Hot Topic: Thermosensing in Plants

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Abstract

Plants alter their morphology and cellular homeostasis to promote resilience under a variety of heat regimes. Molecular processes that underlie these responses have been intensively studied and found to encompass diverse mechanisms operating across a broad range of cellular components, timescales and temperatures. This review explores recent progress throughout this landscape with a particular focus on thermosensing in plants. Direct temperature sensors include the photosensors phytochrome B and phototropin, the clock component ELF3 and an RNA switch. In addition, there are heat-regulated processes mediated by ion channels, lipids and lipid-modifying enzymes taking place at the plasma membrane and the chloroplast. In some cases the mechanism of temperature perception is well understood but in others this remains an open question. Potential novel thermosensing mechanisms are based on lipid and liquid phase separation. Finally, future research directions of high temperature perception and signalling pathways are discussed.

1. Introduction

Heat stress is an increasingly prevalent environmental constraint for plants. Rising average global temperatures, and more frequent temperature extremes have a negative impact on world crop yield (Kang et al., 2009; Lobell & Field, 2007). Warm temperatures can impair plant growth, fertility, development, metabolism, photosynthesis and immunity (Hatfield & Prueger, 2015; Howarth & Ougham, 1993; Janda et al., 2019; Wolf et al., 1991; Xu et al., 1995). In natural environments, plants experience daily and seasonal temperature fluctuations that vary in range, rate and duration. Whether a temperature becomes stressful depends on these variables, as well as coincident stress factors such as drought and salinity. At the cellular level, heat stress perturbs protein folding, membrane fluidity, cytoskeletal organization, transport, and enzymatic reactions, which leads to metabolic imbalances and pernicious accumulation of by-products such as reactive oxygen species (ROS). It is therefore of primary interest for plants to sense temperature alterations and initiate timely adaptive strategies to preserve cell function and viability. Plants respond to different temperature ranges with widely divergent physiological and developmental responses. However much less is known about the sensing mechanisms involved.

At temperatures above the optimum ambient growth temperature, but still within the physiological range (i.e. up to around 28°C for Arabidopsis), many plants undergo a process known as thermomorphogenesis, in which they alter morphology and development (for example, through expanded leaf structure, deeper roots and early flowering) to reduce exposure to potentially damaging temperatures (Fig. 1) (Casal & Balasubramanian, 2019; Crawford et al., 2012; Park & Park, 2019). At higher temperatures, i.e 28-37°C for Arabidopsis, there is still some growth, but several adverse effects become visible. Reproductive development and photosynthesis are affected, and root and shoot growth rates are compromised. At these temperatures, plants employ a variety of acclimation strategies to enhance temperature tolerance, including the production of molecular

chaperones within minutes, and modulating the composition of cell membranes over a period of days. As temperatures rise above 40°C, severe heat stress is experienced, which can result in global injury, malfunction and ultimately, cell death (Fig. 1).

Molecular plant scientists have long questioned how heat is actually perceived and converted into a cellular signal. Since macromolecules are generally affected by heat, many have the potential to serve as thermosensors. The concept of thermosensor needs to incorporate processes akin to ligand-receptor binding coupled to downstream signaling, but also include less well-demarcated processes such as heat-induced increases in membrane fluidity followed by changes in membrane structure and function. This makes it difficult to identify macromolecules that actually perceive temperature and elicit specific signalling events. In recent years, several potential thermosensors and sensing mechanisms have emerged, both of ambient warm and stressful hot temperatures. Here, we summarize that knowledge, focussing on their mode of action, and provide a perspective for future research in this exciting field.

2. Sensing mechanisms of warm ambient temperatures

2.1. Phytochrome B (phyB) as temperature sensor

In recent years, there has been a growing realization that certain molecules originally characterized as photosensors moonlight as thermosensors, fine tuning growth and differentiation in response to moderate temperature changes. Here, the photo/thermo sensor acts as a receptor for a change in temperature, and in doing so initiates downstream signaling processes.

There is extensive crosstalk between light and temperature signalling in plants (Hayes, 2020). Plants perceive light conditions with at least five types of photoreceptors, i.e. (1) phytochromes, (2) cryptochromes, (3) phototropins, (4) zeitlupes and (5) UVR8 (Voitsekhovskaja, 2019). Of these photoreceptors, the temperature sensitive activity of phytochromes is the most well characterised, in particular phytochrome B (phyB). Phytochromes control many aspects of thermomorphogenesis, especially architectural changes, accelerated flowering and senescence (Jung et al., 2016; C. Kim, 2020; Quint et al., 2016).

Phytochromes are red/far red (R/FR) sensitive photoreceptors that absorb light through a phytochromobilin chromophore. The absorption of light by phytochromobilin induces its isomerisation, and this translates to conformational changes in phytochrome structure. R light promotes a shift to the active form of phytochrome (Pfr) whereas FR light promotes reversion to the inactive form (Pr) (Hayes, 2020). Importantly, Pfr can also spontaneously revert to Pr, and this process is temperature dependent. The rate of thermal reversion from Pfr to Pr is accelerated at warm temperatures (Legris et al., 2016), resulting in a reduced pool of active phytochrome (Fig. 2).

When activated by R light and cool temperatures, phytochromes promote the degradation of a family of bHLH transcription factors known as PHYTOCHROME INTERACTING FACTORS (PIFs).When phytochrome function is reduced by FR light or warm temperatures, PIFs accumulate and promote hypocotyl elongation through the enhanced expression of auxin biosynthesis genes (Jung et al., 2016; Koini et al., 2009; Legris et al., 2016). Hypocotyl elongation at warm temperatures is largely driven by *PIF4* , *PIF7* and to some extent *PIF5* (Chung et al., 2020; Fiorucci et al., 2020; Koini et al., 2009). PIF-mediated elongation and hyponasty at warm temperatures results in an open architecture and enhances leaf cooling (Crawford et al., 2012; Park & Park, 2019).

2.2. Other photosensors: phototropin, cryptochrome and UVR8

Phytochrome B is not the only plant photosensor with the ability to perceive temperature. The phototropin of liverwort (*Marchantia polymorpha*) (Mp*PHOT*) is also temperature sensitive (Fujii et al., 2017). Phototropins are membrane bound blue light receptors that respond to positional light cues. They regulate phototropism, leaf flattening and chloroplast positioning. In *Marchantia* , an important phototropin regulated process is the cold avoidance response. At 22°C, blue light induces the movement of chloroplasts to the cell surface, in order to maximise photosynthesis. In contrast, at 5°C blue light induces the movement of

chloroplasts to the periclinal cell walls. This process is known as cold avoidance and is thought to protect the photosynthetic machinery in suboptimal temperature conditions (Fujii et al., 2017).

