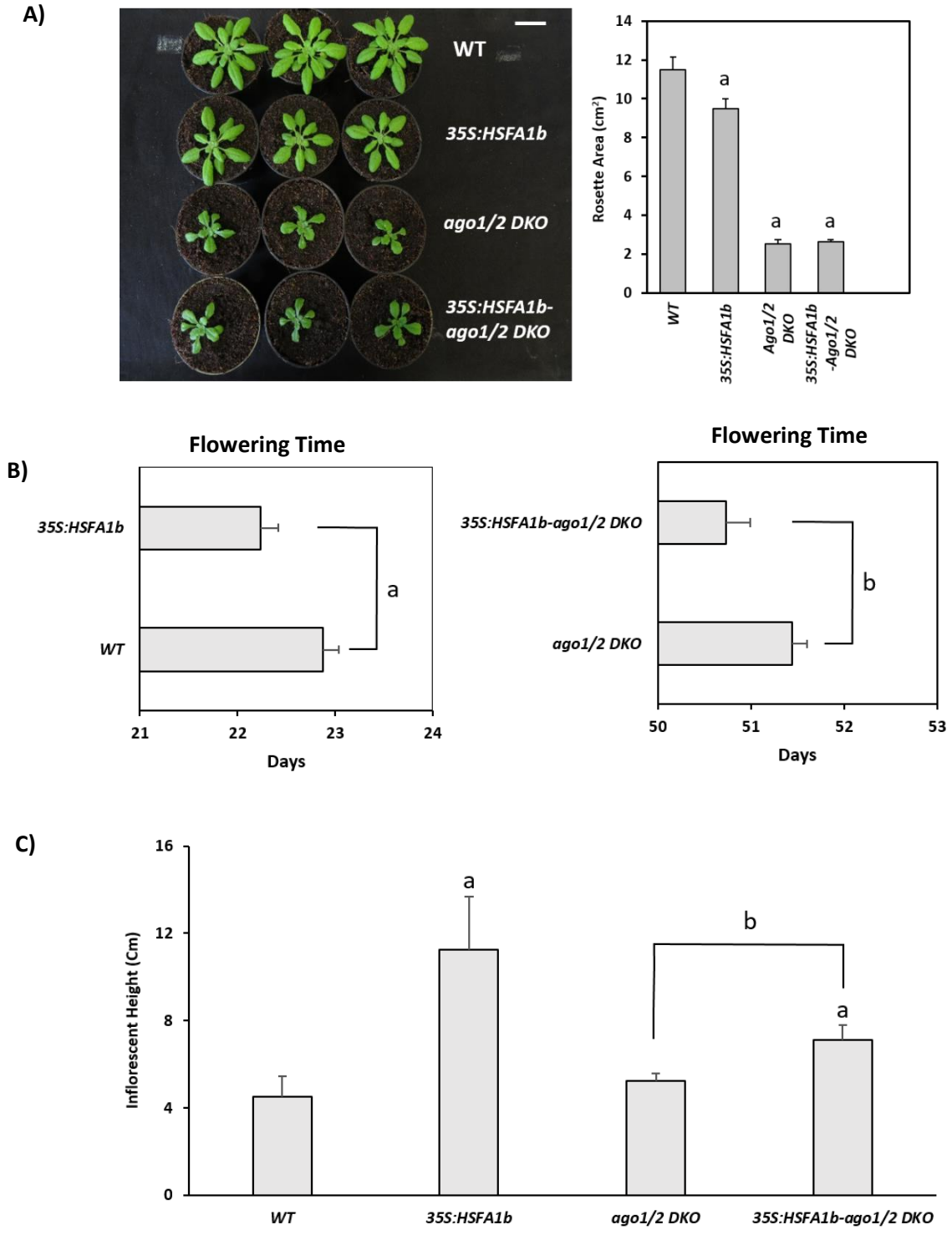


Figure 5. 4 . Growth Phenotype of different plant genotypes. **A)** Rosette area of WT and *35S:HSFA1b, ago1/2 dko* and *35S:HSFA1b-ago1/2 dko* grown in short day on soil at 22°C (n=12). Measurement were taken 5 weeks post germination (left panel and right panel). Scale bar = 2cm. **B)** Flowering time (i.e. number of days flowering bolt ≥ 1cm) of WT and *35S:HSFA1b* (left panel); ago1/2 dko and 35S:HSFA1b-ago1/2 dko (right panel) grown on soil in LD conditions at 22°C (n=50). **C)** Inflorescent height of WT and *35S:HSFA1b*, *ago1/2 dko* and *35S:HSFA1b-ago1/2 dko*. Plants were grown in soil under SD conditions for 4 weeks and then transferred to LD to induce flowering at 22°C Inflorescence was measured when half the number of plants (n = 20) had open flowers. (n=20). 'a' means significant difference to WT; 'b' means significant difference to *ago1/2 dko* (*p value* <0.05; ANOVA and Tukey's HSD post hoc test).

5.2.4 *35S:HSFA1b-ago1/2 dko* Plants Have Increased Oomycete Resistance

Resistance to the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) had been previously reported in the *35S:HSFA1b* mutant compared to wildtype (Bechtold *et al.,* 2013). Similarly, miRNAs and ARGONAUTE proteins have also been implicated in the resistance of viral, bacterial as well as oomycete pathogens (Qu *et al.,* 2008; Schuck *et al.,* 2013; Wong *et al.,* 2014; Garcia-Ruiz *et al.,* 2015). Therefore, it was imperative to determine if the mechanism of resistance to *Hpa* in the *35S:HSFA1b* mutant was attained via miRNAs. To achieve this, 2-week-old plants of *35S:HSFA1b-ago1/2 dko, ago1/2 dko, 35S:HSFA1b* and wildtype were infected with virulent *Hpa* WACO9 (See methods chapter 2.3.24) to determine basal level of resistance. Spores from all infected mutants were collected in a known volume and countered using a haemocytometer. Due to the variability in spore counting, the experiment was repeated 3 times. Results showed that *35S:HSFA1b* was more resistant to the pathogen compared to the wildtype due to the reduced number of oospores counted (42% reduction; Fig. 5.5). However, similar result was also observed between *35S:HSFA1b-ago1/2 dko* compared to *ago1/2 dko* with an 88% reduction in spore count (Fig 5.5). When compared to wildtype (Col-0), spore count was reduced in both *ago1/2 dko* and *35S:HSFA1b-ago1/2 dko* by 73% and 97% respectively suggesting that the mutants are less susceptible to *Hpa* infection. These results also suggest that the mechanism/pathway for pathogen resistance in *35S:HSFA1b* plants are separate from that of *ago1/2 dko* plants and might not involve miRNAs.

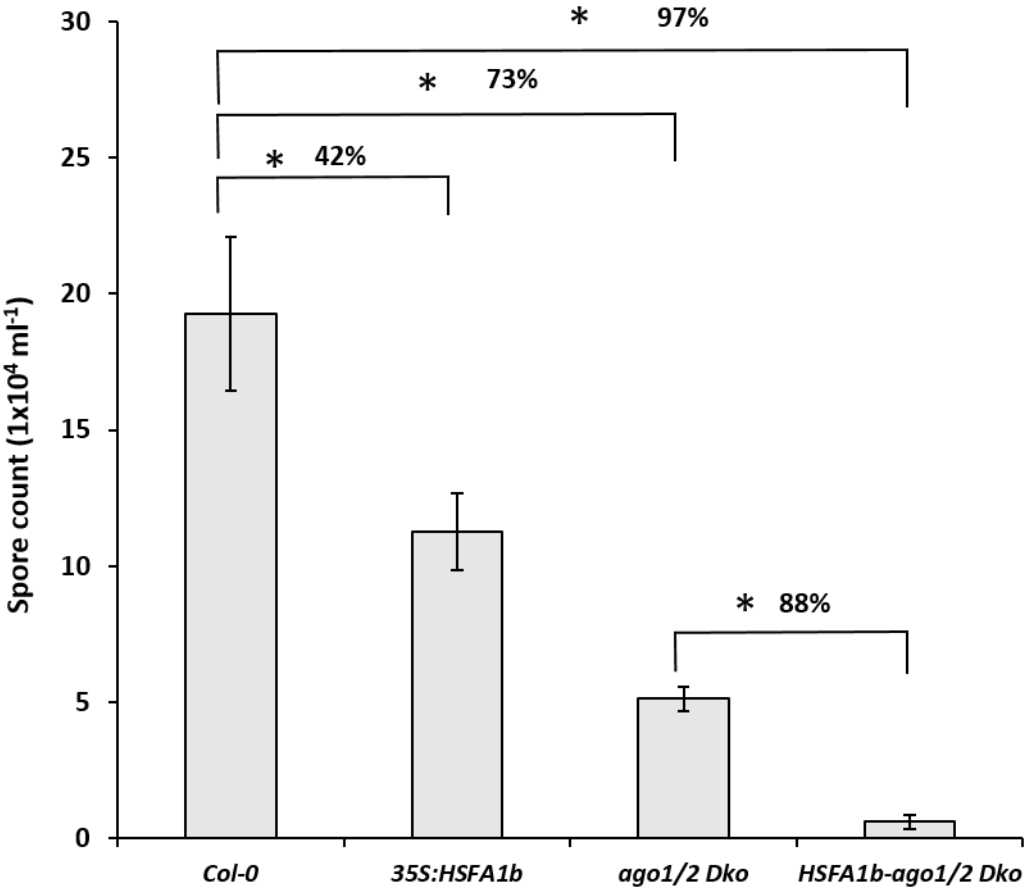


Figure 5. 5 . Analysis of oomycete Resistance in the different plant genotypes. 2 week old seedlings grown on soil in SD at 22°C were sprayed with *Hyaloperonospora arabidopsidis* Waco9 spores at $5x10^4$ spores/ml. Spores were counted 7 days after spraying. Numbers represents percentage reduction in spore count. '*' means significant difference (*p value* <0.05; ANOVA and Tukey's HSD post hoc test). Data is a mean of 3 replicates.

5.2.5 Hydrogen Peroxide Concentration Is Altered In *ago1/2 dko* Mutant

The relationship between miRNAs and a change in plants redox state was previously outlined when different transgenic and knockout lines of copper/zinc Superoxide dismutase (Cu/Zn–SOD, also known as CSD) genes, regulated by *miR398*, showed altered H_2O_2 levels under heat stress (Guan *et al.,* 2013). Superoxide dismutase (SOD) is an important reactive oxygen species (ROS)-scavenging enzyme that catalyses conversion of superoxide radicals to H_2O_2 and O_2 (Fridovich, 1995). The Arabidopsis genome encodes three CSD isozymes: *CSD1* in the cytoplasm, *CSD2* in chloroplasts, and *CSD3* in peroxisomes (Kliebenstein *et al.,* 1998). Plants harbouring *miR398*-resistant forms of *CSD1*, *CSD2* and their copper chaperone*; CCS* led to a reduction in H_2O_2 levels compared to wildtype under heat stress while the opposite was observed for *csd1*, *csd2* and *ccs* knockout mutants under the same conditions. Under normal conditions however, H_2O_2 levels remained the same (Guan *et al.,* 2013). As a consequence, H_2O_2 levels in *35S:HSFA1b-ago1/2 dko* and *ago1/2 dko* was determined since it has been shown previously that over-expressing *HSFA1b* plants have increased endogenous H_2O_2 levels compared to wildtype (Bechtold *et al.,* 2013). Results showed as expected a 108% increase in H_2O_2 level in *35S:HSFA1b* plants compared with wildtype confirming previously reported observations (Fig. 5.6A). Interestingly, there was a 29% reduction in H_2O_2 in the *ago1/2 dko* mutant compared with wildtype suggesting a relationship between the plant redox state and miRNAs. In *35S:HSFA1b-ago1/2 dko* plants however, over-expressing *HSFA1b* was sufficient to increase H_2O_2 concentration to within wildtype levels but 20% higher than *ago1/2 dko* mutants (Fig.5.6A) also suggesting a link between the plant redox state regulated in part by *HSFA1b* and miRNA function via AGO1 and/or 2.

Furthermore, it is known that altered redox state affects expression of heat-responsive genes (Volkov *et al.,* 2006). Therefore real-time quantitative RT–PCR analysis was performed to check the expression of some classic heat stress responsive (HSR) genes. *MBF1c, HSP70,* and *HSP90* were significantly reduced in *ago1/2 dko* plants compared to wildtype while they were significantly increased in *35S:HSFA1b* plants (Fig 5.6B). In *35S:HSFA1b-ago1/2 dko* plants however, HSR genes were increased compared with *ago1/2 dko* but comparable to wildtype (within 1-1.8 fold increase). This pattern was also similar to the H_2O_2 concentration observed in those plants suggesting that a change in redox state of the different mutants indeed alters the expression of heat stress responsive genes under normal conditions (Fig. 5.6B).

