Molecular mechanisms governing plant responses to high temperatures

Bingjie Li, Kang Gao, Huimin Ren and Wenqiang Tang

Ministry of Education Key Laboratory of Molecular and Cellular Biology, Hebei Collaboration Innovation Center for Cell Signaling, Hebei Key Laboratory of Molecular and Cellular Biology, College of Life Sciences, Hebei Normal University, Shijiazhuang 050024, China

†These two authors contributed equally
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Abstract The increased prevalence of high temperatures (HTs) around the world is a major global concern, as they dramatically affect agronomic productivity. Upon HT exposure, plants sense the temperature change and initiate cellular and metabolic responses that enable them to adapt to their new environmental conditions.

Decoding the mechanisms by which plants cope with HT will facilitate the development of molecular markers to enable the production of plants with improved thermotolerance. In recent decades, genetic, physiological, molecular, and biochemical studies have revealed a number of vital cellular components and processes involved in thermoresponsive growth and the acquisition of thermotolerance in plants. This review summarizes the major mechanisms involved in plant HT responses, with a special focus on recent discoveries related to plant thermosensing, heat stress signaling, and HT-regulated gene expression networks that promote plant adaptation to elevated environmental temperatures.

INTRODUCTION

Temperature is one of the most important environmental factors affecting the seasonal growth and geographic distribution of plants. From 1951 to 2012, the increased release of greenhouse gases, due to human industrial development, has caused the Earth’s average surface temperature to rise by an estimated 0.72°C. If this business-as-usual scenario continues, it is predicted that the average global surface temperature can increase by 3.7 ± 1.1°C by the end of the 21st century (IPCC 2013). These rising temperatures around the world will affect the life cycle of plants, by promoting seed germination, reducing vegetative growth, promoting early flowering, and disrupting the seasonal growth of certain species (Fitter and Fitter 2002). The populations of species that cannot adjust their flowering time in response to elevated environmental temperatures are already decreasing, potentially leading to local extinction (Willis et al. 2008).

One of the most troubling consequences of increasing global temperatures is the negative effect on agricultural productivity. It is estimated that for every 1°C increase in the mean global temperature, wheat (Triticum aestivum) yields will decrease by 6%, rice (Oryza sativa) by 3.2%, maize (Zea mays) by 7.4%, and soybean (Glycine max) by 3.1% (Zhao et al. 2017). Between 1980 and 2008, global maize and wheat production dropped by 3.8% and 5.5%, respectively, due to global warming (Lobell et al. 2011). It is therefore very important to study the impact of high temperatures (HTs) on plant growth and development to maximize agricultural production and food security in the future.

HT has profound effects on plant growth and development; however, the degree of its effect depends on the intensity of the heat and the duration and speed...
of the temperature increases. The effect of HT varies in different plant species and cultivars, and even at different developmental stages within a species. At the subcellular level, photosynthesis is one of the physiological processes most sensitive to HT. HT can rapidly inhibit photosynthesis by changing the internal structure of the chloroplasts, inactivating ribulose bisphosphate carboxylase/oxygenase (Rubisco), reducing the abundance of photosynthetic pigments, and damaging photosystem II (Crafts-Brandner and Salvucci 2002; Dunn et al. 2004; Allakhverdiev et al. 2008; Sharkey and Zhang 2010). Additionally, HT can affect the stability of proteins and membranes, induce the accumulation of reactive oxygen species (ROS), cause organelles to malfunction, alter phytohormone production and signaling, and induce transcriptomic reprogramming and metabolomic changes (Kotak et al. 2007; Wahid et al. 2007; Hasanuzzaman et al. 2013).

At the whole-plant level, when the temperature rises 5–6 °C above the optimum ambient temperature (for example, around 28 °C for Arabidopsis thaliana), this change accelerates the development of vegetative leaves, promotes early flowering, increases root growth, inhibits seed production, and makes the plants more susceptible to pathogen infection (Alcazar and Parker 2011; Gray and Brady 2016). High ambient temperatures also induce plant thermomorphogenesis, which is characterized by increased hypocotyl elongation, the hyponastic growth of petioles, reduced stomatal density, and decreased leaf thickness (Quint et al. 2016; Ibañez et al. 2017). Thermomorphogenesis is thought to increase the air space between the plant tissues, allowing better airflow and evaporative leaf cooling (Crawford et al. 2012).

As temperatures continue to increase, plants begin to experience heat shock (HS) stress. At this stage (between 28 °C and 37 °C for Arabidopsis), plants can still grow, but obvious deleterious effects begin to emerge, particularly in reproductive development. These include the inhibition of male and female gametophyte development, the inhibition of anther opening, the inhibition of pollen germination and pollen tube growth, disturbances in pollen tube guidance and fertilization, and the abortion of early embryos (Hedhly 2011; Sage et al. 2015). As a result, plant fertility is reduced or completely abolished, potentially leading to significant agricultural losses. Other phenotypic changes caused by HS include reduced seed vigor, decreased seed germination, slow root and shoot growth, decreased flowering, increased leaf senescence and abscission, and reduced grain filling (Wahid et al. 2007; Bahuguna and Jagadish 2015; Sage et al. 2015). If temperatures rise to 40 °C or higher, severe cellular injury may occur, resulting in rapid cell death due to the global malfunction of cellular activities.

Plants pre-exposed to a non-lethal HT for a certain period of time exhibit a significantly increased tolerance to otherwise lethal HTs. This is called acquired thermotolerance (Sung et al. 2003). During this process, plants sense an elevation in the ambient temperature and initiate signal transduction networks that regulate the expression of a series of genes, including those encoding HEAT SHOCK PROTEINS (HSPs) and ROS-scavenging enzymes, to increase their thermotolerance. A deeper understanding of the mechanisms by which plants acquire thermotolerance is therefore vital, as this could facilitate the development of HT-tolerant crops through molecular marker-assisted breeding. In the past decade, significant progress has been made in uncovering the mechanisms through which plants sense and respond to HT, using Arabidopsis as a model system. This review summarizes some of these findings, with a focus on recent discoveries related to thermosensing, the molecular mechanisms that regulate thermostressive growth, and the primary HS signal transduction mechanisms in plants.

**HIGH AMBIENT TEMPERATURE SIGNALING MECHANISMS**

When plants encounter high ambient temperatures, several morphological changes can be observed, including the enhanced elongation of hypocotyls and petioles, early flowering and reduced stomata formation. These changes involve the coordinated regulation of light signaling (Lorenzo et al. 2016), phytohormone signaling (Choi and Oh 2016), and the circadian clock (Nomoto et al. 2012). Mounting evidence suggests that various signaling pathways are integrated to regulate the abundance and/or transcriptional activity of the basic helix-loop-helix transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4), which forms part of the central regulatory hub mediating the diurnal growth of plants under normal and HT conditions (Figure 1).
Perception of ambient HT

PIF4 is a major component of the light signaling pathway. Its protein stability is regulated by light; red light-activated phytochrome B (phyB) phosphorylates PIF4, targeting it for 26S proteasome-mediated degradation (Lorrain et al. 2008). It was recently reported that phyB functions as a warm temperature sensor in Arabidopsis (Jung et al. 2016). HT speeds up the reversion of phyB from its active Pfr state to the inactive Pr state in the dark, causing the accumulation of PIF4 and enhancing cell elongation at night (Jung et al. 2016; Legris et al. 2016). This HT-mediated inactivation of a chromophore also exists for other photoreceptors. For the blue light receptor PHOTOTROPIN (PHOT), increased temperatures caused the rapid dissociation of its chromophore, a flavin mononucleotide, from its binding light/oxygen/voltage (LOV) domain in the fern Adiantum capillus-veneris. This dissociation inactivates the kinase activity of PHOT and regulates blue-light-dependent chloroplast movement (Fujii et al. 2017).