MpPHOT contains two LOV (light, oxygen or voltage) domains that are responsible for light sensing. In darkness, each LOV domain contains a non-covalently bound flavin mononucleotide (FMN) chromophore. Blue light absorption by FMN triggers its covalent attachment to the LOV domain. This in turn causes structural re-arrangement of the phototropin molecule into its active form. Importantly, the covalent bond that links FMN and the LOV domain spontaneously degrades over time, resulting in inactivation of *MpPHOT* . This degradation rate increases with temperature, meaning that MpPHOT remains more active at cooler temperatures. As a result, MpPHOT promotes the relocation of chloroplasts to the cell periphery when temperatures are too low for efficient photosynthesis (Fujii et al., 2017). The phototropins of Arabidopsis were also recently implicated in temperature signalling (Kostaki et al., 2020). Warm temperatures promote guard cell opening in a cell autonomous manner. Curiously, this process is dependent on blue light and phototropins (Kostaki et al., 2020). This would seem to imply that in contrast to MpPHOT (Fujii et al., 2017), the activity of phototropin in Arabidopsis guard cells is actually enhanced at warm temperatures. Further investigation into the potential temperature sensitivity of Arabidopsis phototropins should help to resolve this point.

Zeitlupes are another class of blue light photoreceptor, which act to accelerate the pace of the circadian clock. Zeitlupes contain a LOV domain with a similar activation mechanism to phototropins (Pudasaini et al., 2017). If the rate of zeitlupe inactivation is increased at warm temperatures, this could potentially reduce the pace of the clock at warm temperatures (a process known as temperature compensation) (Hayes, 2020). Several years ago, zeitlupe was identified as a quantitative trait locus for natural variation in temperature compensation in Arabidopsis (Edwards et al., 2005) and so it would be interesting to experimentally test this hypothesis. Other plant photosensors, such as the blue light sensing cryptochrome (cry) and UV-B sensing UV Resistance Locus 8 (UVR8), could potentially also function as thermosensors (Figure 2). Cryptochromes undergo thermal reversion in a similar manner to phytochromes and phototropins. If cryptochrome thermal reversion is enhanced at warm temperatures, it is feasible that cryptochrome would also exhibit higher activity at cool temperatures (Hayes, 2020). UVR8 exists as a homodimer in the dark, but undergoes monomerization after absorbing UV-B. The active UVR8 monomer then reverts back to the inactive dimer, in a process that is mediated by Repressor of UV-B Photomorphogenesis 1 (RUP1) and RUP2. The RUP-mediated reversion of the UVR8 monomer to the UVR8 dimer seems to be influenced by temperature (Findlay & Jenkins, 2016), but the details of this process are currently unclear. Whether zeitlupe, cryptochrome and UVR8 functions are truly temperature sensitive remains to be investigated.

2.3. RNA thermosensors

Temperature sensing through RNA structure has earlier been shown in bacteria and animals (Vu et al., 2019). Recently, an RNA based temperature 'switch' was also identified in plants. Chung *et al.*(2020) used ribosome profiling to identify genes that show an increase in translational efficiency at warm temperatures. Interestingly, one of the genes that showed enhanced translation was *PIF7* . *PIF7*mRNA contains a hairpin structure in its 5'-UTR and the structure of this hairpin is temperature dependent (Fig. 2). It has been proposed that at low temperatures the hairpin forms and blocks *PIF7* mRNA translation. At warm temperatures, the hairpin can no longer form and PIF7 can be translated (Chung et al., 2020; Fiorucci et al., 2020). As PIF7 is one of the key transcription factors regulating hypocotyl and petiole elongation at warm temperatures, the temperature-dependent folding of its mRNA can be considered a temperature sensor.

2.4. Temperature-dependent action of the Evening Complex

A large proportion of the genome is regulated by the circadian clock. To ensure robust rhythmicity, the clock is entrained to daily cycles of light and temperature. The evening complex (EC) is a group of core clock components that show peak expression in the early evening. In recent years it has become apparent that the EC is one of the key points at which light and temperature signals enter the clock (Ezer et al., 2017). The EC consists of three components, the scaffold protein EARLY FLOWERING 3 (ELF3), the transcription factor

LUX ARRYTHMO (LUX), and a protein of unknown function, ELF4. Together they act as a transcriptional repressor that directly binds DNA (Ezer et al., 2017; Huang et al., 2016; Nusinow et al., 2011). Besides its function in the clock, the EC also represses the expression of thermomorphogenesis promoting genes such as *PIF4* , limiting the period of temperature-induced growth (Box et al., 2015).

Consistent with its importance in conveying temperature information to the clock, binding of the EC to DNA is temperature dependent. At cool temperatures, the EC binds to DNA much more strongly than at warm temperatures (Ezer et al., 2017). Phytochromes play an important role in regulating the EC (Ezer et al., 2017; Huang et al., 2016) and so temperature sensitivity of DNA binding could potentially be ascribed to increased thermal reversion of phyB (Legris et al., 2016). Intriguingly however, EC DNA binding is also temperature dependent *in vitro* , implying that EC activity is directly modulated by temperature (Silva et al., 2020).

ELF3 contains a prion-like domain (PrD) with a high proportion of glutamine residues (Jung et al., 2020). The PrD shows variable length between species, with *Arabidopsis thaliana* ELF3 (AtELF3) containing a much longer PrD than *Brachypodium distachyon* ELF3 (BdELF3). Replacing the PrD of AtELF3 with the corresponding region from BdELF3 abolished the temperature-dependent DNA binding of AtELF3 (Jung et al., 2020). At high temperatures, AtELF3 forms speckles within the nucleus and this is also dependent on the PrD. Importantly, these PrD-dependent speckles also form at high temperatures when AtELF3 is expressed in yeast cells, in the absence of other evening complex components. Furthermore, the purified PrD from AtELF3 spontaneously and reversibly forms liquid droplets when in solution, in a temperature-dependent manner (Jung et al., 2020). Warm temperature-dependent self-coalescence of ELF3 through its PrD domain therefore likely constitutes a temperature sensing mechanism in plants (Fig. 2).

Direct temperature sensing by ELF3 may help to explain a curious observation about phyB at warm temperatures. Under R light, active phyB accumulates in several large sub-nuclear foci known as photobodies (Hahm et al., 2020). In R + FR light, phyB is inactivated and disperses to numerous smaller foci. Warm temperature also inactivates phyB and so we might expect it to lead to a similar change in phyB photobodies. However, in direct contrast to FR light, warm temperature appears to promote the aggregation of phyB into fewer, larger photobodies (Hahm et al., 2020). Whether there is a link between phyB aggregation and ELF3 PrD-mediated condensation at warm temperatures remains to be investigated. However if this is the case, it presents an attractive mechanism whereby plant cells could distinguish between FR and warm temperature signals, based on the inactivation of phyB alone or the inactivation of phyB *and* ELF3 in conjunction.