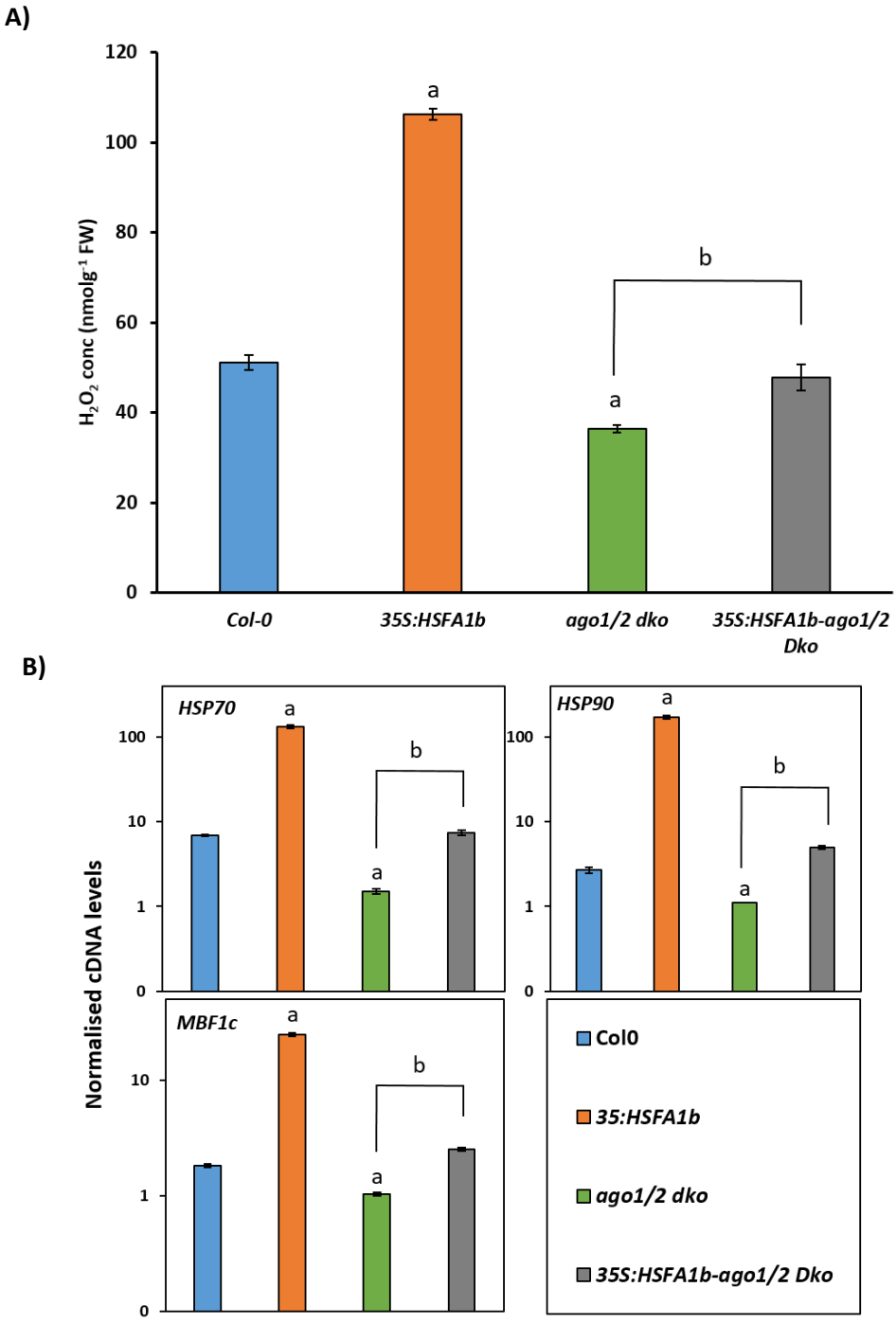


Figure 5. 6. Redox state of different plant genotypes correlates with expression of Heat stress responsive genes. A) 2 week old seedlings grown on soil in SD at 22°C were pooled, flash frozen and ground in buffer with amplex red. H_2O_2 was then measured from the supernatant against a H_2O_2 standard curve. **B)** Gene expression (Log10) of heat responsive genes in 3 week old seedlings grown on ½ MS media supplemented with 1% glucose in SD condition at 22°C. Expression was normalised against PP2AA3 with 3 biological replicates used to determine transcript level. 'a' means significant difference to Col-0; 'b' means significant difference to *ago1/2 dko* (*p value* <0.05; ANOVA and Tukey's HSD post hoc test).

5.2.6 MIR398b Induction under Heat Stress Is Regulated By HSFA1s

According to Guan, *et al*. (2013), *miR398* is rapidly induced in Arabidopsis subjected to heat stress, down-regulating 3 of its targets: *CSD1, CSD2* and *CCS*. Transgenic plants expressing *miR398*-resistant versions of *CSD1, CSD2* or *CCS* are hypersensitive to heat stress, and expression of many heat stress-responsive genes (HSF and HSP genes) under high-temperature conditions is reduced in these plants. They also identified two HSFs (*HSFA1b* and *HSFA7b*) that were responsible for heat induction of *miR398* which in turn regulates CSD expression. They conclude that HSF genes, *miR398* and its target genes *CSD1, CSD2* and *CCS* are essential for plant thermotolerance. The expression of 2 CSD genes (*CSD1* and *CSD2*) and its miRNA precursor (*MIR398b*) in all mutants under no stress were examined since the *ago1* mutants are deficient in post transcriptional gene silencing. Results show an increase in *CSD1* and 2 transcripts; and a decrease in expression of its miRNA precursor *MIR398b* in both *ago1/2 dko* and *35S:HSFA1b-ago1/2 dko* (Fig. 5.7A). The transcript level of *MIR398b* in WT and QK2 under normal and heat stress was also examined to determine if any member of the HSFA1s regulated its expression. Results show a reduction in *MIR398b* transcript level in QK2 compared to WT in both conditions (Fig. 5.7B) agreeing with the observations made by Guan and colleagues.

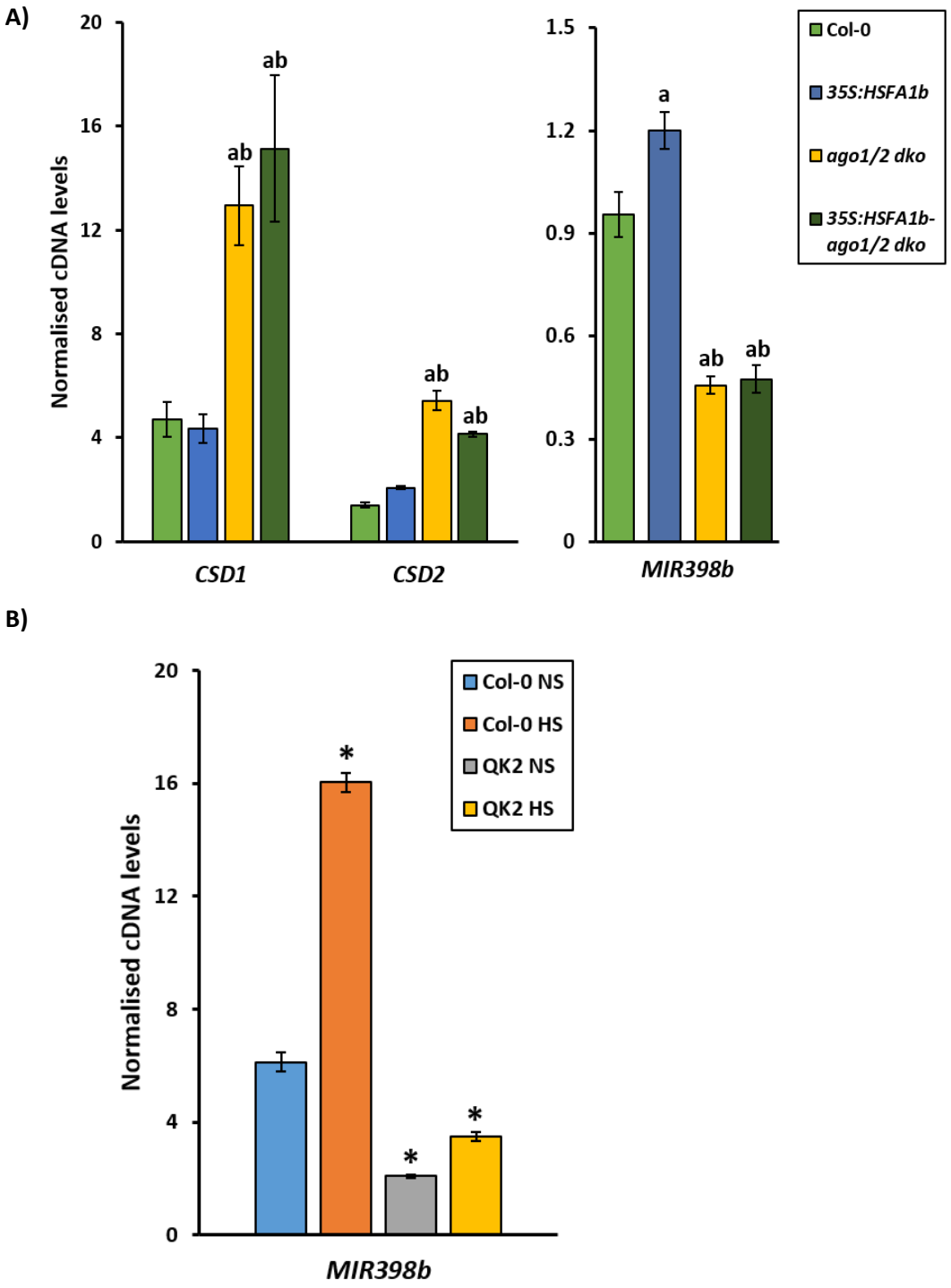


Figure 5. 7. CSD Transcript Expression in different genotypes and treatment. **A)** CSD and its pri-miRNA (MIR398b) transcript expression was determined using qRT-PCR on cDNA transcribed from RNA of 3 week old seedlings grown on soil in SD at 22°C of the different genotypes, Col-0 (WT); *35S:HSFA1b*, *ago1/2 dko* and *35S:HSFA1b-ago1/2 dko* under no stress. **B)** qRT-PCR of MIR398 pri-miRNA in 5 week old Col-0 and QK2 mutant grown on soil in SD at 22°C (NS) before subjecting to 30 mins of heat stress at 37°C (HS). Expression was normalised expression against *PP2AA3* and *TIP41-like*. Average of 3 biological replicates were used to determine transcript level. NS, No stress (22°C); HS, Heat stress (37°C for 30 mins). 'a' means significant difference to Col-0; 'b' means significant difference to *35S:HSFA1b;* '*' means significant difference to Col-0 NS (*p value* <0.05; ANOVA and Tukey's HSD post hoc test).

5.2.7 *HSFA1b* Could Indirectly Regulate Gene Expression via microRNAs

Since results above show that heat inducible *MIR398b* expression is regulated at least by members of the clade A1s, focus was shifted to identify other microRNAs that could potentially be regulated by the clade. Rather than the shotgun approach of directly testing all heat inducible miRNAs in the QK2 mutant, a more tactical approach was employed. Firstly, all upregulated transcripts (≥1.5 fold) from whole genome tiling array analyses in 15-day-old seedlings of hypomorphic *ago1-25* was obtained (*ago1-25*; Kurihara *et al.,* 2009) and overlapped with *HSFA1b* indirectly regulated developmental (IRD) and non-developmental genes (IR; Figure 5.8). Upregulated transcripts of *ago1-25* were used because defects in miRNA-mediated gene silencing was linked with increased accumulation of their target transcripts. Therefore, there is a high probability that *HSFA1b*-regulated genes, especially those indirectly regulated, whose transcripts overlap with *ago1-25* upregulated set have a potential miRNA target. Results showed that 18 IRD and 101 IR transcripts significantly overlapped with *ago1-25* upregulated genes (Figure 5.8A, B). The overlapped transcripts were then grouped together and using a plant small RNA prediction tool (psRNATarget; Dai *et al*., 2018), checked to determine the likelihood of a potential miRNA. Using the default parameters, a number of predicted miRNAs were determined using the scoring schema cut-off of ≤3 (See methods chapter 2.3.22; Table 5.1).

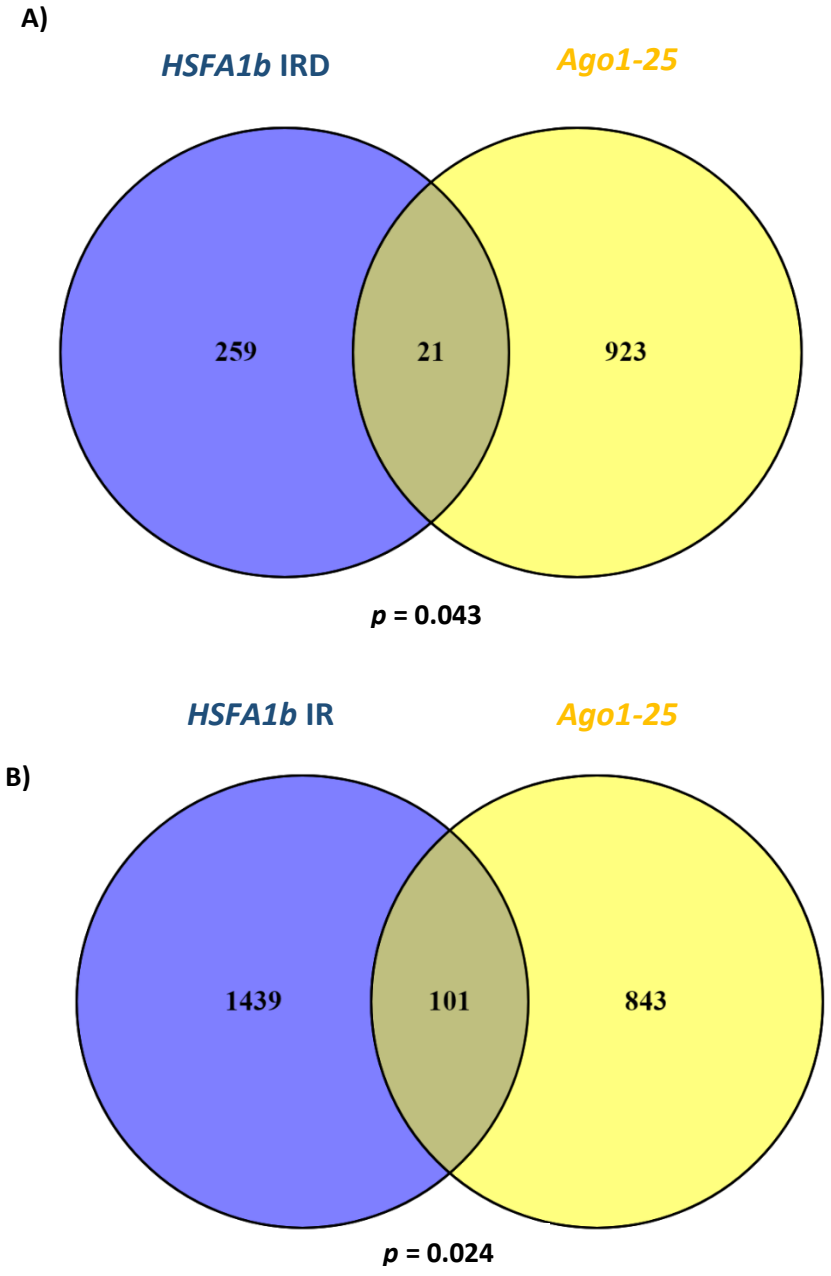


Figure 5. 8. Venn diagram showing overlap between *HSFA1b* indirectly regulated developmental/non-developmental genes and *Ago1-25* upregulated transcripts. Overlap between *HSFA1b* indirectly regulated developmental genes (IRD; **A**) and non-developmental genes (IR; **B**) with upregulated transcripts of *ago1-25.* IRD, indirectly regulated developmental; IR, Indirectly regulated non-developmental. *p* value determined by Hypergeometric test.