Although there is no evidence that they function as thermosensors, the blue light receptor CRYPTOCHROME 1 (CRY1) and ultraviolet (UV) light receptor UV RESISTANCE LOCUS 8 (UVR8) have been shown to regulate HT-mediated hypocotyl elongation in Arabidopsis. Blue light inhibits thermoresponsive growth by promoting CRY1 to directly interact with PIF4 to inhibit its transcriptional activity (Ma et al. 2016). UV light can abolish thermoresponsive hypocotyl elongation and decrease both the transcript and protein abundance of PIF4. UV light may also inhibit the transcription activity of PIF4 by stabilizing its inhibitory interacting protein LONG HYPOCOTYL IN FAR RED (HFR1), possibly via the inhibition of the HFR1-degradation-promoting ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Yang et al. 2005; Hayes et al. 2017).

High ambient temperature can trigger the nuclear import of COP1, via an unknown mechanism (Park et al. 2017), which may promote the expression of PIF4 and enhance its protein stability, as well as targeting the PIF4 antagonist, ELONGATED HYPOCOTYL 5 (HY5) for degradation (Gangappa and Kumar 2017). HY5 may inhibit PIF4-mediated hypocotyl elongation, via two distinct mechanisms: it could bind to the PIF4 promoter and inhibit its transcription (Delker et al. 2014), or compete with PIF4 for binding to the promoters of
the PIF4-target genes, inhibiting their expression (Gangappa and Kumar 2017).

Plants generally encounter higher temperatures during the day; therefore, the ability to sense and adapt to high daytime temperatures could be vital for plants to cope with their ever-changing environment. It has been proposed that a histone variant, H2A.Z, mediates the thermosensing of high ambient temperatures during the day in Arabidopsis (Kumar and Wigge 2010). The H2A.Z occupancy of genes that are rapidly upregulated by HT is significantly decreased when plants encounter high ambient temperatures (Cortijo et al. 2017). However, this HT-mediated H2A.Z eviction is dependent on a class of HEAT SHOCK FACTOR (HSF) family transcription factors, the HSFA1s, suggesting that the HSFA1s function upstream of H2A.Z to mediate plant responses to high ambient temperatures (Cortijo et al. 2017). Meanwhile, a recent study reported that plant basal thermotolerance varies diurnally, peaking during the day. This light-dependent regulation of plant thermotolerance might be initiated by ROS signals, generated from the chloroplasts (Dickinson et al. 2018), which could diffuse into the nucleus (Exposito-Rodriguez et al. 2017) to activate the transcription activity of the HSFA1s, regulating the expression of heat-responsive genes such as HSP70 to increase the basal thermotolerance of plants (Dickinson et al. 2018).

The PIF4-dependent ambient HT responses
PIF4-mediated thermoresponsive growth is regulated by an extensive network involving light and phytohormone signaling. Decreasing internal auxin concentrations or inhibiting polar auxin transport abolishes hypocotyl elongation under HT, suggesting that auxin is critical for thermo-responsive growth (Gray et al. 1998). HT can dramatically increase the binding of PIF4 to the promoters of auxin-biosynthesis-related genes such as YUCCA 8 (YUC8), TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1), and cytochrome P450, CYP79B2, activating their expression (Franklin et al. 2011; Sun et al. 2012). In addition, PIF4 interacts with the auxin signaling transcription factor AUXIN RESPONSE FACTOR 6 (ARF6) to synergistically promote the expression of genes required for hypocotyl elongation (Oh et al. 2014). During prolonged exposure to HTs, the over-accumulation of auxin can induce abnormally elongated stems and leaves. The RNA-binding protein FLOWERING CONTROL LOCUS A (FCA) can directly interact with PIF4, causing it to dissociate from the YUC8 promoter and maintain a balanced level of auxin in plants experiencing HT (Lee et al. 2014a).

In addition to interacting with the auxin pathway, PIF4 can integrate brassinosteroid (BR) and gibberellin (GA) signaling by interacting directly with central biosynthesis and signaling components of the two hormones. Evidence shows that PIF4 binds directly to the promoters of DWARF4 (DWF4) and BRASSINOSTEROID-6-OXIDASE 2 (BR6ox2) to promote the expression of these BR biosynthesis genes (Wei et al. 2017), as well as forming a complex with ARF6 and the BR signaling transcription factor BRASSINAZOLE RESISTANT 1 (BZR1) (Oh et al. 2012, 2014). Together, the PIF4/ARF6/BZR1 complex functions interdependently as a central regulatory hub to control cell elongation (Oh et al. 2012, 2014).

In contrast, GA signaling components, known as the DELLA proteins, may negatively regulate PIF4- and BR signaling-mediated cell elongation by binding to PIF4, ARF6, and BZR1, inhibiting their DNA-binding and transcriptional activities (Lucas et al. 2008; Bai et al. 2012; Li et al. 2012a; Oh et al. 2014). In the presence of the GA biosynthesis inhibitor, paclobutrazol (PAC), thermoresponsive hypocotyl growth is completely inhibited; however, a d ella pentuple mutant was shown to be insensitive to PAC-inhibited hypocotyl elongation under HT, suggesting that the DELLA proteins are negative regulators of plant thermomorphogenesis (Koini et al. 2009).

As a cell elongation-promoting plant hormone, BR plays an important role in HT-regulated hypocotyl elongation, but HT-induced hypocotyl elongation was completely abolished by the mutation of the PIF1/3/4/5 genes; therefore, the regulation of plant-thermosensitive growth by BRs is dependent on PIF4 (Oh et al. 2012). Meanwhile, HT induces the nuclear translocation of BZR1, enabling it to bind directly to the promoter of PIF4 to regulate its expression. This produces a feed-forward loop that amplifies thermoresponsive growth (Ibañez et al. 2018). Phosphorylated BZR1 cannot bind to DNA (He et al. 2005); however, HT cannot induce the dephosphorylation of BZR1 in vivo (Oh et al. 2012; Ibañez et al. 2018), raising the intriguing question of how phosphorylated BZR1 regulates the expression of PIF4 at 28 °C.

In addition to BZR1, PIF4 is regulated by another BR signaling component, BR-INSENSITIVE 2 (BIN2). BIN2 is
a serine/threonine protein kinase, which inhibits cell elongation by negatively regulating BR signaling. However, BR can induce the degradation of BIN2 to relieve its inhibitory effect (Li and Nam 2002; Peng et al. 2008; Zhu et al. 2017). A recent study showed that BIN2 can phosphorylate PIF4 and target it for proteasome-mediated degradation, especially during the maximal growth of the plant. The expression of a mutant form of PIF4, which cannot be phosphorylated by BIN2, resulted in hyper-elongated petioles and stems (Bernardo-García et al. 2014), indicating an additional level of thermoresponsive regulation of growth.

The diurnal growth of plants, under natural conditions, is regulated by crosstalk between the circadian clock and environmental temperature fluctuations. At 22°C, hypocotyl elongation is generally inhibited during the day and early night. As the night progresses, this inhibition is ameliorated and maximum growth occurs before dawn (Nozue et al. 2007). HT relieves the inhibition of hypocotyl elongation, promoting hypocotyl growth throughout the night (Box et al. 2015).