2.5. Epigenetic regulation of thermomorphogenesis

In a screen for mutants with enhanced temperature response, Kumar & Wigge (2010) discovered ARP6, a component of a chromatin remodelling complex. Mutants lacking ARP6 show constitutive warm temperature phenotypes. ARP6 is required for the deposition of a specific histone variant H2A.Z. It was shown that at warm temperatures, there is a reduction in H2A.Z occupancy at the promoters of warm-temperature induced genes and it was initially proposed that H2A.Z could play a role in temperature sensing (Kumar & Wigge, 2010). More recent evidence has however cast doubt on this hypothesis. It was shown that at warm temperatures, the binding of HSFA1a to heat responsive genes precedes the H2A.Z eviction and the activation of transcription (Cortijo et al., 2017). The eviction of H2A.Z is facilitated by HISTONE DEACETYLASE 9 (HDAC9), and is required for the full transcriptional response to elevated temperatures (van der Woude et al., 2019), but it appears that this occurs mostly downstream of temperature perception.

3. Sensing mechanisms of moderate to severe heat stress

3.1. The heat stress response is partly based on unfolded protein sensing

Heat stress triggers adaptive responses which protect macromolecular structures, restore cellular homeostasis and prevent damage. The composite response likely involves the action of multiple parallel sensors that activate signaling pathways in different cellular compartments with different dynamics. Cellular defense mechanisms are activated by monitoring protein, DNA and membrane damage (Balogh et al., 2013; Ding

et al., 2020; Niu & Xiang, 2018). The expression of Heat Shock Proteins (HSPs) is induced by elevated temperatures in a large variety of eukaryotes. HSPs act as molecular chaperones to promote the correct folding of proteins in the cell and are crucial for tolerance to high temperatures in plants. HSPs can also contribute to the thermomorphogenic response. For example, HSP90 promotes the stability of the auxin receptor TIR1 and in doing so, promotes root and shoot elongation at warm temperatures (R. Wang et al., 2016).

Studies in yeast have suggested a mechanism by which HSPs are induced at warm temperatures. HSPs bind to a class of transcription factors known as Heat Shock Factors (HSF), which are thus kept in an inactive state. At warm temperatures, the binding of HSPs to HSFs is disrupted. HSFs are then free to travel to the nucleus to induce the expression of HSPs and other heat stress genes. This increased abundance of HSPs then sequesters the free HSFs, thus reaching another equilibrium.

Currently it is unclear whether the HSP:HSF module functions in the same manner in plants, but similar mechanisms are known to activate the unfolded protein response (UPR). When a plant experiences heat stress, unfolded and misfolded proteins can accumulate to such levels that they overload the protein quality control system, leading to ER stress. When unfolded proteins accumulate in the ER, they are bound by binding protein (BiP), an HSP70 chaperone. In the absence of unfolded proteins, BiP binds to the ER membrane-tethered transcription factors (MTTFs) bZIP17 and bZIP28. When unfolded proteins sequester BiP, bZIP17 and bZIP28 are activated by translocation and proteolytic cleavage of their membrane anchors at the Golgi. These transcription factors then travel to the nucleus to promote the expression of chaperones and foldases to assist in protein folding, a process known as the unfolded protein response (UPR). Unfolded proteins also interact with the lumenal domain of the transmembrane sensor IRE1b (inositol-requiring enzyme1), inducing unconventional splicing of bZIP60, which then activates the ER stress response genes. IRE1b splicing activity is induced by heat (Deng et al., 2011). Moreover, IRE1 was found to be inducible by lipid bilayer stress in yeast (Ernst et al., 2018; Halbleib et al., 2017), and to regulate the degradation of specific mRNA's, shaping the stress transcriptome. Interestingly, the transcription factor ELONGATED HYPOCOTYL 5 (HY5) was recently found to compete with bZIP17 and bZIP28 to repress the UPR (Nawkar et al., 2017). HY5 abundance is reduced at warm temperatures (Park et al., 2017), potentially through the photo/thermo sensing mechanism described above. Reduced HY5 abundance may help to promote the UPR under heat stress.

High temperature and light conditions can also activate UPR responses through a signal from the chloroplast, methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP, Fig. 3), a retrograde signaling metabolite (Rivasseau et al., 2009; Walley et al., 2015). This intermediate of the methylerythritol-4-phosphate (MEP) pathway of isoprenoid synthesis accumulates as a direct consequence of a heat stress-induced metabolic bottleneck, and induces CAMTA3-dependent transcription of IRE1a and bZIP60 and other genes with a *rapid stress response element* (Benn et al., 2016). Hence, the MEP pathway is considered an important stress sensor (Xiao et al., 2012). The functions of retrograde signals in heat stress have been reviewed elsewhere (Sun & Guo, 2016). Clearly, different sensory modules feed into the UPR response.

Under heat stress, unfolded proteins also accumulate in chloroplasts. The small heat shock protein (sHSP) of Chlamydomonas, HSP22E/F, forms high-molecular weight complexes with them to prevent proteotoxicity (Rütgers, Muranaka, Mühlhaus, et al., 2017). Heat-inactivation of specific thermolabile proteins in the chloroplast, and their sequestration into complexes with sHSP, is considered an adaptive mechanism to directly regulate metabolism and signaling processes in response to heat.

AtNTL4 and OsNTL3, which are MTTFs of the NTL (NAC transmembrane transcription factor-like) group, also appear critical in regulating heat stress responses. The basis for their thermosensitive proteolytic activation remains however, unresolved (Lee et al., 2014; X. Liu et al., 2020). Evidence of their ER localization (Liang et al., 2015) and partial convergence with ER-stress responses (Yang et al., 2014), suggests that they could signal heat-induced protein and lipid perturbations at the ER.

3.2. Regulation based on physical changes in the membrane

The membrane is the most thermally sensitive macromolecular structure in the cell (Balogh et al., 2013; Niu & Xiang, 2018). With increasing temperature, the rotational motion, lateral diffusion, and fatty acid disorder of the lipid bilayer increase, while the headgroup packing density decreases. These four parameters are different aspects of the commonly used term 'membrane fluidity'. Changes in membrane fluidity affect the folding, mobility, and activity of membrane proteins. These changes can have deleterious effects on cell functions, but at moderate levels can also serve as a basis for thermosensing. Plants, like other non-homeothermic organisms, actively maintain an almost constant membrane fluidity upon shifts in temperature (Higashi et al., 2015; Los & Murata, 2004). The increased fluidity under heat stress is counteracted by the incorporation of fluidity-decreasing saturated fatty acids, a process known as homeoviscous adaptation. In bacteria, membrane thickness is measured through the membrane protein DesK and in yeast, lipid packing density is measured by Mga2. These sensors support membrane homeostasis by the transcriptional activation of lipid desaturases when temperature drops (Ballweg et al., 2020; Covino et al., 2016; Cybulski et al., 2010).