miRNA	Target	Schema cut-off	miRNA_aligned_fragment	Inhibition
miR5658	AT2G39710	0.5	AUGAUGAUGAUGAUGAUGAAA	Cleavage
miR171c-5p	ATAPR3	1.5	AGAUAUUGGUGCGGUUCAAUC	Cleavage
miR163	PXMT1	2	UUGAAGAGGACUUGGAACUUCGAU	Cleavage
miR157a-5p	AT2G39710	2.5	UUGACAGAAGAUAGAGAGCAC	Cleavage
miR157b-5p	AT2G39710	2.5	UUGACAGAAGAUAGAGAGCAC	Cleavage
miR157c-5p	AT2G39710	2.5	UUGACAGAAGAUAGAGAGCAC	Cleavage
miR394a	AT3G58570	2.5	UUGGCAUUCUGUCCACCUCC	Cleavage
miR394b-5p	AT3G58570	2.5	UUGGCAUUCUGUCCACCUCC	Cleavage
miR395a	CSLG3	2.5	CUGAAGUGUUUGGGGGAACUC	Cleavage
miR395d	CSLG3	2.5	CUGAAGUGUUUGGGGGAACUC	Cleavage
miR395e	CSLG3	2.5	CUGAAGUGUUUGGGGGAACUC	Cleavage
miR5641	AtBBE-like 13	2.5	UGGAAGAAGAUGAUAGAAUUA	Cleavage
miR414	CDC45	2.5	UCAUCUUCAUCAUCAUCGUCA	Cleavage
miR5015	LPR2	2.5	UUGGUGUUAUGUGUAGUCUUC	Cleavage
miR171b-5p	ATAPR3	3	AGAUAUUAGUGCGGUUCAAUC	Cleavage
miR395b	CSLG3	3	CUGAAGUGUUUGGGGGGACUC	Cleavage
miR395c	CSLG3	3	CUGAAGUGUUUGGGGGGACUC	Cleavage
miR395f	CSLG3	3	CUGAAGUGUUUGGGGGGACUC	Cleavage
miR414	LECRK1	3	UCAUCUUCAUCAUCAUCGUCA	Cleavage
miR5021	MRP4	3	UGAGAAGAAGAAGAAGAAAA	Translation
miR5021	PRH26	3	UGAGAAGAAGAAGAAGAAAA	Cleavage
miR5658	AT3G16660	3	AUGAUGAUGAUGAUGAUGAAA	Cleavage
miR5664	AKR4C9	3	AUAGUCAAUUUUAUCGGUCUG	Cleavage
miR8181	AT4G18250	3	UGGGGGUGGGGGGGGUGACAG	Cleavage
miR8182	AT3G61280	3	UUGUGUUGCGUUUCUGUUGAUU	Cleavage
miR826a	AST91	3	UAGUCCGGUUUUGGAUACGUG	Cleavage
miR168a-5p	**AGO1**	**3**	**UCGCUUGGUGCAGGUCGGGAA**	**Cleavage**
miR168b-5p	**AGO1**	**3**	**UCGCUUGGUGCAGGUCGGGAA**	**Cleavage**

Table 5. 1. List of predicted miRNAs of transcripts overlapped with *Ago1-25* (Figure 5.7) using psRNATarget with a schema cut-off of 3 (https://plantgrn.noble.org/psRNATarget/). Default parameters were used for miRNA prediction (see methods chapter 2.3.22).

AGO1 is known to be regulated by *miR168* (Vaucheret, 2004) and as a proof of concept it was expected to be predicted by psRNATarget. As seen in Table 5.1, *AGO1* (bold) was correctly predicted to be a target of *miR168a* and *miR168b*. This result suggests that the indirect regulation of some genes by *HSFA1b* occurs via miRNAs. To this effect, all *HSFA1b* IRD and IR group of genes were checked for potential miRNA target using psRNATarget since there is a possibility that some transcripts that are not upregulated ≥ 1.5 fold in *ago1-25* are overlooked. Therefore, all *HSFA1b* IRD and IR group of genes (1820) were checked for potential miRNA regulation. psRNATarget predicted 121 miRNA families which could regulated some of the 1820 genes indirectly regulated by *HSFA1b*, of which a few have been experimentally confirmed (Fig 5.9A). *TCP10* expression, which was established to be regulated by miR319, was confirmed by qRT-PCR (Fig 5.9B). Compared to wildtype expression *TCP10* expression was significantly reduced in *35S:HSFA1b* plants but remained the same when *ago1/2 dko* was compared to *35S:HSFA1b-ago1/2 dko* suggesting that miRNA is necessary for the indirect regulation of *TCP10* by *HSFA1b* (Fig 5.9B).

A)

miRNA Family	Gene symbol	Reference
miR169	*NF-YA5*	Li et al., 2008
miR159	*MYB65*	Li et al., 2016
miR858	*MYB65*	Sharma et al., 2016
miR164	*ANAC092*	Kim et al., 2009
miR319	*TCP10*	Koyama et al., 2017
miR319	*MEE33 (TCP4)*	Koyama et al., 2017
miR391	*ACA10*	Xia et al., 2013
miR826	*AOP2*	He at al., 2013
miR163	*PXMT1*	Chung et al., 2016
miR781	*AKR4C8*	Siqueira, 2015

B)

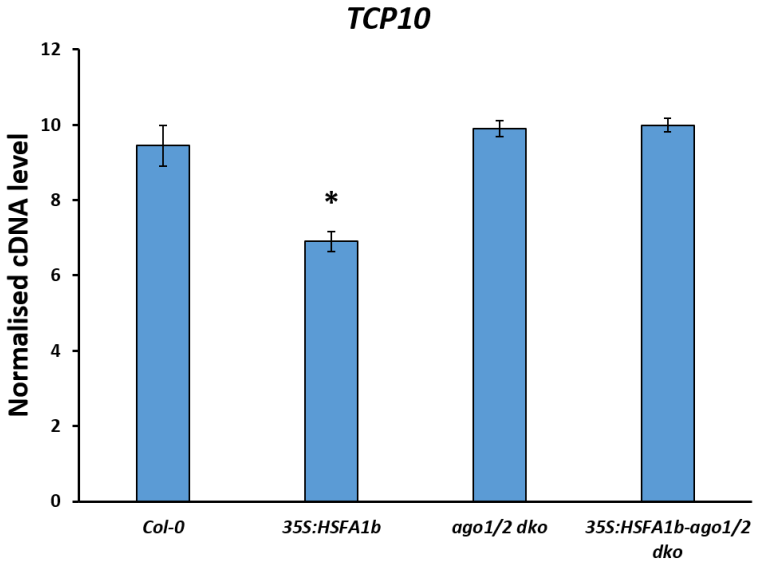


Figure 5. 9. *HSFA1b*-indirectly regulated genes regulated by miRNA. A) List of genes indirectly regulated by *HSFA1b* with published miRNA targets. **B)** Gene expression of *TCP10* in 3 week old seedlings grown on soil in SD conditions at 22°C of the different genotypes, Col-0 (WT); *35S:HSFA1b*, *ago1/2 dko* and *35S:HSFA1b-ago1/2 dko* under no stress. Expression was normalised against *PP2AA3* and *TIP41-like*. Average of 3 biological replicates were used to determine transcript level. '*' means significant difference to Col-0 (*p value* <0.05; ANOVA and Tukey's HSD post hoc test).

5.2.8 MiRNA Precursors Are Perturbed in QK2 Mutant under Normal and Heat Stress

A few miRNAs identified above were selected and checked for altered expression in QK2 compared to wildtype. A number of different methods have been proposed to query miRNA expression in different samples including using Two-tailed RT-qPCR (Androvic *et al.,* 2017). After several tries without success, attention was shifted to amplifying miRNA precursors which had been previously successful with qRT-PCR (Fig 5.7B). Primers used for amplification were obtained from published papers (Kurihara *et al.,* 2009) to ensure target accuracy. Results show that the miRNA precursors tested are perturbed in the QK2 mutant. Under normal conditions *MIR156a* was upregulated in the QK2 mutant compared to wildtype and increased further under heat stress although not up to wildtype heat stress levels. A similar but reversed pattern was observed in *MIR396a*. On the other hand, while there was no difference in expression observed in *MIR158a* and *MIR172a* transcripts between wildtype and QK2 under normal conditions, upregulation and downregulation of *MIR158a* transcripts was observed during heat stress respectively (Fig 5.10). A few other miRNA precursors were tested but amplification was not successful.

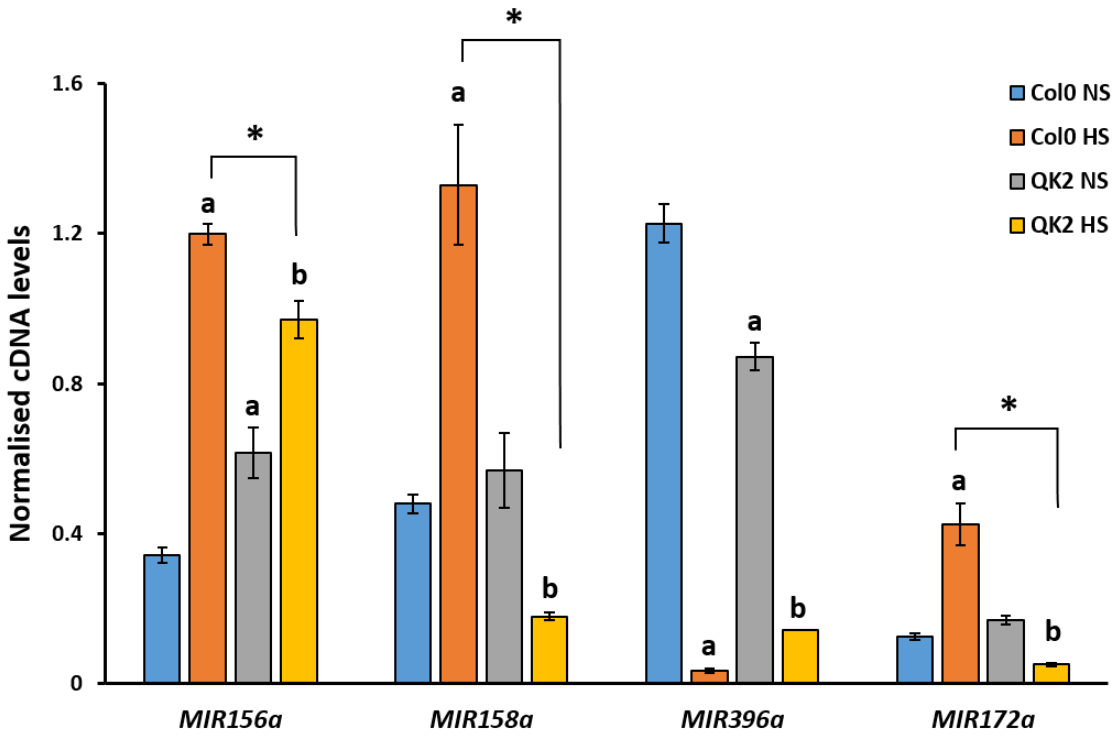


Figure 5. 10. Expression of several pri-miRNA in normal and heat stress condition. Gene expression of pri-miRNAs in 5-week old Col-0 and QK2 mutant under different stress conditions. Plants were grown on soil in SD at 22°C (NS) before subjected to 30 mins of heat stress at 37°C (HS). Expression was normalised against *PP2AA3* from 3 biological replicates. 'a' means significant difference to Col-0; 'b' means significant difference to *ago1/2 dko;* '*' means significant difference (*p value* <0.05; ANOVA and Tukey's HSD post hoc test). NS, No stress (22°C); HS, Heat stress (37°C for 30 mins).

5.3 DISCUSSION

5.3.1 AGO1 and 2 May Be Involved In *35S:HSFA1b* Developmental Phenotype

Identifying *AGO1* and *AGO2* as *HSFA1b* direct targets using ChIP-SEQ and RNA-SEQ data (Albihlal *et al.,* 2018) was interesting as it highlighted the possibility of both ARGONAUTE genes to be regulated by heat stress through HSFA1s. Their regulation was confirmed when both *AGO1* and *AGO2* transcripts were upregulated during heat stress in wildtype plants and failed to accumulate in the HSFA1 quadruple knockout mutant (QK2; Fig 5.2A) as well as the presence of HSE elements on the promoter of both genes (Fig 5.2B). Though the presence of intact HSEs extends regulation to other class A HSFs in the expression of AGO1 and 2 under stress conditions, *HSFA1b* and/or other members of the clade appear to be direct regulators. Furthermore, since both ARGONAUTE genes are known to be involved in the regulation of genes that function in development as well as biotic and abiotic stress via miRNAs (Saini *et al.,* 2012), the possibility of *HSFA1b* (and HSFA1s) to regulate development via *AGO1*, *AGO2* and miRNAs was investigated under biotic and abiotic stress.

In order to determine if the *35S:HSFA1b* transgenic plant phenotype was achieved via the interaction of *HSFA1b* with *AGO1* and 2, *ago1/2* knockout plants were crossed with *35S:HSFA1b* plants and the resulting progeny phenotyped. The idea was to determine if the observed phenotype of *35S:HSFA1b* plants were not retained in the *35S:HSFA1b-ago1/2 dko* plants. Unfortunately, this was not the case as *35S:HSFA1b-ago1/2 dko* plants had a combined phenotype of both parental genotypes (Fig 5.4) which could mean that the effect of *HSFA1b* and *AGO1* are non-additive to those developmental traits measured. However, the smaller rosette area of *35S:HSFA1b* plants compared to its control (Col-0)

was not observed in *35S:HSFA1b-ago1/2 dko* plants compared to its control (*ago1/2 dko*). This might be due to the *ago1* KO effect on plants which generally have smaller rosettes compared to wildtype, due to fitness costs, masking that observed in the *35S:HSFA1b* plants. This, therefore, suggests *AGO1* and/or 2 is involved in the reduction in rosette size of *35S:HSFA1b* plants.