Recent studies have shown that interaction between PIF4 and several central clock components controls the periodic growth of plants under normal and high ambient temperatures. For example, the circadian clock component EARLY FLOWERING 3 (ELF3) inhibits the expression of PIF4 during the early night to prevent cell growth (Nusinow et al. 2011). Additionally, ELF3 and the clock proteins TIMING OF CAB EXPRESSION 1 (TOC1) and PSEUDO-RESPONSE REGULATOR 5 (PRR5) interact directly with PIF4, preventing it from activating its target genes (Nieto et al. 2015; Zhu et al. 2016). PRR5 levels peak in the late afternoon, while TOC1 peaks in the evening and ELF3 peaks at night (Nusinow et al. 2011; Hsu and Harmer 2013); thus, PRR5, TOC1, and ELF3 sequentially repress expression and transcriptional activity of PIF4, inhibiting plant growth from the afternoon to the early night.

Toward the end of the night, the expression of TOC1 and ELF3 begins to decrease, allowing PIF4-regulated growth. HT promotes phyB inactivation and prevents the binding of ELF3 to the promoters of its target genes, thus causing an increase in PIF4 expression and thermoresponsive growth during the night (Nomoto et al. 2012; Box et al. 2015; Jung et al. 2016). As it takes hours for HT to reduce the binding of ELF3 to its target promoters (Box et al. 2015), the mechanisms by which HT alters ELF3 and TOC1 activity during the night are not clear.

Much evidence indicates that a mechanism exists that mediates a trade-off between plant growth and defense responses. Increased ambient temperatures can stimulate plant growth, but make the plant more susceptible to pathogen infection; the mechanism that coordinates these two processes is not well understood (Alcázar and Parker 2011). A recent study revealed that, in addition to mediating plant thermoresponsive growth, PIF4 plays a critical role in suppressing the plant defense responses in response to HT (Gangappa et al. 2017). Indeed, many defense-related genes are downregulated in PIF4-overexpressing plants and upregulated in the pif4 mutant. Consistent with this, pif4 mutants are more resistant to Pseudomonas syringae pv. tomato (Pto) DC3000 infection, while PIF4-overexpressing plants are more susceptible to Pto DC3000, compared with wild type. Mutants or accessions with reduced phyB activities also have enhanced hypocotyl growth but reduced resistance to Pto DC3000 (Gangappa et al. 2017). Based on these results, a phyB-PIF4 thermosensory signaling module was proposed to play an essential role in balancing plant growth and defenses during the response to HTs (Gangappa et al. 2017).

PIF4 also regulates thermoresponsive flowering and stomatal development. It binds directly to the promoters of FLOWERING LOCUS T (FT) and SPEECHLESS (SPCH) to regulate their expression and promote early flowering and restrict stomatal development, respectively, under HT conditions (Kumar et al. 2012; Lau et al. 2018).

PIF4-independent ambient HT responses
PIF4 plays a central role in mediating HT thermoresponses, but other PIF4-independent mechanisms also regulate plant growth, flowering, and the transition of seedlings to an autotrophic habit under HTs. A HT treatment dramatically increased the abundance of HSP90, which interacts directly with the auxin co-receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) to prevent its degradation and enable its regulation of auxin-mediated plant growth (Wang et al. 2016b). HT also stimulates early flowering by inducing the degradation of SHORT VEGETATIVE PHASE (SVP), which otherwise forms a flowering-repressing complex with FLOWERING LOCUS M (FLM) (Lee et al. 2013, 2014b).
FCA can act independently of PIF4 to regulate the expression of the *PROTOCHLOROPHYLLIDE OXIDOREDUCTASES* (PORs) and suppress the degradation of their encoded proteins by a currently unknown mechanism. The resulting increase in PORs stimulates chlorophyll biosynthesis and suppresses ROS production under HT (Ha et al. 2017; Lee et al. 2017). FCA can also regulate the processing of miRNA172 in the thermosensory flowering pathway (Jung et al. 2012). However, it is still unclear how these PIF4-independent processes cooperate with the PIF4-dependent processes to regulate the various thermoresponses in plants.

**HEAT SHOCK SIGNALING MECHANISMS**

**HSFAi-dependent transcriptional regulation networks**
Emerging evidence supports the existence of a HS-activated transcriptional network that regulates thermoresponsive gene expression (Figure 2). Within this network, the HSFs are considered to play conserved and central roles in animals, yeasts, and plants (Scharf et al. 2012). The 21 HSFs encoded in the *A. thaliana* genome can be divided into three classes: HSFA, HSFB, and HSFC (Scharf et al. 2012). Reducing the expression of HSFA2, HSFA1a, 1b, 1d and 1e, HSFA3, HSFA6b, or HSFB1 and

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**Figure 2. Signaling networks regulating plant heat shock responses**
Heat stress might simultaneously affect membrane fluidity, cause the malfunction of chloroplasts and mitochondria, and denature proteins in the cytosol or endoplasmic reticulum, resulting in increased cytosolic calcium, reactive oxygen species (ROS) and nitric oxide (NO) levels and activation of cytoplasmic protein response (CPR) and unfolded protein response (UPR) responses. Interdependently, calcium, ROS and NO can activate multiple signaling pathways to alter the activity of heat shock factors (HSFs), via post-translational modification. HSFA1s are master transcription factors affecting plant heat shock responses. HSFA1i activation stimulates the expression of a number of transcription factors that participate in a critical transcriptional regulatory cascade underlying the acquisition of thermotolerance in plants.
B2b using T-DNA insertions or RNA interference significantly alters the thermotolerance of *Arabidopsis* (Schramm et al. 2008; Ikeda and Ohme-Takagi 2011; Liu et al. 2011; Liu and Charng 2013; Huang et al. 2016). Recent studies have also shown that the HSFA1s function as master regulators of HS-regulated gene expression in plants (Liu et al. 2011; Yoshida et al. 2011). For more than 65% of the genes up-regulated during HS, including those encoding transcription factors/co-activator with essential roles in regulating plant thermotolerance (e.g., HSFA2, HSFB1, HSFB2a, MBF1c, DREB2a, and bZIP28), this up-regulation was abolished in the *hsfa1a/b/d/e* quadruple mutant (Liu et al. 2011).

Of the HSFA1s-targeted transcription factors/co-activators, the expression of HSFA2, a key regulator of plant thermotolerance (Chang et al. 2007; Ogawa et al. 2007), exhibited more than a 10,000-fold up-regulation by HS in wild-type *Arabidopsis* (Busch et al. 2005). HSFA2 interacts directly with HSFA1s to synergistically activate the expression of HS-regulated genes (Chan-Schaminet et al. 2009). Overexpressing HSFA2 rescued the developmental and HS-hypersensitive phenotypes of the *hsfa1a/b/d/e* mutant, suggesting that HSFA2 can function downstream of the HSFA1s to regulate plant HS responses (Liu and Charng 2013). HSFA2 overexpression in the *hsfa1a/b/d/e* mutant only rescued the expression of a subset of HS-regulated genes, suggesting that additional transcription factors are specifically targeted by the HSFA1s and function in coordinate with HSFA2 to regulate plant HS responses (Liu and Charng 2013).

In addition to activating HS-regulated genes, HSFA2 functions in regulation of chromatin state to condition long-term gene expression. For example, HSFA2 is required to maintain high levels of specific histone modifications (H3K4me2 and H3K4me3) at the 5′ regions of ASCORBATE PEROXIDASE 2 (APX2), HSP22, and HSP18.2. These modifications are critical for plants to maintain prolonged acquired thermotolerance, a phenomenon also known as HS memory (Chang et al. 2007; Lämke et al. 2016). HSFA2 does not possess DNA methyltransferase activity; therefore, how it recruits DNA methyltransferases to specific loci to sustain the activation of these HS memory-related genes remains unknown.