In plants, such membrane property sensors have not been identified. Instead, heat was found to directly inhibit the activity of critical desaturases, simply by virtue of their heat-instability. The plastidial FAD8 enzyme is responsible for the synthesis of α-linolenic acid (18:3), a component of the main thylakoid lipid, monogalacosyldiacylglycerol (MGDG). FAD8 contains a labile autoregulatory domain, that destabilizes the protein upon a temperature shift from 22°C to 27°C (Matsuda et al., 2005) (Fig. 3). Reduced FAD8 stability resulted in decreased accumulation of 18:3 and reduced membrane fluidity. The importance of this is clear from the finding that mutants with low 18:3 content in their MGDG showed improved heat tolerance (Murakami, 2000). This may be because 18:3-MGDG is prone to oxidative damage and perturbs membranes. The ER desaturases FAD2 and FAD3 also display thermolability. Upon transfer to warm temperatures, FAD2 and FAD3 are targeted for ubiquitin-mediated or ER-associated degradation, respectively (O'Quin et al., 2010; Tang et al., 2005). Currently the mechanism by which these desaturases are inactivated upon heat stress is unknown; they may either be direct thermosensors or act downstream of temperature perception.

Adjustment of membrane fluidity through changes in membrane desaturation is a slow process and can take several takes days (Falcone et al., 2004). In the case of acute heat stress, alternative mechanisms are employed to secure bilayer integrity. These sense-and-respond mechanisms are based on heat-induced, biophysical changes in membrane properties. In thylakoid membranes, heat induces packing defects in the lipid headgroups. These defects provide a spatial cue for docking of proteins with membrane-protecting functions, such as sHSP (Heckathorn et al., 1998) and vesicle-inducing protein in plastids 1 (VIPP1, Fig. 3) (Theis et al., 2019; L. Zhang et al., 2016). The inducible association of these proteins with membranes likely follows the sensing of the membrane status through their amphipathic α-helices.

Acute heat also induces the aggregation of light-harvesting complex II (LHCII) proteins in the thylakoid membranes. MGDG is normally associated with LHCII, and upon aggregation of LHCII, excess MGDG gets extruded to the lumen (Jahns et al., 2009; Schaller et al., 2010) (Fig. 3). Due to MGDG's non-bilayer propensity, extruded MGDG forms a so-called inverted hexagonal phase (HII) (Garab et al., 2017; Krumova et al., 2008). Thylakoid membranes are always close to HII phase transition and, as HII phases emerge, they must be controlled to avoid damage. HII phases are however key to chloroplast heat acclimation because when they emerge under stress, they recruit and activate the xanthophyll cycle enzyme, violaxanthin de-epoxidase (VDE). VDE synthesizes zeaxanthin which quenches excess excitation energy and enhances membrane stability. The HII phases serve the sequestering of excess MGDG and promote the diffusion of xanthophylls (Latowski et al., 2002) (Fig. 3).

Another membrane feature that can undergo rapid stress-induced modification are microdomains. Most lipids within a membrane exist in liquid-disordered phase, often envisioned as a two-dimensional fluid. Lipids can however also exist in the liquid-ordered phase known as nano- and microdomains (Jaillais & Ott, 2020; Saenz et al., 2012). Microdomains form coherent, dynamic platforms for proteins with functions in sensing, signaling, membrane integrity maintenance and transport. Even mild changes in temperature can result in altered microdomain fluidity and consequently, redistribution and modified activity of these proteins (Török et al., 2014). Based on studies of membranes and Molecular Dynamics simulation, microdomains are

speculated to act as dynamic reservoirs of fluidity-decreasing lipids. Heat may trigger increased partitioning of these lipids from microdomains to the bulk fluid phase (Nickels et al., 2019). This simple buffering effect that can occur in complex membranes is based on thermodynamics of phase separation and could be far more responsive than the metabolic responses of homeoviscous adaptation (Ernst et al., 2018).

Some plasma membrane microdomains are tethered to the underlying cortical ER at so-called ER-plasma membrane contact sites (EPCSs) through synaptotagmins (SYT1 and SYT3) (Ruiz-Lopez et al., 2020). SYT1/3 are ER proteins that bind (via C2-domains) to phosphatidylinositolphosphate (PIP)-containing microdomains of the plasma membrane (Fig. 4). The close proximity of the two membranes allows for exchange/removal of detrimental lipids, e.g. diacylglycerol that is formed at the plasma membrane during phospholipase C (PLC) signaling (*see below*). In yeast, EPCSs are important for plasma membrane integrity maintenance under heat stress (Collado et al., 2019), and they appear to function similarly under stresses in plants (Ruiz-Lopez et al., 2020; Yan et al., 2017).

The biophysical changes in membranes under heat stress can be sensed by altered protein activity and/or location. Moreover, the alternative lipid phases allow for prompt, thermosensitive responses, and thereby provide structural and functional flexibility that is of vital importance under heat stress. Notably, this suggests that homeoviscous adaptation does not necessarily involve a sensor of membrane fluidity. Whether fluidity sensing underlies other heat stress responses remains unknown. Many studies have attempted to probe the effect of membrane fluidization using pharmacological and genetic interventions, but it is becoming clear that these techniques have indirect effects on proteins and gene expression (Rütgers, Muranaka, Schulz-Raffelt, et al., 2017; Vu et al., 2019).

3.3. The primary roles of Ca^{2+} and H_2O_2 signaling

Intracellular Ca^{2+} increases are a feature of all plant stress responses, and heat stress is no exception (Finka et al., 2012; Saidi et al., 2011). Ca^{2+} increases were found to be required for the induction of HSPs through Ca^{2+}/calmodulin-dependent kinases and for the acquisition of heat tolerance (H.-T. Liu et al., 2003, 2007). It has been reasoned that Ca^{2+} channels could function as thermosensors at moderate temperature elevations and many attempts have been made to identify such channels (Ding et al., 2020; Horvath et al., 1998; Saidi et al., 2011).

Using Arabidopsis seedlings expressing the Ca^{2+}reporter aequorin, Lenzoni and Knight (2019) were unable to detect a heat-induced cytosolic Ca^{2+} response. Instead, they found a Ca^{2+} increase in the chloroplast stroma (Lenzoni & Knight, 2019). Temperatures of >35°C induced rapid Ca^{2+} responses, with higher temperatures provoking higher and faster peaks. The response was not influenced by the rate of warming, but was determined by the absolute temperature. The thylakoid membrane Ca^{2+} sensor CAS was required for full induction of stromal Ca^{2+} in response to heat (Fig. 3). CAS amplified the Ca^{2+} signal, but what governs the initial signal is still unknown.