5.3.2 Oomycetes Resistance Pathway Triggered by *HSFA1b* Is Independent Of miRNAs But Involves H_20_2 Signalling.

Plants elicit an immune response to defend themselves against pathogen attack which relies on the recognition of Pathogen- or Microbe-Associated Molecular Patterns (PAMPs or MAMPs) sensed by Pattern-Recognition Receptors (PRRs, Couto and Zipfel, 2016). This is closely followed by an initiation of a defense mechanism referred to as PAMP-triggered immunity (PTI) characterised by phosphorylation of the PRRs leading to downstream signalling cascades involving production of reactive oxygen species (ROS), activation of mitogen-activated-protein- kinases (MAPKs) and changes in gene expression (Couto and Zipfel, 2016). This response, also known as basal immunity, is the first layer of defense in plants. Pathogens have also evolved to circumvent basal immunity by successfully delivering effector proteins into the plant cytoplasm that supress PAMP-triggered defense response allowing promotion of pathogen growth (Abramovitch *et al.,* 2006). Plants also defend against this form of attack by recognising effector proteins mediated by R genes. There is a specific R gene that recognises a specific pathogen effector and their interaction triggers a defense response known as Effector- Triggered Immunity (ETI) characterised by a hypersensitive response resulting in localised cell death (Boller and Felix, 2009). The

recognition of effectors by R genes results in inhibition of pathogen, thus restricting the progression of disease. Accordingly, as both PTI and ETI defense response involve transcription reprogramming, studies have shown a direct interaction between miRNAs and defence response (Jin, 2008; Peláez and Sanchez, 2013), suggesting a key role of post-transcriptional gene silencing (PTGS) in regulating the plant immune system. For example, several Arabidopsis mutants defective in PTGS and miRNA biogenesis (*ago1, dcl4, dcl1, hen1, hyl1*) have been shown to be essential for Arabidopsis anti-bacterial immunity (Yi and Richards, 2007; Navarro *et al.,* 2008; Li *et al.,* 2010). A recent study also showed how the bacterial effector, HopT1-1 supressed the activity of *AGO1* which in turn supressed PTI response and triggered a potent ETI-like activation observed in *ago1* knockouts correlated with an over-accumulation of salicylic acid-dependent defense responses (Thiebeauld *et al.,* 2017). It is well established that *ago1 KO* mutants are constitutively expressing SA-dependent defense genes (Mason *et al.,* 2016) therefore *ago1/2 dko* resistance to *H. arabidopsidis* infection is expected to be higher than wildtype (Fig 5.5) since mutants with SA accumulation have heightened disease resistance (Bowling *et al.,* 1994; Yi and Richards, 2007; Bechtold *et al.,* 2010). However, the increased resistance of *35S:HSFA1b-ago1/2 dko* plants compared to *ago1/2 dko* plants highlights miRNA independent regulation of disease resistance. It also reaffirms, previous observations which suggest that the disease resistance pathway triggered by over-expression of *HSFA1b* is SA-independent (Bechtold *et al.,* 2013).

Bechtold *et al.,* (2013) showed that plants overexpressing *HSFA1b* in non-stressed conditions had increased H_2O_2 levels (Fig 5.6A) which has been shown to be correlated with promoting basal resistance (Custers *et al.,* 2004; Torres *et al.,* 2005; Bechtold *et al.,* 2010). This suggests that the increased *H. arabidopsidis* resistance of *35S:HSFA1b-ago1/2*

dko plants compared to *ago1/2 dko* plants could be a result of the increased H_2O_2 levels (Fig 5.6A) further agreeing with the premise that the pathways involved in *H. arabidopsidis* resistance of *HSFA1b* is independent of miRNAs. Additionally, because H_2O_2 levels in *35S:HSFA1b-ago1/2 dko* plants was restored to control levels (Fig 5.6a), it can be argued that the drop in H_2O_2 observed in *ago1/2 dko* mutant led to reduced *H. arabidopsidis* resistance of the mutant compared to *35S:HSFA1b-ago1/2 dko* plants further implicating H_2O_2 in promoting basal resistance. In summary, the additive combination of increased H_2O_2 as a result of *HSFA1b* overexpression and the constitutive upregulation of SA-dependent defensive genes as a result of *ago1* KO lead to the increased *Hpa* resistance of *35S:HSFA1b-ago1/2 dko* plants.

5.3.3 HSFA1 knockout Changes miRNA Precursor Accumulation Which Might Contribute to the QK2 Phenotype in Normal and Heat Stress Condition

Despite several attempts to quantify miRNA levels in the QK2 mutant without success, the switch to their precursors (pri-mRNAs) was easily attainable which may or may not correlate with their respective mature miRNAs in terms of accumulation at the genome wide level (Barciszewska-Pacak *et al.,* 2015), but others have reported a directly correlation of individual pri-miRNAs with their mature miRNAs (Rodriguez *et al.,* 2010; Guan *et al.,* 2013; Stief *et al.,* 2014). Unfortunately, this discrepancy could be due to differences in experimental setup, length of stress, methods of extraction, extraction fraction used (either total RNA or miRNA fraction) or developmental stage of the plant which factor into results generated. These reasons do not only justify obtaining qRT-PCR primers for pri-miRNA transcript from published work but also explain the failure to amplify several pri-miRNA transcripts in this work. Nevertheless, a few pri-RNA transcripts were perturbed in the QK2 mutant compared to control under normal and heat stress

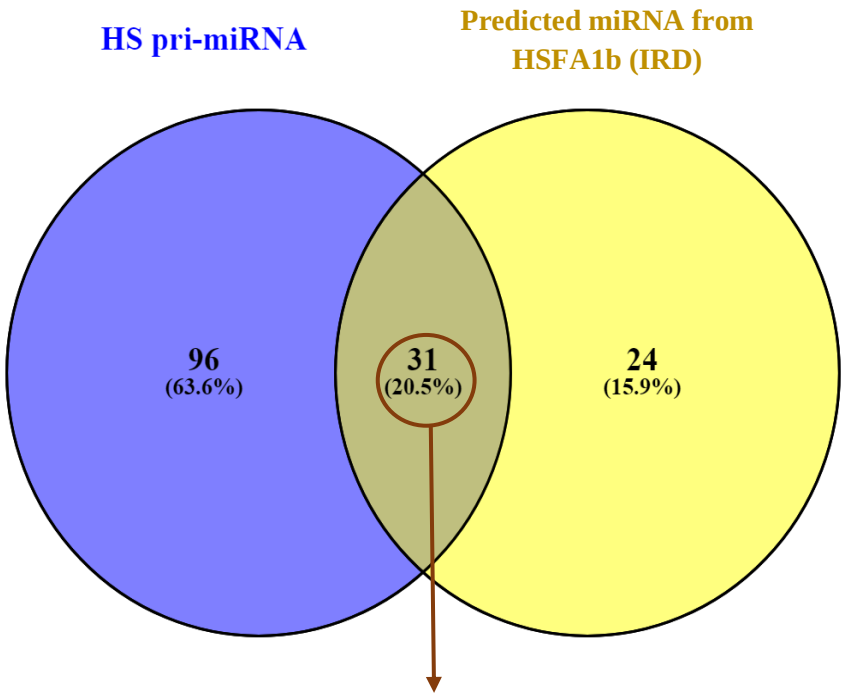
which suggests a possible regulation of miRNAs by HSFA1s (Fig 5.7b, 5.10). The idea is not farfetched as *HSFA1b* has been previously shown to be involved in the heat stress induction of *miR398* which is essential for establishing thermotolerance in Arabidopsis (Guan *et al.,* 2013). Furthermore, *miR156* isoforms, which are highly induced after heat stress, are important for tolerance to reoccurring heat stress (Stief *et al.,* 2014). In both cases, the pri-miRNAs with their mature miRNAs were upregulated which resulted in the downregulation of their target transcripts. In agreement, *MIR156a, MIR172a* and *MIR398b* are upregulated after heat stress in wildtype plants but altered in QK2 mutants (Fig 5.7b, 5.10) suggesting a possible regulation by HSFA1s, at least during heat stress.

While results discussed above show miRNA regulation under heat stress, a number of miRNAs are also perturbed under various other abiotic stresses and are termed general stress-regulated miRNAs which included *miR156, miR159, miR165, miR167, miR168, miR169, miR171, miR172, miR319, miR393, miR394, miR396, miR397 and miR408* identified via microarray-based approach (Liu *et al*., 2008b). *miR165, miR319*, and *miR393* were up-regulated by both high salinity and cold temperatures, *miR167* was induced by both high salinity and drought stress, while *miR168, miR171*, and *miR396* responded to drought, salinity and cold stresses. These miRNA families also possessed known stress – related *cis* elements upstream of their precursor genes which are target for transcription factor binding further extending regulation of gene expression via miRNAs in response to stress. For example, *miR156, miR167, miR171, miR319* and *miR396* precursors possessed HSE elements on their promoter regions which are crucial for HSF binding leading to heat responsive gene regulation. Other stress-regulated elements identified include ABA-response elements (ABREs), anaerobic induction elements (AREs), auxin response elements (AuxRE) and low-temperature-responsive elements (LTRs) amongst others (Liu

et al., 2008b). Considering that miRNAs play a vital role in regulating several abiotic stresses, it is tempting to speculate that HSFA1 are actively involved in this process since they are the master regulators of abiotic stress response in Arabidopsis (Liu *et al.,* 2011).

Furthermore, in the context of development, which this study is based on, some of these "general stress responsive" or "heat inducible" miRNAs regulate targets which have developmental functions. For example *miR156*, which is upregulated during heat stress (Fig 5.10), controls developmental phase transitions in plants (Huijser and Schmid, 2011). *miR156* is one of the most abundant miRNAs in Arabidopsis and targets 11 of the 17 *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* transcription factors. Overexpressing *miR156* delays flowering and produces larger number of leaves with juvenile features (Wu and Poethig, 2006). During heat stress however it is thought that *miR156* delays the transition to flowering in order to prevent damage to the reproductive machinery during high temperatures. Together with SPL transcription factors, *miR156* mediates the tolerance to recurring heat stress which is suggested to integrate stress responses with development (Stief *et al.,* 2014). Likewise, the control of cell proliferation in Arabidopsis leaves is mediated by *miR396* downregulation of 7 members of the GROWTH-REGULATING FACTOR (GRF) family of transcription factors (Rodriguez *et al.,* 2010). Overexpression of *miR396*, which is also heat inducible, results in a dwarf plant with highly reduced cell number in leaves. In the same vein, heat inducible *miR319* targets 5 Arabidopsis TCPs and its overexpression causes crinkled shaped leaves (Palatnik *et al.,* 2003). These highlights a possible mechanism by which *HSFA1b* (and HSFA1s) indirectly regulate developmental genes through the action of heat inducible miRNAs (Fig 5.7b, 5.10).

In addition, several heat inducible pri-miRNA were identified 4 and 52 hours after heat acclimation (Stief *et al.,* 2014). Overlapping the miRNA families of both heat stress inducible pri-miRNA and predicted miRNAs from *HSFA1b* IRD group of genes reveal that 20.5% of the predicted developmental miRNAs have precursors that are potentially heat stress inducible (Fig 5.11). Therefore, it can be argued that the *HSFA1b*-indirectly regulated developmental genes are altered by the action of heat inducible miRNAs. If this holds true, it can be speculated that the aberrant expression of miRNAs in the QK2 mutant in normal and heat stress condition contributes to its phenotype. To this effect, a survey of all miRNA expression (miRNA-SEQ) in QK2 would be needed for confirmation.



miR156	miR172	miR393	miR415	miR829	miR834	miR854	miR867
miR164	miR1886	miR400	miR472	miR831	miR838	miR858	miR868
miR168	miR319	miR403	miR773	miR832	miR843	miR859	miR870
miR169	miR391	miR414	miR780	miR833	miR846	miR865	

Figure 5. 11. Venn diagram showing overlap between *HSFA1b* developmentally associated predicted miRNA and heat stress regulated pri-miRNA. Overlap between predicted *HSFA1b* indirectly regulated developmentally associated miRNAs (HSFA1b IRD) and heat stress pri-miRNA.

5.4 CONCLUSION

This chapter was set up to find out if *AGO1* and *AGO2* via miRNAs were responsible for the developmental phenotype of *HSFA1b* overexpressing plants since their expression was perturbed and found to be bound by the TF. Although the effect of both *HSFA1b* and *AGO1/2* were non-additive developmentally due to *35S:HSFA1b-ago1/2 dko* retaining all but one of the developmental phenotype associated with both *35S:HSFA1b* and *ago1/2 dko* plants, it possible that *HSFA1b* regulates other aspect of stress and development indirectly via AGO1/2 through the actions of miRNAs. Furthermore, the regulation of *miR398b* by HSFA1s under both normal and heat stress suggested a potential role for HSFA1s in miRNA regulation in both conditions. Exploring this possibility revealed indeed that several miRNAs, at least their precursors, can be regulated by HSFA1s. Consequentially, some of these heat inducible miRNAs have targets that are developmentally associated which could explain the differential expression of *HSFA1b*-indirectly regulated developmental genes and by extension be involved in the overall phenotype of the QK2 mutant under non-stress condition. In conclusion, a mechanism by which HSFA1s indirectly regulate development through the action of miRNAs is as follows: when heat stress is encountered, HSFA1s (*HSFA1b*) binds and increases the expression of several heat inducible pri-miRNAs which are then processed via the miRNA pathway. At the same time, HSFA1s (*HSFA1b*) also binds and increases the expression of *AGO1/2* in order to cope with the increase in miRNA transcripts. These miRNAs are then loaded into the RISC complex (AGO1/2) for target mRNA repression ultimately affecting development (Fig 5.12).