In addition to HSFs, the ERF/AP2 family transcription factor DREB2A also functions in HS-mediated transcriptional regulatory networks and DREB2A is a direct target of HSFA1. Under normal conditions, the level of intracellular DREB2A protein is maintained at a very low level, possibly due to DREB2A-INTERACTING PROTEIN 1 (DRIP1) and DRIP2-mediated proteasome degradation (Qin et al. 2008). HS can rapidly up-regulate the expression of DREB2A; 30 min after exposure to 37°C, the level of DREB2A transcripts peaked at a 150-fold higher abundance than in non-HS-treated control plants (Sakuma et al. 2006). Knocking out the expression of DREB2A made plants more sensitive to HS treatment, and plants overexpressing a constitutively active form of DREB2A showed enhanced thermotolerance, demonstrating the importance of DREB2A in regulating plant HS responses (Sakuma et al. 2006).

DREB2A forms a complex with NUCLEAR FACTOR Y, SUBUNIT C10 (NF-YC10), NF-YB3, and NF-YA2; this complex binds to the promoters of specific genes, including HSFA3 and the HSPs, regulating their expression (Sato et al. 2014).

Another direct target of HSFA1 is MULTIPROTEIN BRIDGING FACTOR 1C (MBF1c), which encodes a conserved transcriptional co-activator. MBF1c accumulates within 20 min of HS exposure and is translocated into the nucleus (Suzuki et al. 2008). There, MBF1c binds to a CTAGA motif and regulates the expression of HS-related genes such as DREB2A, HSFB2a, and HSFB2b (Suzuki et al. 2011b). These genes are also direct targets of the HSFA1s; therefore, it would be interesting to investigate whether the HSFA1s interact with MBF1c to regulate their common target genes.

Although the HSFA1s play central roles in regulating HS-induced gene expression, the molecular mechanisms by which HS activates HSFA1 are unclear. Studies of animal cells indicate that, under normal conditions, HSF1 is inactive and forms a complex with HSP70 and HSP90. HS increases the cytosolic concentration of unfolded proteins, which compete for binding to HSP70 and HSP90, releasing HSF1 from its inert complex. The free HSF1 protein is then phosphorylated and forms an active, homo-trimeric transcription complex (Vabulas et al. 2010). This activated HSF1 complex binds to a conserved HS element (HSE), nGAAnTTCn, and alters the expression of the HS-regulated genes (Vabulas et al. 2010).

Mounting evidence indicates that similar mechanisms might exist in plants; for example, HSP70 can bind to HSFA1 and inhibit its DNA-binding ability under normal conditions in tomato (*Solanum lycopersicum*)
(Hahn et al. 2011). Through sequential deletion studies, a temperature-dependent repression (TDR) domain was identified in Arabidopsis HSFA1d, which allows it to interact with HSP70 and HSP90. Mutant HSFA1d lacking the TDR domain constitutively localized to the nucleus and could induce expression of the HSPs in the absence of HS (Ohama et al. 2016).

HSF-BINDING PROTEIN 1 (HSBP1) is another negative thermotolerance regulator that binds to HSFA1, and HS induces HSBP1 expression and HSBP1 nuclear translocation (Hsu et al. 2010). HSBP1 can bind directly to HSFA1a, HSFA1b, and HSFA2, and inhibit the DNA-binding capacity of HSFA1b, but the mild reduction of HSP expression in the hsbp1 mutant suggests that HSBP1 may not be as important as expected in regulating plant HS responses (Hsu et al. 2010).

Post-translational modifications play important roles in regulating protein stability, DNA-binding ability, nuclear localization, and transcriptional activation/suppression of HSF1 in animal cells (Gomez-Pastor et al. 2018). However, whether HS activates HSFA1s via post-translational modifications, in planta, is still an open question. The cyclin-dependent kinase CDC2a, calmodulin (CaM)-binding protein kinase CBK3, and PROTEIN PHOSPHATASE 7 (PP7) might modulate the phosphorylation status of the HSFA1s and regulate their DNA-binding ability (Reindl et al. 1997; Liu et al. 2007a, 2008). Additionally, the phosphorylation of Tyr271 in HSFA1d or Tyr294 in HSFA1a can reverse the auto-inhibitory effect of the TDR domain on HSFA1s (Ohama et al. 2016). However, further genetic and molecular data are required to reveal whether in vivo phosphorylation is important for the function of HSFA1s during plant HS responses. Besides phosphorylation, HSFA1 can be SUMOylated in vivo (Miller et al. 2010). HS can significantly increase protein SUMOylation, and knocking out the expression of SLIZ1, which encodes a SUMO E3 ligase, increases plant sensitivity to heat stress (Yoo et al. 2006; Zhang et al. 2017a); however, the regulatory effect of SUMOylation on the function of the HSFA1s during plant HS responses requires further elucidation.

**HSFA1-independent transcription regulation networks**

The observation that only 65% of HS-upregulated genes were regulated by the HSFA1s suggested that additional transcription factors regulate gene expression in response to HS (Liu et al. 2011). Although studies have revealed a number of transcription factors the expression levels of which in vivo are correlated with the thermotolerance of plants (Davletova et al. 2005; Li et al. 2011; Hsieh et al. 2013; Wang et al. 2013; Guan et al. 2014; Lee et al. 2014c; Chao et al. 2017; Liao et al. 2017), it is not clear whether these transcription factors are directly regulated by the HS signaling pathway, or merely the targets of unknown primary transcription factors. Of these transcription factors, the activity of a NAC transcription factor, NAC019, during HS is regulated by protein dephosphorylation (Guan et al. 2014). RCF2, a protein phosphatase, interacts with and dephosphorylates NAC019 under HS conditions, thereby increasing the binding of NAC019 to the promoters of HSFA1b, HSFA6b, HSFA7a, and HSFC1 (Guan et al. 2014). Despite this, the HS-induced expression of the HSFs and the HSPs was only mildly altered in rcf2 and naco19 mutant plants (Guan et al. 2014); therefore, the role of NAC019 in the plant HS response, especially in the HS signaling pathway, requires further investigation.

**Calcium (Ca$^{2+}$) signaling**

Of all the characterized HS-induced cellular responses, the rise in the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]) is thought to be the most rapid one. HS causes a significant increase in [Ca$^{2+}$] in wheat epidermal cells within 90 s, peaking at around 5 min after the HS treatment (Liu et al. 2003). This increase in cytosolic [Ca$^{2+}$] is mediated by plasma membrane-localized CYCLIC NUCLEOTIDE GATED CHANNEL (CNGC) family proteins in Physcomitrella patens and Arabidopsis (Finka et al. 2012; Gao et al. 2012). Another membrane-localized Ca$^{2+}$ channel, ANNEXIN 1 (ANN1), also contributes to the HS-induced rise in cytosolic [Ca$^{2+}$], although it is not responsible for the initial HS-induced influx of Ca$^{2+}$ (Wang et al. 2015). Instead, ANN1 may become activated following a CNGC-induced increase in cytosolic [Ca$^{2+}$] to help maintain the high [Ca$^{2+}$] required to stimulate downstream signaling events during HS (Wang et al. 2015).