Cyclic nucleotide-gated channels (CNGCs) have also been shown to mediate heat-induced cytosolic Ca^{2+} increases in*Physcomitrella patens* and in Arabidopsis. A temperature increase from 22°C to 28°C in moss quickly triggered a transient inward electrical current, likely reflecting Ca^{2+} influx through PpCNGCb (Finka et al., 2012; Saidi et al., 2009). Based on the use of channel blockers and chelators, the heat-activated Ca^{2+} current was attributed to CNGC6 in Arabidopsis (Finka et al., 2012; F. Gao et al., 2012) (Fig. 4). CNGC6 is activated by cAMP, which also increases upon heat stress. This has led to the suggestion that an, as yet unidentified, adenylyl cyclase activity could act as membrane-associated temperature sensor (Thomas et al., 2013). Research on CNGC functions is complicated by the fact that CNGC subunits make various combinations to form heterotetrameric channels. These channels differ in ion conductance, physiological function and mode of regulation, and mutations in CNGC subunit genes often lead to pleotropic mutant phenotypes (Dietrich et al., 2020).

Identifying the primary heat-activated Ca^{2+} channel remains a challenge. The animal heat-activated Transient Receptor Potential channel, TRPV1, is a mechanosensitive and voltage-gated cation channel (Benítez-Angeles et al., 2020). It likely responds to forces transmitted via microtubules (Bavi et al., 2017; Prager-

Khoutorsky et al., 2014). Plants lack TRP channel homologs, but possess other mechanosensitive channel types that may function in heat signaling, including OSCA1 (reduced hyperosmolality-induced [Ca^{2+}] increase1), MCA (Mid1-complementing activity), which was implicated in cold sensing (Mori et al., 2018), and Small Conductance Mechanosensitive Ion Channel (MscS)-Like (MSL) proteins (Ackermann & Stanislas, 2020).

Annexins are another class of membrane proteins that may play a role in heat-induced Ca^{2+} signaling. Proteomic and forward genetic approaches identified several Arabidopsis annexins that enhanced heat- and oxidative stress-induced Ca^{2+} responses and the expression of HSPs and HSFs (Liao et al., 2017; X. Wang et al., 2015). In the presence of Ca^{2+}, annexins bind to anionic lipids, such as phosphatidic acid (PA) and phosphatidylserine (PS) in the plasma membrane (Yadav et al., 2018). Heat stress rapidly induces ANNEXIN1 (ANN1) membrane association, but this may be a downstream effect of Ca^{2+} and/or PA accumulation (see below), rather than a direct effect of temperature on ANN1.

In addition to Ca^{2+}, H_2O_2 levels at the plasma membrane also rise quickly in response to heat stress, a process catalyzed by the NADPH oxidase, RbohD . The plasma membrane H_2O_2 signal is required for the heat stress gene expression and the enhancement of heat tolerance (Suzuki et al., 2012; Volkov et al., 2006). There is also accumulation of H_2O_2 in chloroplasts and mitochondria and this may provide additional priming signals (Sun & Guo, 2016). ANN1 is activated by H_2O_2 (Richards et al., 2014) and so it may be that annexins function to co-ordinate H_2O_2 and Ca^{2+} signals under heat stress (Fig. 4).

RbhoD-derived H_2O_2 has also been shown to accumulate in the apoplastic space in a self-propagating manner. This results in an extracellular ROS wave travelling through the plant at a speed of 8.4 cm/min (Miller et al., 2009; Suzuki et al., 2012). Local heat stress triggered accumulation of H_2O_2 can thereby lead to acclimation of systemic tissues (Miller et al., 2009). H_2O_2 and Ca^{2+} signals are not unique to heat stress and it is presently unclear how these signals lead to particular stress responses. It has been proposed that the relative intensity or timing of these signals confers specificity to the response (Gilroy et al., 2016).

3.4. Heat sensing is mediated by lipid signals

Lipids are the primary structural components of membranes, but also have signaling and regulatory functions, coupling perception of environmental cues to cellular responses (Hou et al., 2015). Lipids such as PA and phosphatidylinositol 4,5-bisphosphate (PIP_2), and their metabolic enzymes, i.e. phospholipases C and D (PLC/PLD), and lipid kinases, diacylglycerol (DAG) kinase (DGK) and phosphatidylinositol-4-phosphate 5-kinase (PIP5K) have a wide range of cellular regulatory functions in environmental stress responses. In Arabidopsis seedlings, heat stress (40°C) triggers increases in PA and PIP_2 abundance within 2 min (Mishkind et al., 2009).

This extremely rapid response suggests that the synthesis of these signaling lipids is closely tied to thermosensing, but as of yet it is unknown how increases in temperature activate these lipid modifying enzymes (Fig. 4). High-temperature induction of PA is largely dependent on membrane lipid hydrolysis by PLD (Mishkind et al., 2009; Shiva et al., 2020) (Fig. 4). The PLD enzyme is localized at the plasma membrane and associated with microtubules, where it regulates their membrane-anchorage (Andreeva et al., 2009). In heat-stressed stomatal cells, apoplastic H_2O_2 enters the cytosol through aquaporins. H_2O_2oxidizes cysteine residues in the C2 domain of PLDδ. The modified cysteine residues promoted Ca^{2+} binding to PLDδ, which resulted in depolymerization of microtubules (Song et al., 2020; S.-S. Zhang et al., 2017). Blocking microtubule depolymerization by chemical stabilizers inhibited the upregulation of HSP70 and the induction of MAPK activity under heat stress (Sangwan et al., 2002; Suri & Dhindsa, 2007), which suggests that this pathway acts to promote thermal acclimation. Curiously though, mutants lacking $\Pi\Lambda\Delta\delta$ were more tolerant to heat stress, which suggests the opposite. The fact that PLDδ requires Ca^{2+} and $H_2O_2$for its activation hints that, despite its rapid activation, phospholipid signaling occurs downstream of primary thermosensing.

Glyceraldehyde-3-phosphate dehydrogenase (GAPC) may also play a role in heat stress signaling. GAPC was shown to translocate to the nucleus under heat stress, where it activates the transcription factor NF-YC10. Activated NF-YC10 then promotes the expression of genes that confer thermotolerance (S.-C. Kim

et al., 2020). The mechanism the promotes GAPC nuclear translocation is as yet unresolved. When in the cytosol, GAPC can directly bind PLDδ and positively promote its activity (Guo et al., 2012). It has also been shown to bind PA (McLoughlin et al., 2013) but it is unclear how or if these attributes contribute to temperature regulation of GAPC. The involvement of GAPC in PLD/PA signaling raises the possibility that the heat stress response is coordinated with basal cell metabolism.