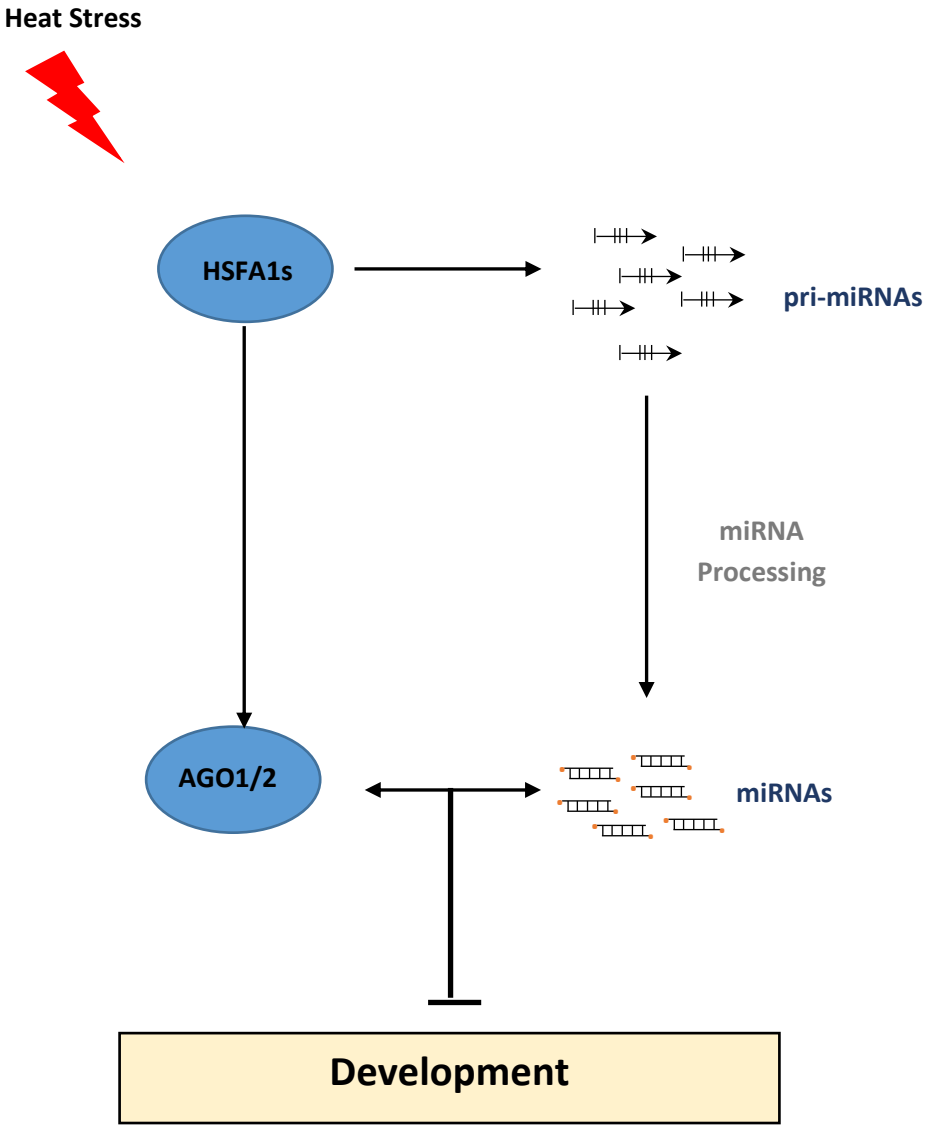


Figure 5. 12. Schematic representation of how HSFA1s could indirectly regulate development via the action of heat inducible miRNAs. HSFA1s binds and increases the expression of several heat inducible pri-miRNAs which are then processed by the miRNA pathway. Similarly, HSFA1s also binds to the HSE elements of *AGO1/2* and increases their expression in order to cope with the increase in miRNA transcripts. These miRNAs are then loaded into the RISC complex (AGO1/2) repressing genes involved in development.

CHAPTER 6

General Discussion

Central to abiotic response in Arabidopsis (as in all plants) lies the coordinated interaction between HSFs and HSPs. A great deal of research has been dedicated to elucidate the functional importance of HSFs and HSPs, individually or collectively by mutation and over-expression in the regulation of stress-responsive genes in relation to changes in environmental conditions, most often resulting in stochastic phenotypic morphologies (Queitsch *et al.,* 2002; Ogawa *et al.,* 2007; Sangster *et al.,* 2007; Ikeda *et al.,* 2011; Jungkunz *et al.,* 2011; Liu *et al.,* 2011; Yoshida *et al.,* 2011; Bechtold *et al.,* 2013; Liu and Charng, 2013; Kataoka *et al.,* 2017). Typifying the above statement is the HSP90 family of proteins which are encoded by seven different loci in Arabidopsis. Four are expressed in the cytosol (HSP90.1, HSP90.2, HSP90.3, and HSP90.4) of which HSP90.1 is stress inducible. A further 3 are expressed in organelles like the chloroplast (HSP90.5), mitochondria (HSP90.6) and endoplasmic reticulum (HSP90.7). A reduction by RNAi or null mutation of one or a combination of these family of proteins leads to pleiotropic developmental defects depending on the type of mutation (Sangster *et al.,* 2007). The developmental effects of HSP90 is not unusual as they function as molecular chaperones that stabilise/maintain other important proteins in conformations that would otherwise be targeted for degradation (Schneider *et al.,* 1996). This would not only affect the targeted protein but also either upstream or downstream of the pathway in which they operate. For example, HSP90 is a requirement for R protein-mediated disease resistance, for RISC assembly and loading of small RNA duplexes which requires ATP and in stabilizing auxin co-receptor F-box protein TIR1 in temperature-dependent seedling growth, amongst its other function (Takahashi *et al.,* 2003; Iki *et al.,* 2010; Iwasaki *et al.,* 2010; Wang *et al.,* 2016b). Therefore, it is evident that mutations that affects the proper

functioning or expression of HSP90 either directly or indirectly could potentially lead to developmental effects under normal or stress conditions.

One of such mutations that alter the expression of HSP90 and other heat induced HSF/HSPs is the HSFA1 quadruple knockout mutant (QK; Liu *et al.,* 2011; Yoshida *et al.,* 2011). Due to the high redundancy of these TFs, individual or combinational double and triple knockouts mutants do not show marked phenotypic differences to wildtype. However, the quadruple knockout was shown to exhibit pleiotropic developmental characteristics akin to plants with reduced HSP90 levels (Liu *et al.,* 2011). This is only part of the story as members of clade A1 have been shown to have collective and distinct functions in conferring tolerance to diverse abiotic stresses (Liu and Charng, 2013). With regards to developmental effects however, over-expression of *HSFA1b* has been shown to have a developmental component (Fig 3.2; Bechtold *et al.,* 2013; Albihlal *et al.,* 2018) which implies that alongside HSP90-dependent regulation of development, members of the clade A1 could potentially target/regulate other developmental genes involved in growth and development. Consequently, this research was aimed at identifying developmental targets of HSFA1s in normal and heat stress conditions.

Firstly, the developmental effect of over-expressing *HSFA1b* in Arabidopsis plants not previously identified were determined by growth analysis characterised by early flowering (days until bolting), longer inflorescence and smaller rosettes compared to wildtype (Fig. 3.2). Accordingly, the effect of the small rosette size in *35S:HSFA1b* plants could be attributed to the constitutive expression of *HSFA2* since its over expression has been shown to cause a reduction in rosette size albeit dose dependent (Ogawa *et al.,* 2007); suggesting that the constitutive upregulation of *HSFA2* in *35S:HSFA1b* plants could be one

of the reasons for the reduced rosette size. Furthermore, it appears that the role of *HSFA2* in development hinges upon the presence or absence of the clade A1 HSFs, positively promoting growth in their absence and vice versa. In agreement with the above statement, when Liu and Charng, (2013) overexpressed *HSFA2* in the original QK, rosette size of the progeny was comparable to wildtype which was also dose dependent. However, when overexpressed in wildtype plants, rosette size was smaller. This suggests that the developmental trait observed in *35S:HSFA1b* plants could be acting through *HSFA2*. In order to test the hypothesis, genome-wide transcriptomic changes between *35S:HSFA1b* and *HSFA2* overexpressing plants can be overlapped to observe if they possess common developmental targets. Experimentally, knocking out *HSFA2* in *35S:HSFA1b* plants can be done to determine if rosette size will be reverted to wildtype levels.

Additionally, the developmental changes observed in *35S:HSFA1b* plants strengthened the claim that individual members of the clade A1 could possesses developmental functions. A recent publication highlighted that *HSFA1b* bound and regulated at least 85 developmentally-associated genes as well as indirectly regulating 281 developmentally-associated genes (not bound by *HSFA1b*; Albihlal *et al.,* 2018). Of the directly regulated set, 27 are transcription factors, 8 of which are developmentally associated. 7 of the 27 directly regulated TFs were tested in the QK mutant by qRT-PCR and confirmed to be regulated by *HSFA1b* (Fig 3.4B). Additionally, the bound and indirectly regulated set of developmental genes function at various stages of plant development from germination to flowering and their regulation could be traced upstream to at least one of the 27 directly regulated set of TFs (See chapter 3.3.2). Regulation of long non-coding RNA was another possible explanation for indirect regulation of unbound developmental genes by

HSFA1b. 480 natural antisense transcripts (lncNATs) were also bound by *HSFA1b* and the expression of 3 lncNATs tested were perturbed in the QK either in normal condition or under heat stress (Fig 3.5). Although the use of the QK was sufficient in confirming the developmental targets of *HSFA1b*, its parental background consisting of both Col-0 and Ws-0 introduced an element of complication as the expression of target genes in some cases was only significant when compared to one parent and not the other (Fig 3.4b, 3.5). For this reason the RNA directed endonuclease (CRISPR/Cas9) was employed to knockout *HSFA1a* from a triple knockout mutant (bdeKO) in single parental background, Col-0.

The CRISPR/Cas9 was efficient in introducing a 10bp deletion in the *HSFA1a* allele causing a frame shift disrupting its function. The resulting quadruple knockout called QK2 had delayed germination, reduced seed size and weight, reduced growth at seedling stage as well as sensitivity to elevated temperatures (Fig 4.7, 4.8, 4.9). These phenotypes are similar to the original QK however, the QK2 mutants didn't show some of the other characteristic phenotypes associated with reduced HSP90 expression like extruded root, meristem-less shoot, narrowly-shaped leaves, twisted rosette or concaved cotyledons (Queitsch *et al.,* 2002; Sangster *et al.,* 2007). While this cannot be fully explained, the QK2 also possessed smaller and pointed cotyledons which were almost translucent. At 5-weeks, the cotyledons of QK2 were completely green but still small compared to wildtype (Fig 4.8f). The biggest observable difference between QK2 and wildtype occurs during seedling stage because as the mutant gets older, these differences diminish. At bolting, the QK2 has similar number of leaves and fresh weight compared to wildtype (Fig 4.8h, i) although bolting in the QK2 was delayed by 4 days (casual observation). This suggested that the seed size and delayed germination combined with the small cotyledon of the QK2 puts the plant at a slight disadvantage and left to catch up with wildtype provided that

stress is avoided which therefore suggested that the clade A1 HSFs are important for the proper development of seed and/or embryo under normal conditions. A few lines of evidence can be used to buttress the above hypothesis. Liu and Charng (2013) suggested and showed that over-expression of *HSFA2* could only partially rescue the QK mutant. Though the mutant could withstand different heat stress regimes, it did not improve other abiotic stress and only partially rescued seed abortion compared to the QK. In addition, Kotak *et al.,* (2007) identified *HSFA9* as the only HSF expressed in the late stage of seed development using publicly available microarray data from different stages of Arabidopsis development (AtGenExpress). However, from the same data *HSFA1a, HSFA1b and HSFA1e* had increased expression in the late stage of seedling development. *HSFA1d* (including *HSFA1a*) on the other hand also had increased expression in pollen development (Fig 6.1). Both papers support the notion that members of clade A1 are important for seed development.

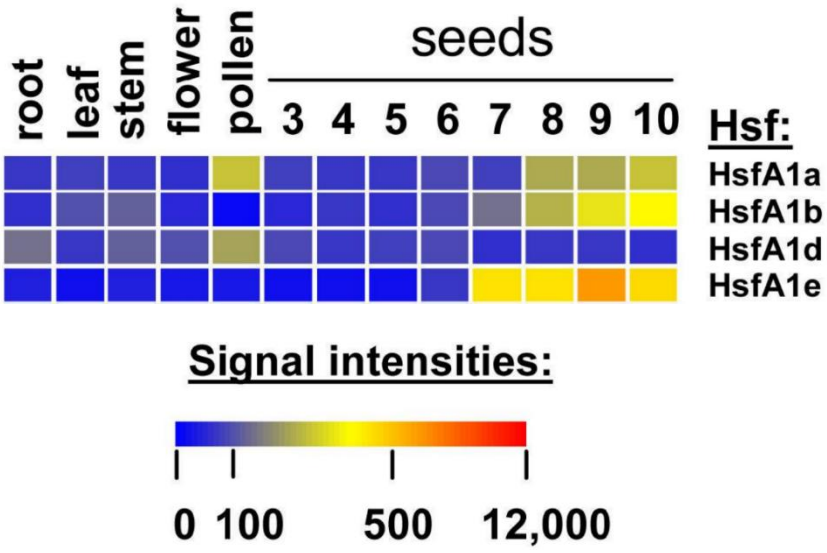


Figure 6. 1. Expression Profiles of HSFA1s in Different Arabidopsis Developmental Stages. Image shows normalized and averaged signal intensities visualized as heat maps with retransformed linear signal intensities using publicly available microarray data from different stages of Arabidopsis development (AtGenExpress). Numbers 3 -10 represents different stages of seed maturation. Images modified from (Kotak *et al*., 2007)

Following above statements, the questions emerge: By what means do the clade A1 HSF affect seed development? Is it a direct regulation of seed-specific genes or an indirect consequence of transcriptomic reprogramming / misregulation of downstream targets? While there are no shortage of questions and hypothesis in terms of how HSFA1s might affect seed development, two plausible situations can be hypothesised; I) improper embryo development resulting in a smaller embryo compared to wildtype or II) lack/reduction of storage lipids and carbohydrate during seed filling causing a dip in the energy reserves necessary for seedling establishment. In support of both hypothesis, *WRINKLED1 (WRI1*), which encodes a transcription regulator of the AP2/EREB family, is of particular interest because it produces wrinkled seeds that are severely depleted in oil accumulation (Focks and Benning, 1998; Baud *et al.,* 2007). In this mutant, maturing embryos are unable to efficiently convert sucrose into Triacylglycerol, the storage form of lipids in Arabidopsis. Although *wri1* seedling were indistinguishable from wildtype (Focks and Benning, 1998), embryos of this mutant showed delay in embryo expansion ultimately resulting in a smaller embryo compared to wildtype in addition to a reduced seed weight (Baud *et al.,* 2007). Therefore, it is plausible that knocking out HSFA1s affects seed development in this manner. Additionally, mutants of other seed maturation genes *abi3, lec1, lec2* and *fus3* as well as mutants disrupted in different steps of storage oil biogenesis and metabolism show reduced accumulation of storage compounds as well as other specific phenotypes (To *et al.,* 2006; Graham, 2008). Hence, it is also plausible that transcriptional reprogramming as a result of HSFA1 knockout indirectly affects seed development owing to their heightened expression during seed maturation/filling (Fig 6.1). So far this is yet to be investigated.