HS can induce rapid changes in the cytosolic [Ca$^{2+}$] in plants, which can induce expression of the HSPs (Saidi et al. 2009); this indicated that CNGCs could function as thermosensors in plants (Finka et al. 2012). Using the artificial membrane fluidizer benzyl alcohol, Finka and Goloubinoff (2014) showed that increasing membrane fluidity made plants more sensitive to HS, and proposed that HS activates the CNGCs...
through changes in membrane fluidity. In the bacterium *Bacillus subtilis*, changes in fluidity, at various temperatures, alters the thickness of membranes, causing the membrane histidine kinase DesK to change from a kinase at low temperatures to a phosphatase at HTs (Mansilla et al. 2004; Inada et al. 2014).

The activity of the CNGCs can also be regulated by the Ca\(^{2+}\) sensor, CaM, as well as cAMP (Borsics et al. 2007; Ma et al. 2009). Gao et al. (2012) showed that the cytosolic level of cAMP was rapidly upregulated by a 60-s HS treatment, and that this accumulation can be blocked by pretreating plants with an adenylyl cyclase inhibitor (Gao et al. 2012). Little is known about what happens within the first 60–90 s of HS treatment; thus, the questions of whether the CNGCs function as the primary thermosensors in plants and whether they are activated by membrane fluidity changes require further investigation.

In addition to promoting the influx of Ca\(^{2+}\), HS can mobilize Ca\(^{2+}\) from intracellular stores. For example, a 1-min treatment of 37°C caused a 2.5-fold increase in the cytosolic level of inositol 1,4,5-triphosphate (IP3) in *Arabidopsis*, reaching a maximum 3.5-fold increase after 2.5 min (Zheng et al. 2012). Preventing the accumulation of IP3, by knocking out the expression of the enzymes involved in its biosynthesis, *PHOSPHOLIPASE C* (PLC)9 and PLC3, impaired the rapid HS-induced accumulation of cytosolic Ca\(^{2+}\) in these mutants, and, thus, their thermotolerance (Zheng et al. 2012; Gao et al. 2014). In contrast to animal cells, the receptor for IP3 has not yet been identified in plants. IP3 can be further phosphorylated into IP6 to mobilize Ca\(^{2+}\) from intracellular stores in plants (Lemtiri-Chlieh et al. 2003); however, no IP6-binding Ca\(^{2+}\) channel has yet been discovered in plants.

Downstream, HS-initiated Ca\(^{2+}\) signaling could be transduced via a number of Ca\(^{2+}\)-binding proteins, such as CaM, the CALCIUM-DEPENDENT PROTEIN KINASES (CDPKs), and the RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs). CaM is one of the most important intracellular Ca\(^{2+}\) receptors. Upon Ca\(^{2+}\) binding, CaM is activated and able to regulate many cellular processes (Ma et al. 2002). CaM also binds S-NITROSOGLUTATHIONE REDUCTASE (GSNOR) to inhibit its activity and increase the cellular levels of nitric oxide (NO) (Zhou et al. 2016), which increases plant thermotolerance (Xuan et al. 2010).

CDPK family proteins are another group of Ca\(^{2+}\)-binding proteins, composed of a CaM-like domain and a protein kinase domain. The binding of Ca\(^{2+}\) with the CaM-like domain of these enzymes activates their kinase activity and initiates a phosphorylation cascade. Treating plants with the CDPK inhibitor W7 inhibits the HS-induced activation of a MITOGEN-ACTIVATED PROTEIN KINASE (MAPK), suggesting that CDPKs might function upstream of the MAPK in transducing HS signals (Sangwan et al. 2002). As W7 is also a CaM antagonist, it is possible that HT may activate the MAPK cascade via CaM instead of the CDPKs; therefore, further investigation is required.

The RBOHs are plasma membrane-localized plant NADPH oxidases, which are important for generating ROS in the apoplastic space under abiotic or biotic stress (Suzuki et al. 2011a). The N-terminal sequence of the RBOHs contains two Ca\(^{2+}\)-binding EF-hand motifs, and their activity is directly regulated by an increase in intracellular Ca\(^{2+}\) (Ogasawara et al. 2008). In addition, several studies have revealed that Ca\(^{2+}\) can activate the RBOHs via CDPK- and CBL INTERACTING PROTEIN KINASE 26 (CIPK26)-mediated phosphorylation (Kobayashi et al. 2007; Drerup et al. 2013; Dubiella et al. 2013); however, whether this regulatory mechanism applies to the HS responses of plants is yet to be determined.

**ROS signaling**

The accumulation of ROS is another major cellular response to HS in plants. A 45 s heat treatment at 42°C can quickly stimulate significant accumulation of intracellular ROS in *Arabidopsis* protoplasts, peaking after around 3 min before gradually decreasing to the basal level 10 min after the HS treatment (Yao et al. 2017). This suggests that ROS are involved in the early HS response and may function as the primary signaling molecules. HS-induced ROS originate mainly from the chloroplasts, mitochondria, peroxisomes, and the plasma membrane (Choudhury et al. 2017). The disruption of photosynthesis and respiration by HS could uncouple the electron transport chain, creating a major
source of ROS (Suzuki and Mittler 2006). HS can also activate plasma membrane-localized RBOHs, partially via the Ca$^{2+}$ signaling pathway. This activation increases ROS production in the apoplastic space; the apoplastic ROS are then imported into the cell, via the aquaporins, to regulate cellular thermoresponses (Bienert et al. 2007).

During HS, if the cellular ROS levels become too high, they can cause oxidative damage by disrupting membranes, proteins, RNAs, and DNA, leading to programmed cell death (Vacca et al. 2006; Petrov et al. 2015). The thermotolerance of plants could therefore be expected to increase following the overexpression of genes encoding the ROS-scavenging enzymes, such as glutaredoxin (Sundaram and Rathinasabapathi 2010) and APX (Shi et al. 2001). Pretreatment of plants with hydrogen peroxide (H$_2$O$_2$) (Wang et al. 2014a) or various phytohormones (Dhaubhadel et al. 1999; Larkindale and Huang 2005) to increase the activity of ROS-scavenging enzymes could also increase plant thermotolerance.

Although high ROS levels are deleterious to cells, lower levels of ROS, especially of H$_2$O$_2$, play a critical role in regulating acquired thermotolerance of plants. For example, Wang et al. (2014b) reported that the HS-induced expression of HSP17.7 and HSP21 was impaired in mutants unable to produce H$_2$O$_2$ (rbohB, rbohD, and rbohB/D), and exogenous applied H$_2$O$_2$ increased the thermotolerance of the plants.

ROS signaling is primarily mediated by the oxidation of cysteine residues, leading to the formation of inter- or intra-molecular disulfide (S-S) bonds on target proteins that cause conformational changes and alter protein functions. This post-translational modification stimulates the cleavage of an extracellular protein, GRIM REAPER (GRI). GRI cleavage produces an 11 amino-acid peptide that binds to a membrane-localized receptor-like kinase (RLK), POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 5 (PRK5), and induces ROS-dependent cell death (Wrzaczek et al. 2015). Additionally, ROS-regulated cysteine modification could directly regulate subcellular protein transport (Klein et al. 2012), as well as altering the activities of the ion channels (Garcia Mata et al. 2010; Seidel et al. 2012), protein kinases (Türkeri et al. 2012), and transcription factors (Shaikhali et al. 2008, 2012).