PLC also appears to have a function in the heat stress response, because PLC9 and PLC3 knock-out seedlings show severely impaired basal and/or acquired heat tolerance, while overexpression improved this (K. Gao et al., 2014; Zheng et al., 2012) (Fig. 4). PLC hydrolyzes PIP and PIP_2 to generate DAG and inositolphosphates. The latter could eventually result in the activation of a Ca^{2+}channel (Munnik, 2014). PLC9 and PLC3, both localized at the plasma membrane, were required for the induction of cytosolic Ca^{2+} and enhanced expression of sHSPs under heat stress.

The heat stress-induced accumulation of PIP_2 displayed interesting dynamics. During heat exposure, PIP_2accumulated first at the plasma membrane, after which it appeared in cytoplasmic punctate structures, followed by accumulation at the nuclear envelope (Mishkind et al., 2009). PIP_2 can function in endocytosis and associates with membrane microdomains (Furt et al., 2010). Microdomains are considered critical in the regulation of early stress signaling, as they contain signaling proteins such as RbohD, HSPs, and CNGCs (Dietrich et al., 2020; Horvath et al., 1998; Niu & Xiang, 2018). Acting very early in the response pathway, PLC3 and PLC9 may be physically close to the thermosensor. Regulation of PLCs is complex, involving calcium, G-proteins and post-translational modifications (Munnik, 2014). Potential protein interactors of PLC3 and PLC9, including two receptor-like kinases, could provide interesting clues as to their heat-responsive mode of activation (Pokotylo et al., 2013).

4.. Conclusions and future challenges

Clearly, our knowledge of thermosensory systems of plants has greatly expanded in the past decade. Important discoveries in ambient temperature signaling include the identification of photo/thermal sensors, an RNA switch and self-coalescence of ELF3 through its prion-like domain. These three systems unambiguously translate high ambient temperatures into altered gene expression. The reprogramming of development by these factors assists plants to avoid damaging high temperatures. In Arabidopsis, the inhibition of phyB activity by warm temperatures has been shown in detail. It is however still unknown if other photoreceptors function in a similar way. Phototropin plays a role in low temperature signalling in Physcomitrella but it is not known whether this temperature-dependent activity also stretches to warm temperatures. Other photoreceptors undergo thermal reversion and so could conceptually also function in warm temperature sensing, but this remains to be demonstrated. The finding that *PIF7* RNA translation is enhanced at warm temperatures opens the possibility that other RNAs act in a similar way. Indeed, the authors of the PIF7 study also show that *HSF2* RNA may also be regulated through a comparable mechanism (Chung et al., 2020). The finding that ELF3 contains a prion-like domain that undergoes temperature-dependent coalescence likely has effects beyond just evening complex transcriptional repression. ELF3 acts as a scaffold protein for large protein complexes (Huang et al., 2016) and directly binds to PIF4 to inhibit its transcriptional activity (Nieto et al., 2015). Both of these functions are likely inhibited at warm temperatures.

Upon moderate temperature increases, plants trigger a heat stress response for acclimation, but the sensing mechanism is still largely unknown. Rather than unfolded proteins, the increased membrane fluidity at high temperatures is speculated to be a molecular basis of sensing. While no fluidity sensor has been found in plants, evidence is accumulating for thermosensory mechanisms based on heat-induced phase changes in lipids and proteins.

Under heat stress, thylakoid membranes locally undergo transition to non-bilayer, HII phases, which are essential for heat acclimation, since they compartmentalize and activate the enzymes of the xanthophyll cycle. At the plasma membrane, microdomains are formed containing lipids in a liquid-ordered phase. These domains harbor potential signaling lipids and proteins, including RbohD, which is activated in response to heat stress. Formation of microdomains and HII phases could constitute a basis for thermosensitive regulation

of enzymes. Simultaneously, they provide potential avenues for the rapid trafficking of lipids between phases, in order to preserve membrane integrity under heat stress. For the latter function, a membrane fluidity sensor would thus not be required.

The regulation of ELF3 has unveiled a novel type of ambient temperature sensing mechanism, based on liquid-liquid phase separation (Jung et al., 2020). Under heat stress, the formation of spherical, condensed liquid phases within a bulk dilute phase can be triggered by the coalescence of proteins through their intrinsically disordered, prion-like domains. The resulting liquid droplets, also called membraneless organelles, constitute compartments that can contain proteins with associated regulatory functions. The reversible process of droplet formation is highly temperature sensitive. Could liquid-liquid phase separation also function, at higher temperatures, in the activation of the heat stress response? Such a function was recently proposed for the yeast RNA-binding protein, Pab1 (poly(A)-binding protein), which displayed self-coalescence and phase separation upon a shift to a temperature that induces the heat-shock response (Riback et al., 2017). The extreme thermosensitivity of this process was quantified using the temperature coefficient Q 10, the ratio of biological properties measured 10°C apart. With a Q 10 of 350, it exceeds by far any other known biological thermosensory process. This indicates the potential of liquid-liquid phase separation of proteins as a thermosensing mechanism. The sharp threshold temperature above which phase separation is triggered, which is determined by the amino acid side chains in the prion-like domain, allows for precise temperature-dependent regulation of responses. Pab1 was speculated to activate the heat stress response by sequestering a negative regulator of HSF in liquid droplets, calling into question the requirement of unfolded proteins for activation (Riback et al., 2017). It seems plausible that similar regulation governs heat stress responses in plants.

The stress-induced clustering of proteins into membrane microdomains could trigger liquid-liquid phase separation in the adjacent cytosol. This could result in coupled lipid and liquid compartments, that assemble selected response components, allowing for specific channeling of sensory signals to downstream responses (Jaillais & Ott, 2020). Similarly, plasma membrane organization could respond to changes in the cell wall, which may also adopt different biophysical states dependent on temperature (Wu et al., 2018). As yet, such potential interactions are unexplored territory.

Identifying plant proteins that could act as thermosensors through liquid-liquid phase separation will be challenging. Previously, heat stress was found to induce relocalization of splicing factors with disordered domains, e.g. serine/arginine-rich protein SR45, into enlarged nuclear speckles (Ali et al., 2003; Reddy et al., 2012), which could underlie alternative splicing of pre-mRNAs. Many of the, approximately, 500 proteins in plants with predicted prion-like domains are transcription factors with potential roles in temperature signalling. There are prion-like domains in HSFA1b, and several PIFs, auxin response factors and ABRE-binding factors (Chakrabortee et al., 2016). Investigating the effect of temperature on the coalescence of these factors *in vitro* could yield interesting results.

Thermosensing appears to be a highly distributed capacity, based on a range of mechanisms which are only just beginning to come to light. Most strikingly, the temperature-dependent behavior of phyB, the PIF7 RNA hairpin, and both lipid and liquid phase separations, provides an impressive spectrum of potential heat sensing and responding modes, essential for plants to acclimate and survive.