Transcriptionally, the changes in gene expression during a change in ambient temperature (22-29°C) is different from that of heat shock (37-45°C). While the role of HSFA1 has been extensively researched during heat stress, an information gap exits during mild changes in temperature. Although it was recently proposed that the ambient temperature transcriptome is dependent upon the HSFA1s causing eviction of H2A.Z-nucleosomes at target genes in Arabidopsis (Cortijo *et al.,* 2017). Developmentally, heat stress is detrimental to growth and vice versa for ambient temperature. Hence the role of HSFA1s in temperature-dependent growth was investigated. Firstly, 5 week-old QK2 plants grown at 22°C did not show the phenotype of wildtype plants when transferred to 27°C characterised by petiole elongation (Fig 4.10C, D). A similar effect was also observed when hypocotyl elongation was hampered in the QK2 compared to wildtype in the same condition (Fig 4.10A). This was interesting because temperature dependent hypocotyl elongation is regulated by PIF4 and auxins (Gray *et al.,* 1998; Koini *et al.,* 2009; Franklin *et al.,* 2011). It also suggests that *PIF4* activity, in regulating auxin levels during mild temperature increase, is dependent on HSFA1s. Interestingly, it has been shown that *PIF4* and *HSFA1b* regulate similar targets during high temperature signalling integrating developmental cues during plant development (Albihlal *et al.,* 2018). Furthermore, because BR have been suggested to act downstream of *PIF4* during thermo-responsive growth (Ibañez *et al.,* 2018), it would also be interesting to see the effect of exogenous application of BR on QK2. Additionally, QK2 seedlings grown at 27°C possessed cotyledons which reverted the glassy phenotype i.e. cotyledon development in QK2 were more normal at 27°C (Fig 4.10B) which suggested a role of HSFA1s in chloroplast biogenesis/development during embryogenesis. While these experiments highlighted the

importance of HSFA1s in temperature-dependent development, it also casually highlighted the role of HSFA1s in cell expansion discussed in chapter 4.3.3.

In line with the above experiment on mild changes in temperature, it was observed coincidentally that adult wildtype plants grown constantly at 27°C wilted more rapidly than QK2 plants grown in similar conditions when water was withheld for several days (Appendix 5A). This is interesting because a combination of drought and heat stress has been shown to negatively impact growth, productivity and yield of crops than other known individual or combinational stresses (Rizhsky *et al.,* 2004; Vile *et al.,* 2012; Suzuki *et al.,* 2014). In this case however, not being able to sufficiently and efficiently respond to mild changes in ambient temperature (QK2) in some way was advantageous in tolerating drought stress. By appearance alone, both QK2 and wildtype did not show detrimental signs when temperature was changed from 22-27°C although only wildtype responded phenotypically with slightly increased leaf area and petiole length but when water was withheld, wildtype plants wilted quicker than QK2. This may not be all too surprising knowing that; if the transcriptomic profiles in mild temperature changes and heat stress are different, therefore the response of a combination of either with drought might equally be different. Furthermore, it was shown that increase in stomatal conductance was associated with heat stressed plants in order to cool the leaves by transpiration (Rizhsky *et al.,* 2004). Since stomatal conductance is a function of stomatal density, stomatal aperture and stomatal size (Lawson and Blatt, 2014), it is plausible that QK2 plants where not efficiently able to increase stomatal conductance compared to wildtype. Consequentially, when drought stress is subsequently encountered in combination, the increased stomatal conductance in wildtype plants might become disadvantageous in combating both stresses hence, leading to rapid loss of water which begs the question;

could not being able to respond to mild changes temperature be an advantage in other stress combinations, for example highlight and/or pathogen infection? As with all hypotheses, experimental validation is required including proper drought stress experiments involving QK2 and wildtype.

Finally, the role of small RNAs cannot be overlooked when investigating changes in growth and development. MicroRNAs in particular have been intensively studied in relation to development as well as stress in plants (Sunkar, 2012), therefore their possible regulation in QK2 plants compared to wildtype in control and heat stress was investigated which would have been experimentally challenging using the original QK due to the variability of miRNA abundance in both parental genotypes (Col-0 and Ws-0). Although directly quantifying target miRNAs using the stem-loop RT-PCR method was unsuccessful in QK2 and control plants, quantifying their precursors yielded better results showing that indeed some miRNAs could be regulated by the HSFA1s. Whether this is a direct or indirect regulation remains unknown. Furthermore, because the biggest observable changes in the QK2 compared to wildtype occurs during seedling development, it is paramount to observe global changes in miRNA and mRNA expression at the genomic level by conducting miRNA-SEQ and RNA-SEQ on 7-day old seedlings. Intersecting both data will give ideas as to how miRNAs are affected in the QK2 mutant and also provide mechanisms as to how HSFA1s regulate development at early stages of development possibly via the regulation of miRNAs.

Concluding Remarks

The results presented in this study highlight the role of HSFA1s in the regulation of growth and development during normal and heat stress conditions. In the wake of increasing global temperatures it is important to investigate the effects of the master regulators of not only heat but other abiotic stress as in the natural environment since increasing heat stress is accompanied by drought and other secondary stresses. Therefore in order to engineer heat resistance crops, it is important to know how development is impacted as this study shows that while HSFs are essential in basal heat tolerance, they also possess developmental components which could be overlooked while "improving" stress tolerance. For instance, although over-expressing *HSFA1b* improved seed yield and harvest index, its growth was reduced due to the constitutive expression of heat responsive genes which is possibly "costly" for the plant (Bechtold *et al.,* 2013). Therefore, since having an increased *HSFA1b* expression is advantageous in terms of seed yield and heat tolerance, a better approach might be to drive *HSFA1b* expression using a heat inducible promoter which could be beneficial to plants or crops growing in the field.

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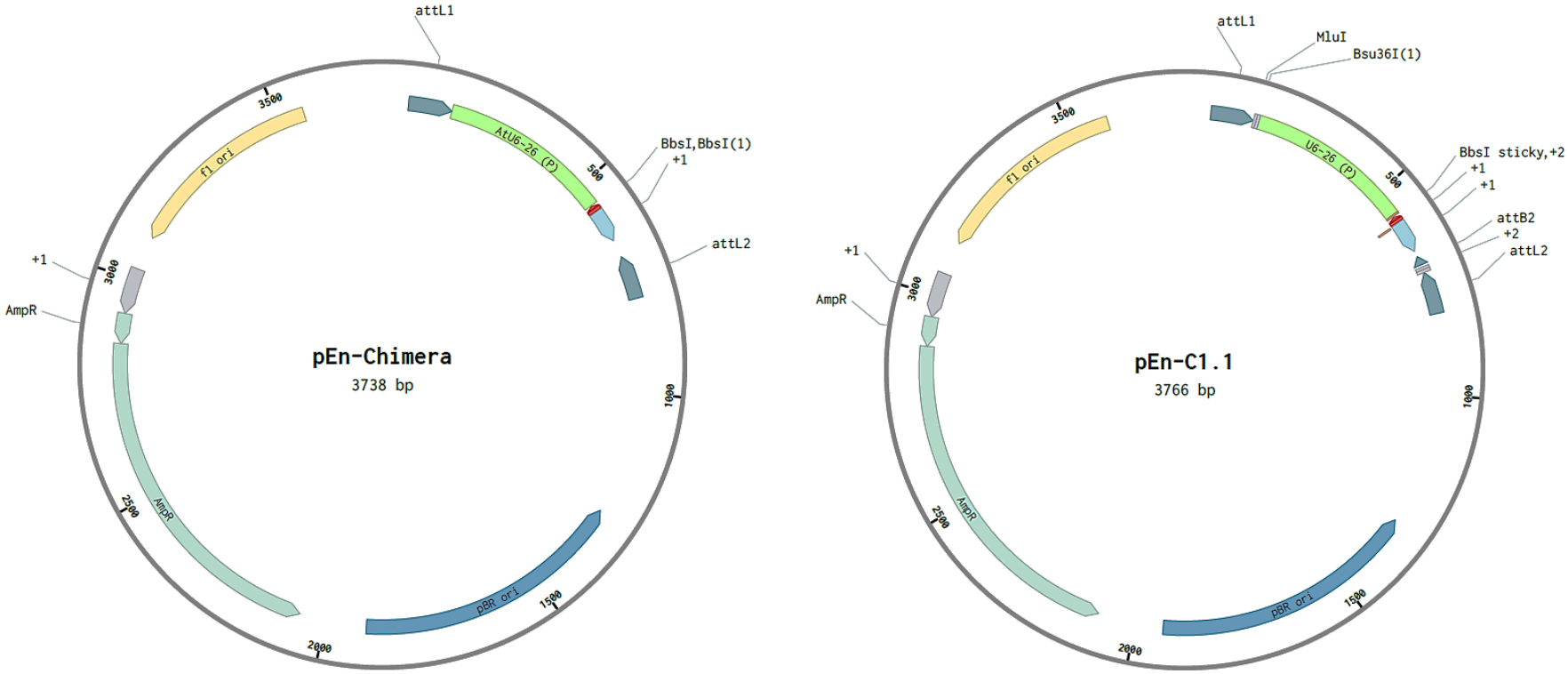
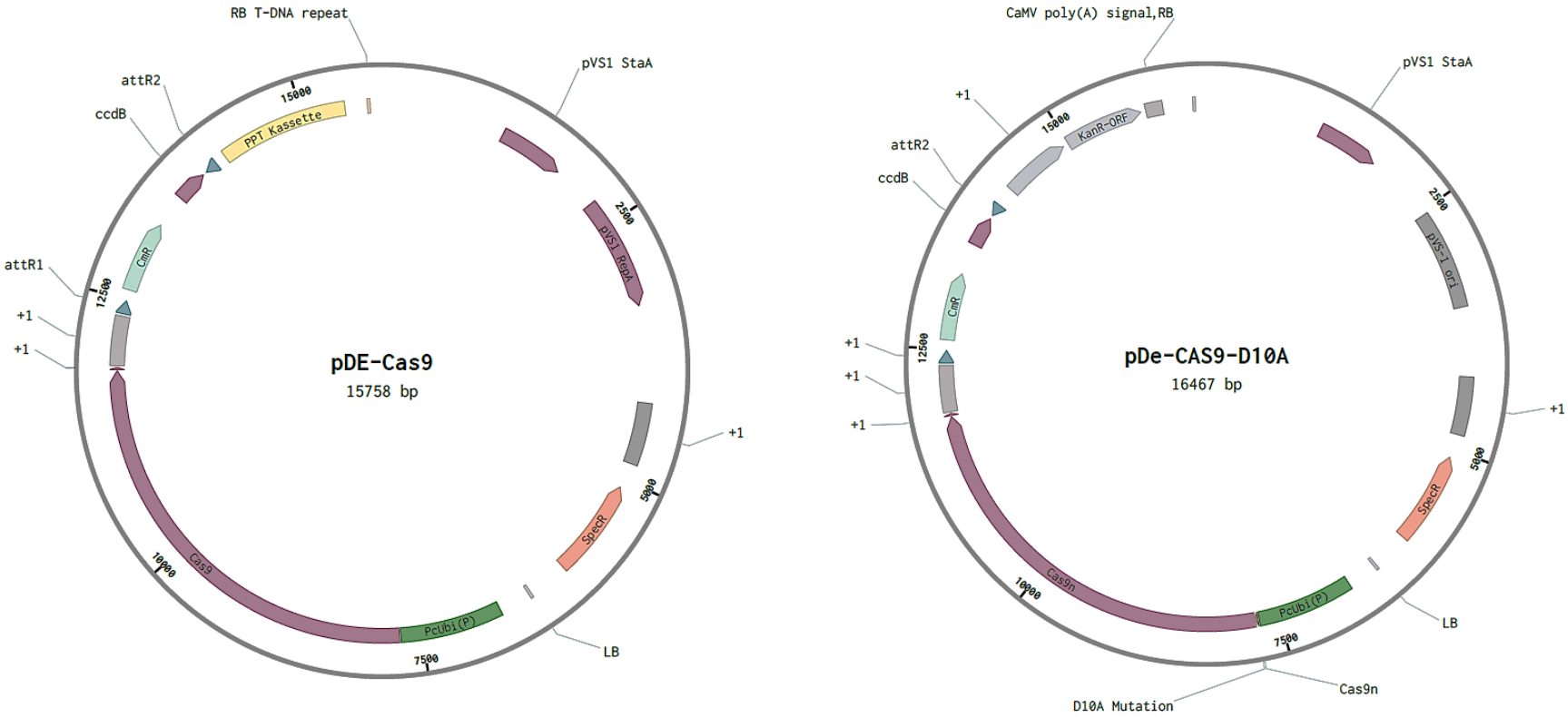
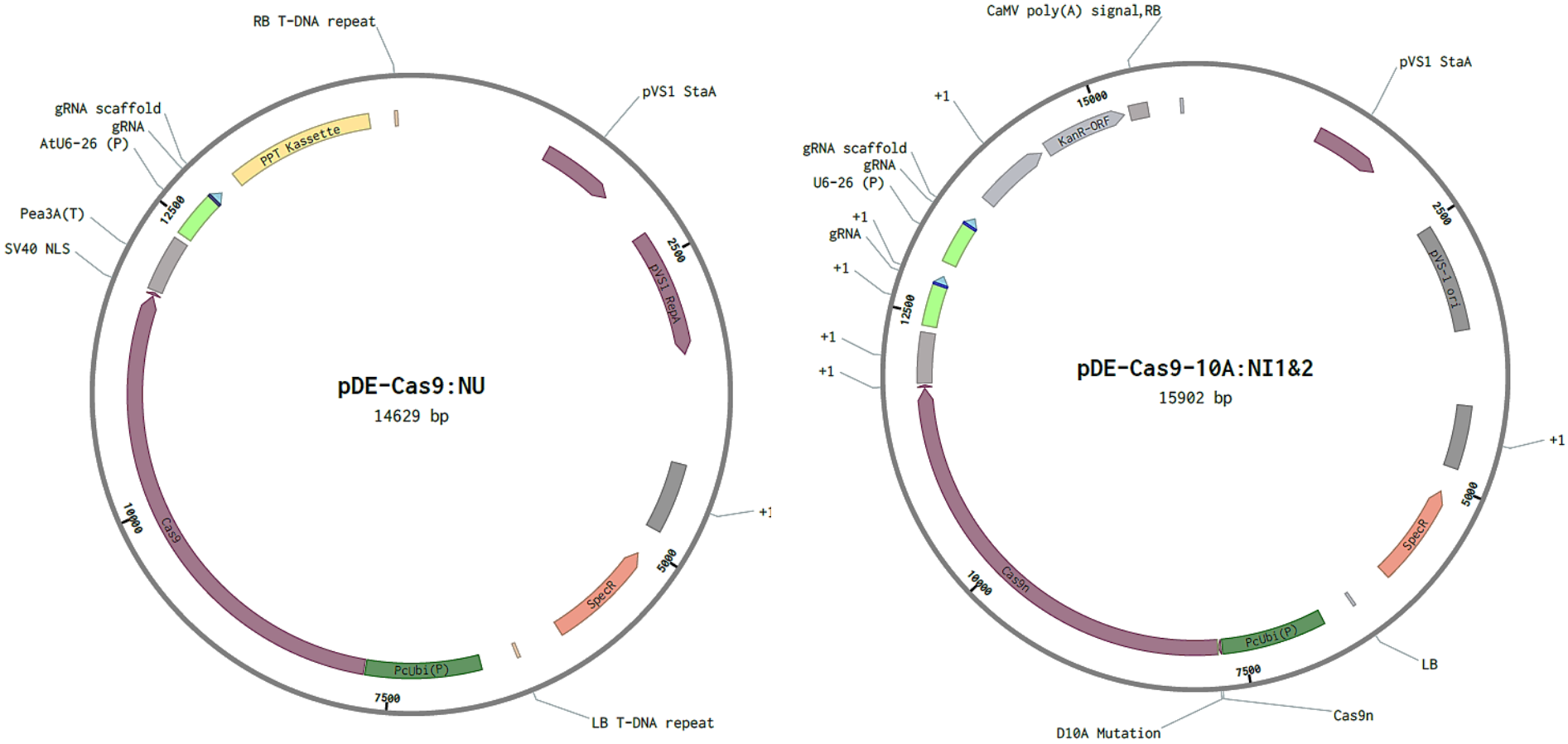
APPENDICES