It was proposed that HSFA4a, HSFA8 and HSFA1s can function as ROS sensors to regulate the expression of HS-induced oxidation-related genes in plants (Miller and Mittler 2006; Dickinson et al. 2018). Mutation of conserved cysteine residues (Cys24 and Cys269 on HSFA8, but Cys153 and Cys357 on HSFA1d) inhibits the H$_2$O$_2$-induced nuclear translocation of HSFA8 (Giesguth et al. 2015), as well as impairs HSFA1d’s ability to activate the expression of APX2 under high-light conditions (Jung et al. 2013). However, HS treatment did not induce the nuclear translocation of HSFA8, suggesting that the redox-sensing function of HSFA8 may not directly regulate HS-related gene expression (Giesguth et al. 2015). Meanwhile, rapid ROS-induced cysteine modification of HSFA8 and HSFA1s during the onset of the HS condition has not been demonstrated in vivo.

In another ROS related mechanism, H$_2$O$_2$ can activate a specific MAPK kinase kinase, Arabidopsis NPK1-LIKE PROTEIN KINASE (ANP1), which initiates a phosphorylation cascade that activates the downstream kinases MAPK3 and MAPK6 (Kovtun et al. 2017). Similarly, the addition of a Ca$^{2+}$ ionophore, induces a transient increase in ROS production within 60 s (Liao et al. 2000). Activated MAPK3 and MAPK6 can phosphorylate HSFA2 and HSFA4a (Evrard et al. 2013; Pérez-Salamó et al. 2014), resulting in the HS-induced nuclear localization of HSFA2 and the transcriptional activation of HSFA4a. Despite this, knocking out/down the expression of AtMPK6 in Arabidopsis and its homologous gene SIMPK1 in tomato increased plant thermotolerance (Li et al. 2012b; Ding et al. 2018). Therefore, the role of the MAPK pathway in regulating the plant HS response and HS signaling is yet to be fully elucidated.

Accumulating evidence suggests that ROS actively can interact with Ca$^{2+}$ signaling to regulate plant development and various stress responses. In one experiment, the application of exogenous H$_2$O$_2$ induced a sharp increase in intracellular Ca$^{2+}$ within 60 s (Liao et al. 2017). Similarly, the addition of a Ca$^{2+}$ ionophore, ionomycin, induces a transient increase in ROS production within 60 s (Kimura et al. 2012); however, there is insufficient evidence to determine whether Ca$^{2+}$ functions upstream of ROS in transducing the primary HS signal, or vice versa.

As already mentioned, the production of ROS by RBOHs could be directly regulated by Ca$^{2+}$ or CDPK-mediated phosphorylation. An increased level of ROS in the apoplastic space can also induce the accumulation
of intracellular Ca\(^{2+}\), possibly by regulating the membrane-localized Ca\(^{2+}\) channel ANN1 (Richards et al. 2014; Liao et al. 2017). The feedback loop between ROS and Ca\(^{2+}\) signaling can therefore generate a self-sustained response to HS in plants. Similar to the pathogen-induced systemic acquired resistance (SAR) pathway, the application of HS to cauline leaves increased the resistance of rosette leaves to HS (Suzuki et al. 2013). This systemic acquired acclimation (SAA) can be blocked by adding a drop of catalase (a H\(_2\)O\(_2\) scavenger) or the RBOH inhibitor diphenylene iodonium, which suggests that extracellular ROS are involved in the SAA to HS (Suzuki et al. 2013).

Based on these and other findings, a ROS/Ca\(^{2+}\) stress signaling hub in plant SAA was proposed (Gilroy et al. 2016). In this model, HS increases the cytosolic [Ca\(^{2+}\)] and activates Ca\(^{2+}\)-dependent protein kinases, which phosphorylate and activate the membrane-localized RBOHs. The activated RBOHs then generate ROS in the extracellular space; the ROS are sensed by the neighboring cells, triggering a new round of Ca\(^{2+}\) signaling. As such, the SAA response would spread through plants at a rate of 8.4 cm/min (Gilroy et al. 2016). Additionally, the ROS-induced production of abscisic acid (ABA) is required for the HS-induced SAA response; however, the mechanism by which ABA regulates the plant SAA response remains to be determined (Suzuki et al. 2013).

**NO signaling**

Besides ROS, NO is another free radical produced in plant cells in response to HS. Using the fluorescent dye, DAF-2DA, as an indicator for NO, HS was shown to induce a transient accumulation of NO within 5 min of treatment (Xuan et al. 2010). NO decreased as the HS treatment continued, but a second wave of NO accumulation began to appear 30 min after the HS treatment, peaking after around 60 min (Xuan et al. 2010). Inhibition of NO biosynthesis was shown to increase the sensitivity of the plants to HS, and the addition of the NO donor, sodium nitroprusside (SNP), increased plant thermotolerance, suggesting that NO plays an important role in regulating plant HS responses (Xuan et al. 2010; Karpets et al. 2015).

The mechanisms by which NO regulates the plant HS responses are currently unclear, but mounting evidence suggests that NO engages in considerable crosstalk with ROS in regulating plant growth and development, as well as the responses to biotic and abiotic stresses (Niu and Liao 2016). The pathogen-induced accumulation of ROS was reduced in the NO-deficient mutant noa/nia1 (Wang et al. 2014b). Similarly, Wu et al. (2015) showed that the NO donors SNP and S-nitroso-N-acetylpenicillamine (SNAP) enhanced the production of intracellular H\(_2\)O\(_2\), as well as the H\(_2\)O\(_2\)-induced accumulation of HSP21, under HS conditions, whereas the NO scavenger 2-(4-phenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO) inhibited it (Wu et al. 2015). On the other hand, a number of reports have shown that H\(_2\)O\(_2\) functions upstream of NO production, possibly through the transcriptional regulation of NO biosynthesis genes (Lin et al. 2012; Shi et al. 2015).

Like ROS, NO is highly reactive and capable of post-translationally modifying proteins at cysteine residues, via S-nitrosylation. This modification can regulate the stability, subcellular localization, and activity of the target proteins (Tada et al. 2008; Lindermayr et al. 2010; Gibbs et al. 2014), causing global changes in gene expression (Hussain et al. 2016). Using shotgun proteomics, a number of S-nitrosylated proteins have been identified (Puyaubert et al. 2014; Hu et al. 2015). Of these proteins, many are related to the plant HT responses, including the HT signaling proteins (CDPK2, CDPK4, CDPK26, CaM, and UVR8), the HSPs (HSP70, HSP90, HSP91, HSP88, and HSP60), and the enzymes that regulate cellular redox levels (catalase, APX, monodehydroascorbate reductase, superoxide dismutase, glutathione peroxidase, glutaredoxin, and glutathione S-transferase) (Hu et al. 2015). NO-mediated S-nitrosylation can regulate the activity of H\(_2\)O\(_2\)-scavenging enzymes (APX) and H\(_2\)O\(_2\)-producing enzymes (RBOHD), demonstrating that NO plays dynamic roles in the regulation of H\(_2\)O\(_2\) production (Yun et al. 2011; Pinto et al. 2013; Yang et al. 2015). Taking these findings together, it is likely that ROS and NO interdependently regulate plant development as well as the stress responses. Whether a similar mechanism applies to HS signaling requires further investigation.