Figure legends

Figure 1. Schematic overview of responses in different warm temperature ranges.

Plants display a wide array of responses when they experience above optimal temperatures. At warm ambient temperatures, up to 30°C, Arabidopsis responds by changes in morphology and development, called thermomorphogenesis, which could aid in avoidance of future heat stress. Thermomorphogenesis features the temperature-sensitive function of phyB. Also in this temperature range, there is thermosensitive regulation of PIF7 mRNA translation. Warm temperatures lead to the loss of a hairpin structure of PIF7 mRNA, which allows for its translation. ELF3 undergoes temperature-dependent phase separation. High temperatures promote the coalescence of ELF3, and the inhibition of ELF3-DNS binding, At temperatures up to

38°C, Arabidopsis initiates acclimation responses that counteract damage to proteins and membranes, and maintain cellular homeostasis. This process involves the activity of HSFA1 master transcriptional factors (H. Liu & Charng, 2013). HSPs/sHSP accumulate to limit misfolding of proteins. and the membrane's lipid composition is adjusted so as to prevent disruption of the bilayer structure due to uncontrolled increases in membrane fluidity. The heat sensors that activate acclimation are unknown. The accumulation, within the first ±15 min of heat stress, of putative signaling components, such as Ca^{2+}, H_2O_2, PIP_2, PA and cAMP suggests their function in heat perception, closely tied to the sensor. Temperatures above 40°C are damaging to Arabidopsis, and all responses in this range are devoted to immediate protection of cellular structures. Mechanisms of clearance and rescue of unfolded proteins, including the UPR, are important for survival of severe heat stress. These heat stress responses rely on the recognition of unfolded proteins in the ER, the cytosol, and diverse organelles.

Figure 2. Schematic overview of thermomorphogenic pathways in arabidopsis.1. Under red light, phyB is converted to a Pfr homodimer that is translocated to the nucleus where it blocks PIF4 and PIF7 activity. High temperatures promote the reversion of phyB back to its inactive state, leaving PIF4 and PIF7 free to transcribe thermomorphogenesis promoting genes. 2. *PIF7* mRNA contains a hairpin near its 5'-UTR sequence. Upon an increase in temperature, this hairpin structure becomes more relaxed. In the relaxed state, *PIF7* mRNA is more easily translated and PIF7 protein levels are increased. 3. At cooler temperatures, ELF3 (as part of the evening complex) represses the expression of *PIF4* . As temperatures rise, a prion like domain in ELF3 promotes its aggregation, thus relieving the transcriptional repression of *PIF4* .

Figure 3. Sensing and signaling of heat stress at the chloroplast.

Exposure to moderate heat has various direct consequences for chloroplast proteins and membranes, which trigger rescue pathways. In the thylakoidal membrane, LHCII proteins aggregate, which leads to an excess of its major constituent, the non-bilayer prone glycerolipid MGDG. As a consequence, a non-bilayer structure (the HII phase) emerges, which consists of MGDG organized in hexagonal, stacked tubules. The xanthophyll cycle enzyme, violaxanthin de-epoxidase (VDE), recruits specifically to the HII phase in the thylakoid lumen, catalyzing the synthesis of zeaxanthin (ZEA) from its precursors, violaxanthin (VIO) and antheraxanthin (ANT). The HII phase remains attached, which allows for free diffusion of the photoprotective xanthophylls to the thylakoid. VIPP1 and sHSP recruit to the thylakoid membrane under heat stress, as they recognize membrane packing defects. They protect thylakoid membrane and PSII integrity. Heat stress is signalled in the chloroplast by a rapid Ca^{2+} increase in the stroma which depends on the activity of the calcium sensor CAS. Furthermore, heat induces breakdown of the envelope desaturase FAD8, responsible for synthesis of polyunsaturated fatty acids. This causes an adaptive decrease in membrane desaturation. The isoprenoid biosynthesis intermediate MEcPP accumulates due to a heat-induced bottleneck in the pathway. MEcPP, together with H_2O_2 resulting from excess excitation energy, and other stress-induced molecules, serve as retrograde signals to regulate heat stress genes in the nucleus.

Figure 4. Sensing and primary signalling events of heat stress at the plasma membrane.

In response to heat stress, several plasma membrane-linked protein activities are triggered which lead to intracellular signals that collectively regulate the heat stress response in plants. 1. Heat perception gives rise to increases in Ca^{2+}, which can enter the cytosol from the apoplast through channels such as CNGC6. This channel might be activated by cAMP, which is generated by a transmembrane adenylyl cyclase (tmAC) perhaps activated under heat stress as membrane fluidity increases. Through association with calmodulin (CaM), Ca^{2+} can negatively regulate CNGC6, and promote the function of HSFs. HSFs are the primary regulators of the heat response leading to transcriptional induction of HSPs and other genes. Apart from CNGC6, Annexin 1 (ANN1) is required for cytosolic increases in Ca^{2+}. 2. The second major factor in the heat stress response is H_2O_2, which is generated by the plasma membrane microdomain NADPH oxidase, RBOHD, whose activity is modulated by several factors, including Ca^{2+} and PA. After H_2O_2 enters the cell, it modifies the PLDδ protein such that it becomes sensitive to activation by Ca^{2+}. 3. PLDδ generates PA, which has a myriad of signaling functions which are mediated by its interaction with cytosolic target proteins.

PLDδ is attached to microtubules and its activity leads to microtubule depolymerization. Moreover, H_2O_2 can activate HSFs through MAPK signaling. 4. PLC3 and PLC9 are required for sHSP induction and thermotolerance. Most likely, they hydrolyze PIP to generate DAG, releasing the inositol-bisphosphate (IP_2) headgroup. DAG can be phosphorylated to PA by diacylglycerol kinase (DGK). In plants, rather than IP_2 or IP_3, inositol's more highly phosphorylated derivatives are the likely inducers of cytosolic release of Ca^{2+}. DAG could associate with synaptotagmin (SYT) in the ER at ER-PM contact sites, which may function to stabilize the plasma membrane under stress, and facilitate the exchange of lipids between the plasma membrane and the cortical ER. 5. Besides PA, also PIP_2 accumulates under heat stress, through PIP kinase (PIPK) activity, first only in the plasma membrane, later also in internal membranes, including the nuclear envelope. PIP_2 regulates effector proteins through specific lipid-binding domains.