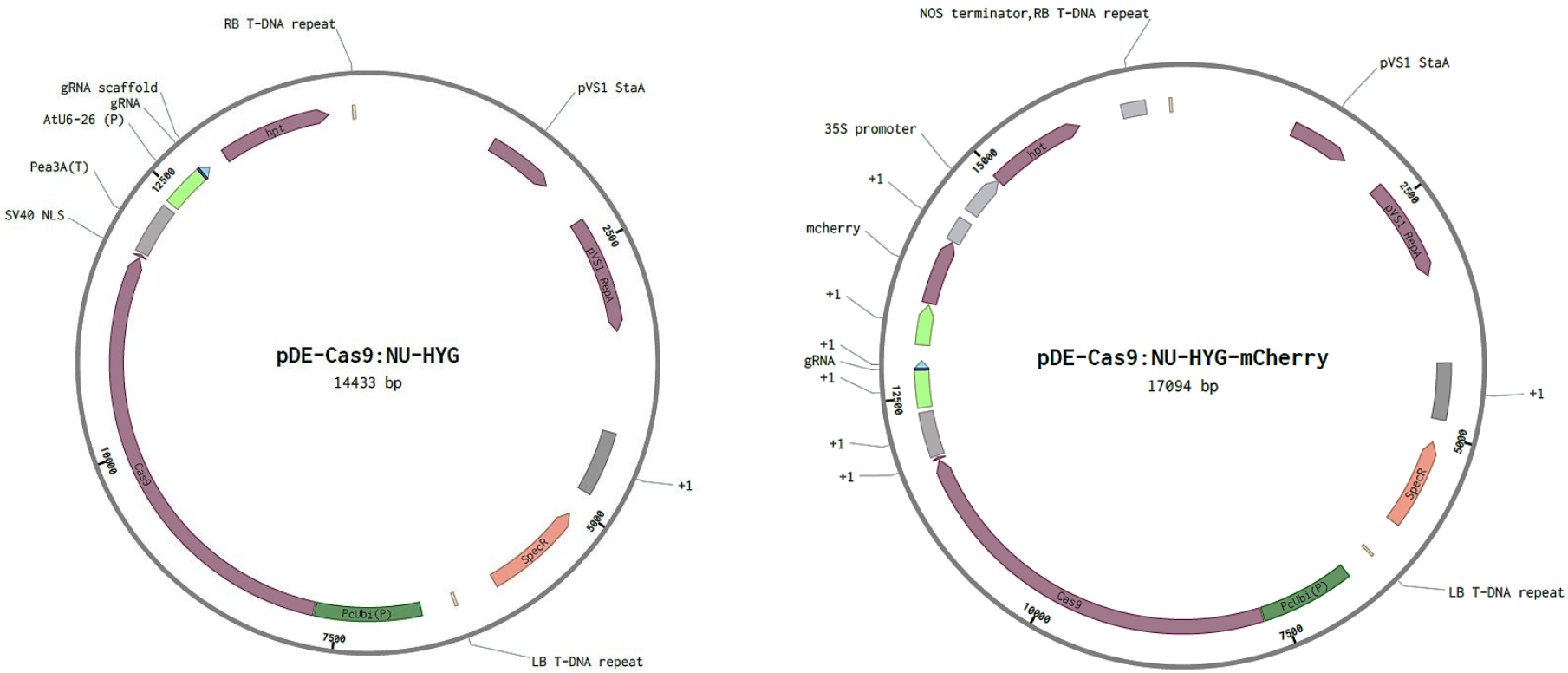
Appendix 1A. Plasmid map of CRISPR/Cas9 entry vectors. pEn-Chimera was used to assemble sgRNA for CRISPR Nuclease construct while pEn-C1.1 was to assemble paired CRISPR Nickases. sgRNA is driven by the Arabidopsis U6-26 promoter (Fauser *et al.,* 2014)**.**



Appendix 1B. Plasmid map of CRISPR/Cas9 Destination vectors**.** pDE-Cas9 (Nuclease) was assembled with pEn-Chimera while pDE-Cas9-D10A was assembled with pEn-C1.1(Nickase). Cas9 is driven by the constitutive Ubi4-2 promotor from parsley (Fauser *et al.,* 2014).



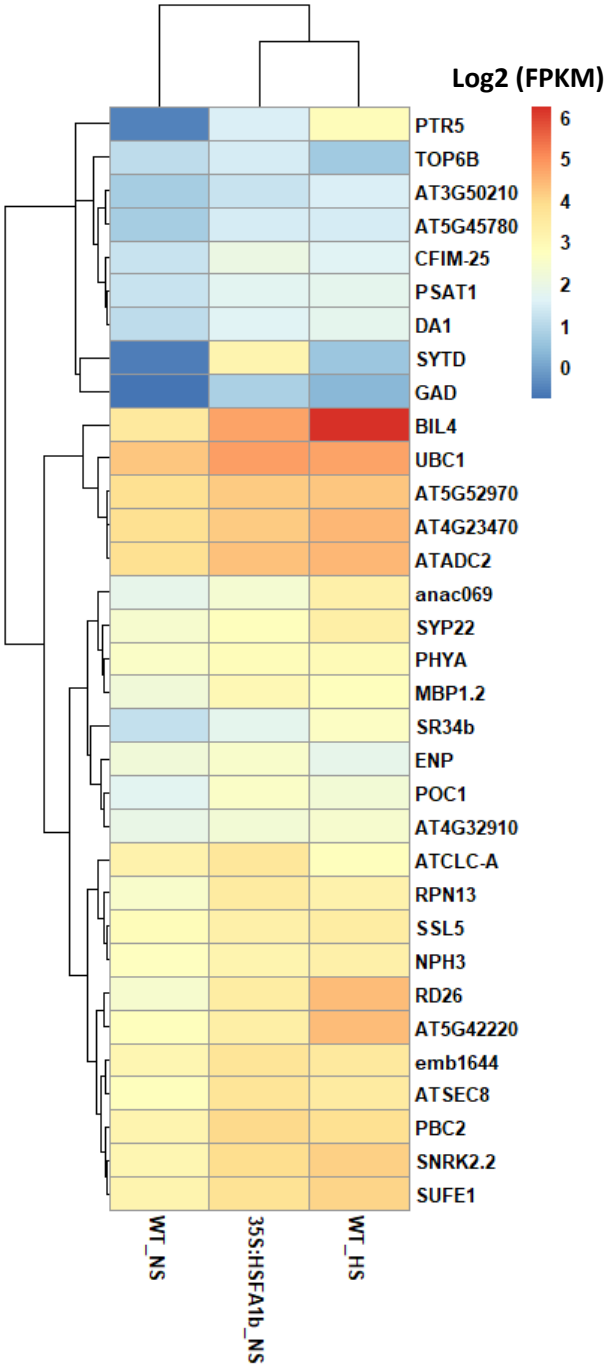
Appendix 1C. Plasmid maps of CRISPR/Cas9 vectors after subcloning the entry vectors into their respective destination vectors. Refer to chapter 2.4.4 for method.



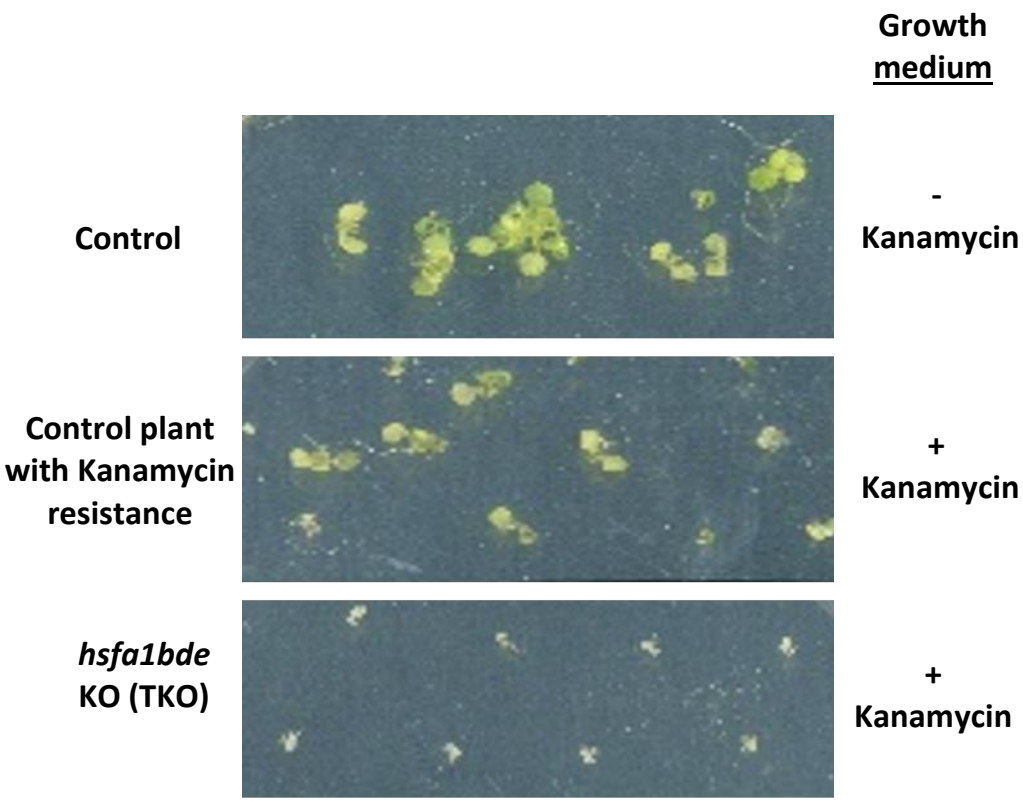
Appendix 1D. Plasmid maps of CRISPR/Cas9 vectors after exchanging a Basta cassette (PPT) with a Hygromycin resistance gene (Hpt) cassette (left) and after inserting an mCHERRY cassette driven by the strong AT2S3 seed coat promoter (Right). Refer to chapter 2.4.5 and 2.4.6 for method.

Gene Name	Developmental Function	Reference
BAM3	Shoot and flower meristem; male gametophyte development	DeYoung, et al., 2006
TCP1	Whole plant development	Guo et al., 2010
AMP1	Seed SAM flower	Griffiths et al., 2011
BRIZ2	Germination and early seedling growth	Hsia & Callis, 2010
ARL	Cell Expansion	Hu,et al., 2006
CTF7	Female gametophyte and embryo development	Jiang,et al., 2010
SKP2A	Cell division in Root	Jurado etal., 2008
ATARP4	Flowering time	Kandasamy et al., 2005
COL2	Flowering time	Kim, et al., 2013
ADAP	Germination and seedling growth	Lee et al., 2009
ALA3	Shoot ovule pollen development	McDowell et al., 2013
CYP707A1	Seed germination and development	Okamoto et al., 2006
BT1	Female gametophyte development	Robert et al., 2009
CDC45	Pollen Development and Female Fertility	Stevens et al., 2004
CML23	Transition to flowering	Tsai et al., 2007
BIL4	Cell elongation	Yamagami et al., 2017
ELF3	Elongation in response to temperature	Box et al., 2015
DWF5	Whole plant and Seed development	Choe et al., 2000
HTA11	Flower and seed development	Coleman-Derr et al., 2012
CYCD3	Cell proliferation and expansion	Dewitte et al., 2007
CDKD;1	Reduced growth	Hajheidari et al., 2012
EMB1075	Embryo development	Yunus et al., 2016
ELF9	Early flowering	Song et al., 2009
GLK2	Chloroplast development	Waters et al., 2009
ATASE2	Chloroplast development	Yang et al., 2015
CRF6	Leaf Senescence	Zwack at al., 2013
UBP14	Seed development	Doelling at al., 2001
MEE3	Regulation of early photomorphogenesis	Hamaguchi at al., 2008
MGP4	Pollen and root growth	Liu at al., 2011
MAP1A	Required for normal growth and development	Ross at al., 2005

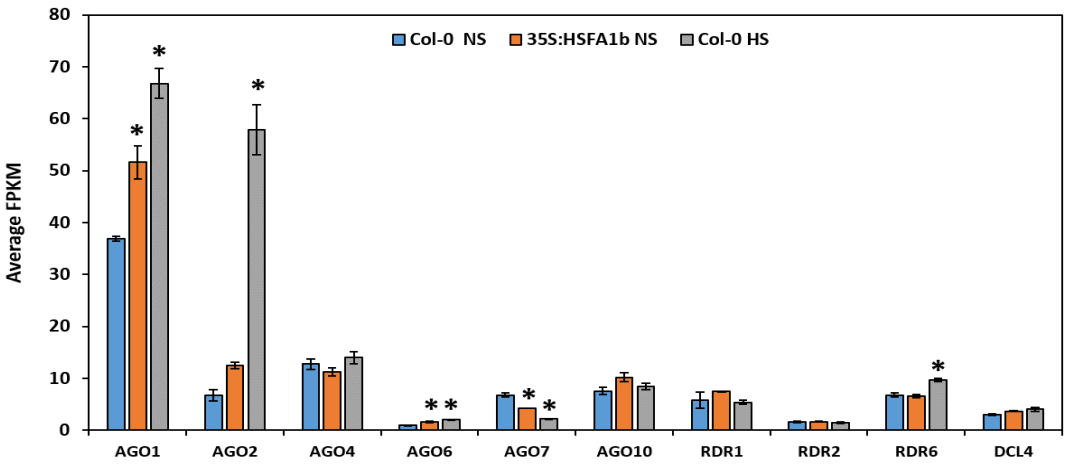
Appendix 2A. List of developmentally confirmed genes whose expression is indirectly regulated by *HSFA1b* overexpression.