In animal cells, NO functions as a secondary messenger to mobilize Ca\(^{2+}\) from intracellular stores (Galione 2006). NO can increase [Ca\(^{2+}\)] in tobacco and Arabidopsis, and this increase in Ca\(^{2+}\) can be inhibited by a pretreatment with c-PTIO, suggesting that the induction is specifically mediated by NO (Lamotte et al. 2006; Aboul-Soud et al. 2009). This NO-induced accumulation of Ca\(^{2+}\) can be significantly blocked by

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inhibitors of the Ca\(^{2+}\) channels in the membrane, including La\(^{3+}\) and Gd\(^{3+}\), and less significantly blocked by the intracellular Ca\(^{2+}\) channel inhibitor 8-bromo cADPR. This suggests that the plasma membrane channel-mediated influx of Ca\(^{2+}\) is the main contributor to the NO-induced elevation in cellular Ca\(^{2+}\) (Lamotte et al. 2006; Aboul-Soud et al. 2009). Considering the time it takes for heat exposure to induce the accumulation of intracellular Ca\(^{2+}\) and NO, it is reasonable to conclude that NO acts downstream of Ca\(^{2+}\) in the regulation of plant HS signaling, suggesting that NO feedback could enhance the Ca\(^{2+}\)-mediated HS signaling, via an unknown mechanism.

**Hydrogen sulfide (H\(_2\)S) signaling**

Under HS or other abiotic stresses, plants accumulate H\(_2\)S (Calderwood and Kopriva 2014). H\(_2\)S is produced by L-cysteine desulfhydrase and D-cysteine desulfhydrase, the activities of which can be upregulated by HS treatment (Chen et al. 2016). An exogenously applied H\(_2\)S donor, NaHS, was shown to increase the thermotolerance of maize (Li et al. 2013). H\(_2\)S increases the activity of the ROS-scavenging enzymes, such as catalase, glutathione peroxidase, superoxide dismutase, and glutathione reductase (Li et al. 2014). H\(_2\)S is thought to regulate plant stress responses via NO and ROS signaling (Hancock and Whiteman 2016). Similar to NO, H\(_2\)S can modify the cysteine residues in a protein; however, whether this modification is responsible for enhancing the thermotolerance of plants is unknown.

**The unfolded protein response (UPR)**

HT, especially extreme HT, can cause protein misfolding and denaturation. Misfolded proteins are highly toxic because they can form aggregates and hamper normal cellular activities. Misfolded proteins trigger the cytoplasmic protein response (CPR) or UPR in the endoplasmic reticulum (ER), stimulating the expression of the HSPs (mostly HSP70 and HSP90), which could help to refold the unfolded proteins (Sugio et al. 2009; Howell 2013). If refolding fails, the unfolded proteins are subjected to protein degradation via the ubiquitin proteasome system or autophagy (Buchberger et al. 2010; Amm et al. 2014). Multiple studies have shown that the ability to clean up unfolded proteins corresponds to the ability of plants to survive exposure to extreme HT. For example, plants deficient in the expression of proteasome subunits (Li et al. 2015), E3 ligases, and autophagy-related genes (Zhou et al. 2014a, 2014b; Liu et al. 2016; Gil et al. 2017) are susceptible to HS. Moreover, transgenic plants overexpressing genes encoding either ubiquitin (Tian et al. 2014) or ubiquitin E3 ligases (Lim et al. 2013; Liu et al. 2014; Liu et al. 2016) exhibit enhanced basal and/or acquired heat tolerance.

Although both outcomes are initiated by unfolded proteins, the molecular mechanism by which gene expression is activated in the CPR is completely different from that of the UPR. The CPR activates HSP expression through an HSF-dependent pathway. Transcriptomic analyses have shown that a significant number of CPR-induced genes contain HSEs, and that the CPR-induced expression of HSP70 is partially regulated by HSFA2 in Arabidopsis (Sugio et al. 2009). In contrast, the UPR activates the expression of stress-related genes via three BASIC LEUCINE ZIPPER (bZIP) transcription factors in two different pathways.

In one pathway, an ER membrane-localized RNA-splicing factor, INOSITOL-REQUIRING ENZYME-1 (IRE1), is activated by unfolded proteins. IRE1 excises a 23 bp segment from the mRNA encoding the membrane-anchored transcription factor bZIP60, resulting in the translation of a new bZIP60 protein without the transmembrane domain, which can translocate to the nucleus to activate gene expression (Deng et al. 2011).

In the second pathway, misfolded proteins in the ER lumen bind with BINDING PROTEIN 3 (BIP3), an HSP70 homolog in the ER, dissociating it from two ER membrane-anchored transcription factors, bZIP17 and bZIP28. These proteins are then transferred to the Golgi body and are cleaved by the proteases SITE-1 PROTEASE (S1P) and SITE-2 PROTEASE (S2P), which release the cytoplasmic domain of bZIP17 or bZIP28 from the membrane to enable them to translocate to the nucleus and promote the expression of the stress-responsive genes (Liu et al. 2007b, 2007c).

The mRNA splicing of bZIP60 and the expression of bZIP28 can be induced by HS (Deng et al. 2011; Liu and Charnig 2012), and bzip28 and bzip28/bzip60 mutant plants are hypersensitive to HT treatments, suggesting an essential role for the UPR in regulating plant thermotolerance (Gao et al. 2008; Zhang et al. 2017b). Interestingly, the heat-induced expression (but not the basal expression) of bZIP28 is dependent on the HSFA1s (Gao et al. 2008), but the heat-induced expression of the bZIP28-target genes is unaltered in the hsfa1a/b/d/e
mutant, possibly due to their basal expression of bZIP28 (Liu and Charng 2012). These results suggest that bZIP28 and the HSFA1s mediate two separate routes of transcriptional regulation under HS conditions. This conclusion is supported by transcriptomic profiling comparisons, which showed that few genes are regulated by both HS exposure and the UPR (Sugio et al. 2009; Zhang et al. 2017b).

The *A. thaliana* genome encodes many other membrane-bound transcription factors, a number of which have been shown to translocate from the ER to the nucleus to regulate gene expression (Iwata and Koizumi 2016; Yang et al. 2016). Of these transcription factors, ER stress can induce the relocation of NAC089, promote the expression of a transmembrane-domain-deletion form of this protein, which can regulate the activity of caspase-like proteins and induce programmed cell death (Yang et al. 2016). Interestingly, the ER stress-induced expression of NAC089 is redundantly regulated by bZIP28 and bZIP60 (Yang et al. 2016), raising the question of how NAC089 coordinates with bZIP28 and bZIP60 to determine when ER stress should initiate programmed cell death under HS conditions.

In addition to the proteolyticable transcription factors affected by HS, the *Arabidopsis* B-CELL LYM-PHOMA 2-ASSOCIATED ATHANOGENE 7 (AtBAG7) protein, which is an ER localized co-chaperone, is also proteolytically processed and translocated into the nucleus in plants treated at 50 °C for 30 min (Li et al. 2017). In the nucleus, AtBAG7 interacts directly with transcription factor WRKY29 following the HS-induced SUMOylation of AtBAG7, enabling it to regulate the expression of downstream target genes (Li et al. 2017).

**FUTURE PERSPECTIVES**

In recent years, significant progress has been made in elucidating the biochemical, physiological, and molecular mechanisms by which plants sense and respond to elevated environmental temperatures; however, many questions concerning HT signaling are still unresolved. Among them, the mechanism by which HT is sensed is the holy grail of the plant HT response research field. As a physical signal, temperature changes can affect every part of a cell almost simultaneously; thus, it is natural to consider the possibility that multiple sensing mechanisms exist. Despite the fact that a number of thermosensors have been proposed, the definition of a thermosensor remains vague. Biochemistry tells us that a raised temperature will increase (or inhibit, if above a certain threshold) enzymatic activity or more generally change a protein’s structure, which may contribute to the acquired thermotolerance of plants. Is it reasonable to have a broad definition that includes all HT-activated or HT-inhibited enzymes or proteins as thermosensors or thermosensing mechanisms?