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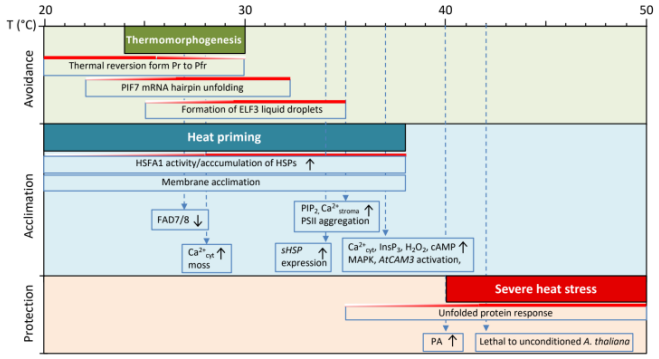

Figure 1. Schematic overview of responses in different warm temperature ranges.

Plants display a wide array of responses when they experience above optimal temperatures. At warm ambient temperatures, up to 30°C, Arabidopsis responds by changes in morphology and development, called thermomorphogenesis, which could aid in avoidance of future heat stress. Thermomorphogenesis features the temperature-sensitive function of phyB. Also in this temperature range, there is thermosensitive regulation of PIF7 mRNA translation. Warm temperatures lead to the loss of a hairpin structure of PIF7 mRNA, which allows for its translation. ELF3 undergoes temperature-dependent phase separation. High temperatures promote the coalescence of ELF3, and the inhibition of ELF3-DNS binding, At temperatures up to 38°C, Arabidopsis initiates acclimation responses that counteract damage to proteins and membranes, and maintain cellular homeostasis. This process involves the activity of HSFA1 master transcriptional factors (Liu & Charng, 2013). HSPs/sHSP accumulate to limit misfolding of proteins. and the membrane's lipid composition is adjusted so as to prevent disruption of the bilayer structure due to uncontrolled increases in membrane fluidity. The heat sensors that activate acclimation are unknown. The accumulation, within the first ±15

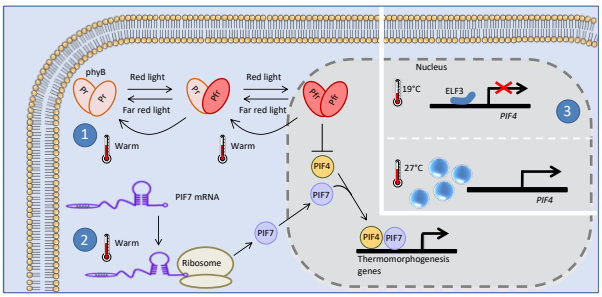

Figure 2. Schematic overview of thermomorphogenic pathways in arabidopsis. 1. Under red light, phyB is converted to a Pfr homodimer that is translocated to the nucleus where it blocks PIF4 and PIF7 activity. High temperatures promote the reversion of phyB back to its inactive state, leaving PIF4 and PIF7 free to transcribe thermomorphogenesis promoting genes. 2. *PIF7* mRNA contains a hairpin near its 5'-UTR sequence. Upon an increase in temperature, this hairpin structure becomes more relaxed. In the relaxed state, *PIF7* mRNA is more easily translated and PIF7 protein levels are increased. 3. At cooler temperatures, ELF3 (as part of the evening complex) represses the expression of *PIF4*. As temperatures rise, a prion like domain in ELF3 promotes its coalescence, thus relieving the transcriptional repression of *PIF4*.

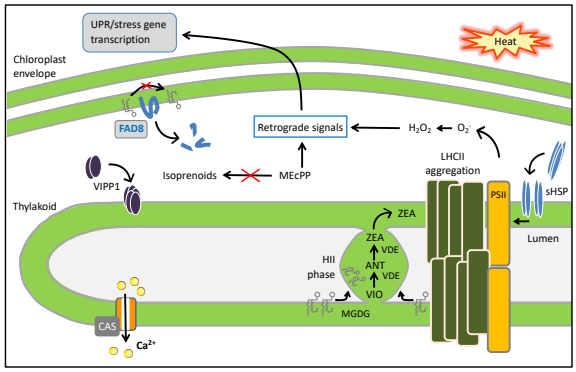

Figure 3. Sensing and signaling of heat stress at the chloroplast.

Exposure to moderate heat has various direct consequences for chloroplast proteins and membranes, which trigger rescue pathways. In the thylakoidal membrane, LHCII proteins aggregate, which leads to an excess of its major constituent, the non-bilayer prone glycerolipid MGDG. As a consequence, a non-bilayer structure (the HII phase) emerges, which consists of MGDG organized in hexagonal, stacked tubules. The xanthophyll cycle enzyme, violaxanthin de-epoxidase (VDE), recruits specifically to the HII phase in the thylakoid lumen, catalyzing the synthesis of zeaxanthin (ZEA) from its precursors, violaxanthin (VIO) and antheraxanthin (ANT). The HII phase remains attached, which allows for free diffusion of the photoprotective xanthophylls to the thylakoid. VIPP1 and sHSP recruit to the thylakoid membrane under heat stress, as they recognize membrane packing defects. They protect chloroplast membrane and PSII integrity. Heat stress is signalled in the chloroplast by a rapid Ca^{2+} increase in the stroma

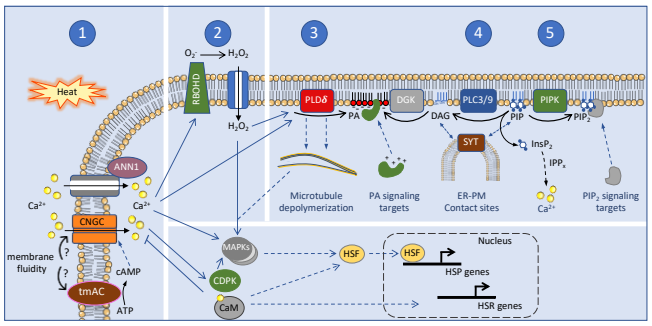

Figure 4. Sensing and primary signaling events of heat stress at the plasma membrane. In response to heat stress, several plasma membrane-linked protein activities are triggered which lead to intracellular signals that collectively regulate the heat stress response in plants. 1. Heat perception gives rise to increases in Ca^{2+}, which can enter the cytosol from the apoplast through channels such as CNGC6. This channel might be activated by cAMP, which is generated by an unknown transmembrane adenylyl cyclase (tmAC), perhaps activated under heat stress as membrane fluidity increases. Through association with calmodulin (CaM), Ca^{2+} can negatively regulate CNGC6, and promote the function of HSFs. HSFs are the primary regulators of the heat response leading to transcriptional induction of HSPs and other genes. Apart from CNGC6, Annexin 1 (ANN1) is required for cytosolic increases in Ca^{2+}. 2. The second major factor in the heat stress response is H_2O_2, which is generated by the plasma membrane microdomain NADPH oxidase, RBOHD, whose activity is modulated by several factors, including Ca^{2+} and PA. After H_2O_2 enters the cell, it modifies the PLDδ protein such that it becomes sensitive to activation by Ca^{2+}. 3. PLDδ generates PA, which has a myriad of signaling functions which are mediated by its interaction with cytosolic target proteins. PLDδ