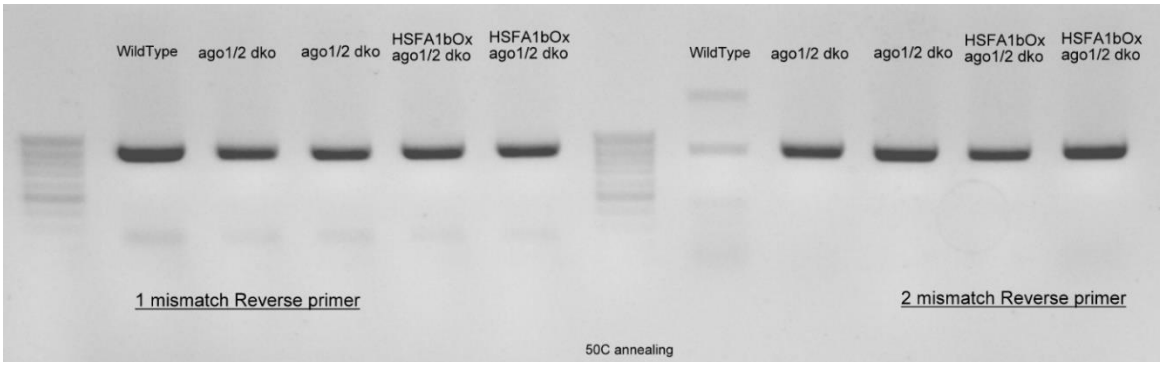
Appendix 2B. Heat map of indirectly regulated developmentally associated genes with perfect/imperfect HSE**.** Upregulated IRDA genes in *35:HSFA1b* plants in no stress compared with Wildtype in both conditions (Heat and no stress). Data from RNA-seq from Albhilal *et al.,* 2018.



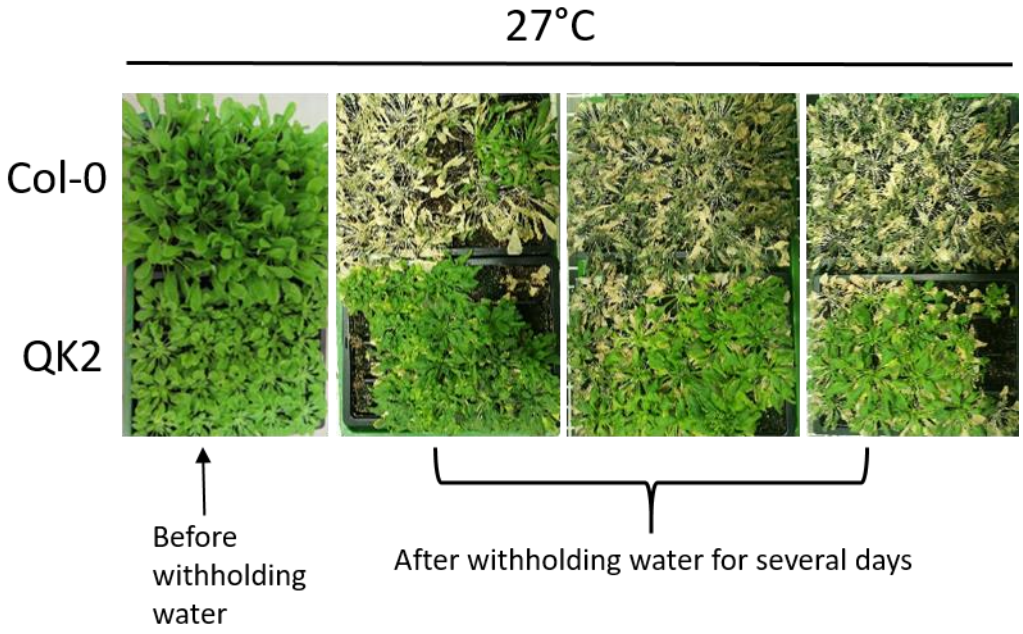
Appendix 3A. Image showing the loss of kanamycin resistance of the *hsfa1bde* triple knockout (TKO) plants. Seedlings grown for 10 days in short day conditions in medium with or without Kanamycin (30mg/l).



Appendix 4A Expression of different genes involved in sRNA biogenesis from RNA-SEQ data (Albihlal *et al.,* 2018)



Appendix 4B. Gel image showing PCR screening of *ago1* allele. 2 reverse primers, each with 1 and 2 mismatches to the mutated *ago1* allele, were designed to discriminate the wildtype *ago1* allele from the mutated. With an annealing temperature of 50°C, the primer with 2 mismatches was suitable to distinguish wildtype allele from mutated.



Appendix 5A. Image of Col-0 and QK2 plant growing at 27C with water withheld for several days. Plants were grown in short day conditions

Appendix 6A

Primers used in this study

Primer	Sequence	Description
qPCR_CSD1-F	TGGTAGTTGTGTGTATCTTCTGGTGTGTG	-
qPCR_CSD1-R	TGAAAGTACCATATCCAGGACTCTTGTTACC	
qPCR_CSD2-F	ATGTCTACTGTGTTGATCTTGTCTG	
qPCR_CSD2-R	ACGCATGATTCATCACAATCTTGAAC	
qPCR_RVE1_F	ATGCACCCAAGGTACGGAAG	
qPCR_RVE1_R	TATTCGTCTCCAAGCTCGCC	
qPCR_RVE7_F	CGCGGAAGAATCTCACAAACCAT	
qPCR_RVE7_R	GCATCCCTGAGTAGTGATTCTCC	
qPCR_MYC2_F	GTAACGCGGTTTGGGTTTCC	
qPCR_MYC2_R	CACTCCTCCTTGCTTAGCCC	
qPCR_SZF1_F	TGTTGCTGGCTGTTCTGTGA	
qPCR_SZF1_R	GCTTTCCTCCTCGGACTAGC	
qPCR_GBF3_F	ATGACGTGGTCATCGTCTTG	
qPCR_GBF3_R	CCAGAGCGAAAAAGAGTTCAG	
qPCR_bZIP28_F	ACGACCAAGTTCGTTGAGCA	
qPCR_bZIP28_R	AAACCCCTTGCTTTCTCGCT	
qPCR_TIP41-like_F	GTGAAAACTGTTGGAGAGAAGCAA	Reference Gene
qPCR_TIP41-like_R	TCAACTGGATACCCTTTCGCA	
NAT_LZF1-F	GGATTAGAGAGGCCATAAACCAG	Long non-coding Natural Antisense Transcript
NAT_LZF1-R	CCAGATGCTTCCTGTACACAC	
NAT_MYB16-F	CATTGCCTGAGAAAGCTGGT	
NAT_MYB16-R	CATCGATGGAGACCTGAGAAGAG	
NAT_CDF1-F	CGCTCACCTTTATTGGTTTCAGT	
NAT_CDF1-R	GTTGGTGAACCAGAGGTTGC	
qPCR_CDF1_F	CAACGTAAACCAACCTCGCC	-
qPCR_CDF1_R	CACTTCTCATGGTCCCACCT	
qPCR_MYB16_F	AGGAAACAGATGGTCAGCGA	
qPCR_MYB16_R	CACTAACCGTTTCTTCAAATGAGTG	
qPCR_LZF1_F	AGGAGATTTTCGGGCTAACCG	
qPCR_LZF1_R	GTTTCATCTTGAGAACGTCTGTCT	
Actin2_F	ACCTTGCTGGACGTGACCTTACTGAT	Reference Gene
Actin2_R	GTTGTCTCGTGGATTCCAGCAGCTT	
qPCR_TCP10-F	ACCACCACCAAACCTTAGCC	-
qPCR_TCP10-R	TCCCGAACGTGTCGAAATGA	
qPCR_HSP90.1-F	TTGTGGACTCTCCCTGCTGT	
qPCR_HSP90.1-R	TCAACGCCTGTGCCTTCATA	
qPCR_PP2AA3-F	GCGTAATCGGTAGGGAGTGAT	Reference Gene
qPCR_PP2AA3-R	CGATAAGCACAGCAATCGGG	
qPCR_HSP70-F	TGCCGGTGGTGTTATGACTG	-

qPCR_HSP70-R	TTTGTTCGTGCCCTCTCTCC	
qPCR_HSFB2b-F	GGGGTTTCTATTGGGGTCAA	
qPCR_HSFB2b-R	CCATTGGCTCTGCCTTAACA	
qPCR_HSFB2a_F	CGATGGGAGTTTTCAAACGA	
qPCR_HSFB2a_R	ACAACCATCGTCTGGTTTCG	
qPCR_HSFA2-F	TTGCTGTTGCCTCAACCTAACTAC	
qPCR_HSFA2-R	GTGTTGAGGTTGGGCAATACG	
qPCR_MBF1c F	GACGATGCCGAGCAGATACC	
qPCR_MBF1c R	TTTCGGATCGCGTAGGTCTT	
qPCR_MIR398b_F	CATGAAGGTAGTGGATCTCGACAG	miRNA Precursor Primers
qPCR_MIR398b_R	GGTAAATGAGTAAAAGCCAGCC	
qPCR_MIR172a-F	GAGCCACGGTCGTTGTTGG	
qPCR_MIR172a_R2	TCATAGAGAACTTTGTGGAGAGTGA	
qPCR_MIR156a-F	CATCTTGTAGATCTCTGAAGTTGGACT	
qPCR_MIR156a-R	GAGATTGAGACATAGAGAACGAAGACA	
qPCR_miR396a-F	GGCGGTTCCACAGCTTTCTT	
qPCR_miR396a-R	TGGTGCAGGGTCCGAGGTATT	
qPCR_MIR158a-F	GTGATGACGCCATTGCTCTTT	
qPCR_MIR158a-R	TGTGACTTTAGATGCCCTTGTTCA	
CRISPR Primers		
CRISPR(Nu)_A1a-F	ATTGGGGAGGAACGAATATCGGCG	Nuclease sgRNA
CRISPR(Nu)_A1a-R	AAACCGCCGATATTCGTTCCTCCC	
CRISPR(Ni)_A1a-F1	ATTGGTGTGACGGCGCCACCACCG	Nickase sgRNA 1
CRISPR(Ni)_A1a-R1	AAACCGGTGGTGGCGCCGTCACAC	
CRISPR(Ni)_A1a-F2	ATTGGCCGATATTCGTTCCTCCGC	Nickase sgRNA 2
CRISPR(Ni)_A1a-R2	AAACGCGGAGGAACGAATATCGGC	
HSFA1a_F	TTTCCATCGGACGCAAGTGA	PCR primers for screening with T7EI
HSFA1a_R	TCCTCTCTGAGTCTCCGCTT	
Cas9_F2	GGAAACCATCACCCCTTGGA	Cas9 primer
Cas9_R	AGCGTAGGTCTTGAGCCTTT	
HSFA1a_ATG_F	ATGTTTGTAAATTTCAAATACTTC	PCR primers for High Resolution Melting
HSFA1a_R2	ATTGTCTTGCTAAGGAAAGGGGG	
AGO Screening		
AGO1_F	TATGAACTTGATGCCATCCG	Screening Primers for AGO1-27 amino acid substitution
AGO1 - 1 mis_R	TGGCTCCATGTAGAATCGAA	
AGO1-2 mis_R	TGGCTCCATGTAGAAGCGAA	
AGO2_F	GATAAAGGCTCGTAATGGAC	AGO2 Screening primers
AGO2_R	CGAGAAGCTTCATCTATCACG	
SALK_LBb1.3	ATTTTGCCGATTTCGGAAC	Salk insert T-DNA primer
RFP_F	ATGGCCTCCTCCGAGG	monomeric Red Fluorescent protein primers
RFP_R	GGCGCCGGTGGAGT	

qPCR_AGO1_F	GGATATGGGCAACCACCACA	-
qPCR_AGO1_R	TACCCTCCTCGACCTCCTTG	
qPCR_AGO2_F	AATAATGATGGAAGTGATAA	
qPCR_AGO2_R	AAGAGTGTAGTAATGAGT	
QK2 Screening		
CRISPR(Nu)_A1a-F	ATTGGGGAGGAACGAATATCGGCG	Primers for screening the quadruple knockout (QK2) mutant
HSFA1a_R	GTGTTCTGTTTCTGATGTGAGA	
HSFA1b_F	ATGGAATCGGTTCCCGAATC	
HSFA1b_R	TTTCCTCTGTGCTTCTGAG	
HSFA1d_F	ATGGATGTGAGCAAAGTAAC	
HSFA1d_R	TCAAGGATTTTGCCTTGAGA	
HSFA1e_F	ATGGGAACGGTTTGCGAAT	
HSFA1e_R	TTTTCTGAGAGCATCTGATGTG	