HT-induced changes in membrane fluidity might also alter the activity of transmembrane proteins other than the CNGCs, including the RLKs. It was recently established that ERECTA, a membrane-localized RLK, regulates the thermotolerance of plants (Shen et al. 2015). There are more than 600 RLKs encoded in the *A. thaliana* genome, many of which are membrane-localized (Shiu and Bleecker 2001). In a large-scale mutant screen, we analyzed the basal and acquired thermotolerance of 151 RLK mutants in *Arabidopsis*, revealing that more than a dozen had significantly altered thermotolerances (Table 1). Additionally, physiological analyses showed that exogenously applied BRs or ethylene significantly improved plant thermotolerance (Dhaubhadel et al. 1999; Larkindale and Huang 2005). Because the receptors for BRs and ethylene are also membrane-localized RLKs, the exact roles of the above-mentioned RLKs in plant thermosensing and thermoresponding processes need future investigations.

Many published studies have shown that altering the expression of a gene can alter the thermotolerance of a plant, but whether these genes are directly involved in regulating plant thermoresponses remains to be determined. Altering the expression of a gene may change the basic state of a cell, by disturbing its cytoplasmic homeostasis or metabolite dynamics, making it more difficult (or easier) for plants to adjust to a new equilibrium under HT conditions and increasing (or decreasing) their susceptibility to HT. The expression of these genes might therefore be related to the healthy state of the plant, rather than being directly related to a thermotolerance acquiring process.

As such, in future plant HS studies, especially studies of HT-related signaling mechanisms, it is important to distinguish the direct effects on plant thermoresponses (i.e., the primary response) from the indirect effects.
This can be facilitated by the identification of a series of specific cellular responses and molecular markers directly related to the plant thermoresponses, which could then be used to evaluate whether the gene of interest is directly involved in the response to HT conditions.

Another critical factor that must be considered is the time a gene of interest takes to affect HT response-related traits following heat exposure. In general, chemical signal-based signal transductions happen rapidly; for example, the dephosphorylation of BZR1 in response to BR exposure occurs within 5 min (Wang et al. 2016a). This fast response enables plants to better acclimatize to ever-changing environmental signals. Similarly, plant HT responses can happen quickly; HT can induce increases in intracellular $\text{Ca}^{2+}$ and ROS within seconds or minutes (Liu et al. 2003; Wang et al. 2014b; Yao et al. 2017). Even HT-induced transcriptional regulation can rapidly happen. For example, heat shock treatments lasting 2.5 min induce gene expression in cultured mouse (Mus musculus) cells (Mahat et al. 2016). In comparison, a 15 min high ambient temperature treatment significantly upregulated the expression of several heat-responsive genes in plants (Cortijo et al. 2017). Therefore, for the chosen cellular thermoresponse studies, the duration of the heat treatment should be as short as possible to limit the influence of the gene of interest on the primary thermoresponse. Otherwise, it can become complicated to judge whether a gene of interest is important in regulating HT signaling, since long-term heat exposure could allow it to indirectly alter thermotolerance through sequential transcriptional regulation or crosstalk of various signaling pathways.

Compared with other signal transduction studies (e.g., hormone signaling), HT-related signal transduction studies are relatively fragmented. For many studies, although mutant and transgenic plants have been used to demonstrate that a gene of interest is involved in regulating plant thermotolerance, solid genetic and biochemical evidence demonstrating that a gene of interest functions upstream or downstream of a particular HT signaling pathway is largely lacking. For the HT-regulated gene expression studies, while it has been shown that the HSFA1s are master transcription factors in plant HS responses, the expression levels of 35% of the HS-responsive genes were similar in HS-treated hsfa1a/b/d/e mutant and wild-type Arabidopsis plants (Liu et al. 2011), suggesting that additional transcription factors are directly activated by the HS signal transduction pathway. Identifying these additional transcription factors and their target genes will further elucidate the HS-induced transcription regulatory network, and help us understand how thermotolerance is acquired.

### Table 1. Heat shock responses of specific receptor-like kinase mutants

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Germplasm</th>
<th>Subfamily</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g11340</td>
<td>SALK_009816C</td>
<td>SD-1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>At1g19390</td>
<td>SALK_122472C</td>
<td>WAKL</td>
<td>Insensitive</td>
</tr>
<tr>
<td>At1g34110</td>
<td>SALK_126772C</td>
<td>LRR XI</td>
<td>Sensitive</td>
</tr>
<tr>
<td>At1g61370</td>
<td>SALK_087761C</td>
<td>SD-1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>At1g79680</td>
<td>SALK_132887C</td>
<td>WAKL</td>
<td>Insensitive</td>
</tr>
<tr>
<td>At2g23450</td>
<td>SALK_085371C</td>
<td>WAKL</td>
<td>Insensitive</td>
</tr>
<tr>
<td>At2g27060</td>
<td>SALK_086912C</td>
<td>LRR III</td>
<td>Sensitive</td>
</tr>
<tr>
<td>At2g41820</td>
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<td>LRR X</td>
<td>Sensitive</td>
</tr>
<tr>
<td>AT3g56370</td>
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<td>LRR VII</td>
<td>Sensitive</td>
</tr>
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</tr>
<tr>
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<td>SD-2</td>
<td>Insensitive</td>
</tr>
<tr>
<td>AT5g45780</td>
<td>SALK_123502C</td>
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<td>Sensitive</td>
</tr>
<tr>
<td>AT5g63710</td>
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</tr>
<tr>
<td>AT5g66790</td>
<td>SALK_078591C</td>
<td>WAKL</td>
<td>Insensitive</td>
</tr>
</tbody>
</table>

The high temperature response was assessed based on the measurement of root and hypocotyl growth at 37 °C, and basal and acquired thermotolerance at 42 °C.
Plant HT-response studies are currently separated into ambient HT and HS studies. Although phenotypic studies reveal few connections between these two thermo-induced processes, plants would naturally encounter ambient HT first, before facing HS during the diurnal or seasonal growth cycle; therefore, it is tempting to further investigate whether interactions exist between the two response pathways. A transcriptomic analysis using plants exposed to a HT of 27 °C revealed that, of the 1,035 genes in which expression was altered by this treatment, 270 (26%) were also up- or downregulated by a HS treatment at 37 °C (Cortijo et al. 2017). These genes co-regulated by 27 °C and 37 °C treatments include HSFA7b, HSFB2a, DREB2A, and the HSPs, which are direct targets of the HSFA1s. These results suggest that the HSFA1s might play important roles in regulating both ambient HT- and HS-induced gene expression in plants. Because PIF4 is considered a central player in the ambient HT responses, whether HSFA1 interact with PIF4 to regulate plant thermomorphogenesis will be an interesting topic for future research.

It is currently unclear whether HT and HS sensing share the same mechanisms. There are 20 CNGC genes encoded in the A. thaliana genome, so it is possible that the different CNGCs might sense and respond to different temperature changes in Arabidopsis, in a manner similar to the thermoreceptors in mammalian cells (Vay et al. 2012). Meanwhile, further studies are required to fully elucidate whether the CNGCs are true plant thermosensors.

Additionally, petiole and hypocotyl elongation are just some of the phenotypes observed in plants exposed to ambient HT; however, the mechanisms by which elevated temperatures regulate other thermomorphogenesis-related traits, such as leaf area and thickness (Quint et al. 2016), require further investigation. Such findings will help to build a complete blueprint of how plants perceive and respond to high environmental temperatures, which could enable the development of HT-tolerant crops, via molecular marker-assisted breeding.

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Plant high temperature response mechanisms


