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Review

How do plants feel the heat and survive?

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Climate change is increasingly affecting the quality of life of organisms on Earth. More frequent, extreme, and lengthy heat waves are contributing to the sixth mass extinction of complex life forms in the Earth's history. From an anthropocentric point of view, global warming is a major threat to human health because it also compromises crop yields and food security. Thus, achieving agricultural productivity under climate change calls for closer examination of the molecular mechanisms of heat-stress resistance in model and crop plants. This requires a better understanding of the mechanisms by which plant cells can sense rising temperatures and establish effective molecular defenses, such as molecular chaperones and thermoprotective metabolites, as reviewed here, to survive extreme diurnal variations in temperature and seasonal heat waves.

Highlights

As the sun rises, the temperature rapidly increases and, by noon, heat may damage labile macromolecular complexes and impair the vital biological functions of plants.

Plants have a heat shock response (HSR), which is activated via fluidity changes in the plasma membrane and heat-responsive cyclic nucleotide-gated ion channels (CNGCs), which use Ca^{2+} and reactive oxygen species (ROS) as messengers to mediate a signaling pathway, leading to the upregulation of heat-induced mRNA in minutes, and to the accumulation of protective heat shock proteins (HSPs) and metabolites in hours.

While some studies have been conducted on plant molecular chaperones, their precise role in acquired thermotolerance and the identity of their thermolabile protein substrates remain unknown.

Based on 'omics analyses, plant HSR is a multigenic trait and new thermoresistant crops should harbor complementary mechanisms combining HSP chaperones and enzymes producing thermo- and ROS-protecting metabolites.

Plant heat sensing and signaling to build up effective molecular defenses

All organisms on Earth are facing rapid climate change. Indeed, the Intergovernmental Panel on Climatic Change estimates a 0.3°C rise in global mean temperature per decade [1], and a greater and more frequent occurrence of heat waves is expected to compromise human food security [2,3]. This alarming situation is calling for crop breeders to better understand how some wild plants have been able to adapt to harsh environments by ameliorating existing molecular strategies to protect their heat-labile macromolecules during **noxious heat stresses** (HS; see Glossary). HS can disrupt ecosystems, especially when combined with other stresses, such as drought [4]. Excessive heat can alter the structure and function of thermo-labile macromolecular ensembles and accelerate water loss, resulting in heat-aggregated proteins deprived of their dedicated biological activities. Misfolded proteins trigger the production of **reactive oxygen species** (ROS), which can damage lipids in membranes, cause DNA mutations, and result in leaf shedding, apoptosis, and, ultimately, plant death [5]. Here, we review recent advances in our understanding of heat-sensing and heat stress-related responses leading to the onset of molecular defenses against heat damage in land plants. Data from omics approaches are discussed, highlighting the central role of heat-induced proteins, among which **heat shock transcription factors** (HSFs), heat-signaling proteins, heat-induced metabolic enzymes producing thermoprotective and ROS-scavenging metabolites, and heat-induced chaperones, commonly but confusingly called **heat shock proteins** (HSPs), which can prevent and revert protein aggregation. In addition, we discuss the central role of plant heat-sensory calcium channels that can progressively respond to increasing temperatures by way of reacting to changes in the fluidity of the plasma membrane [6].

At sunrise, most land plants face a rapid increase in ambient temperature of up to 20°C over the following 12 h [7]. This can occur more suddenly when winds chase sun-veiling clouds. Thus, a seemingly harmless increase in the ambient temperature at dawn induces an optimal heat signal, leading to the synthesis of metabolic enzymes, which, by noon, can result in the production of dozens of millimolars of thermoprotective metabolites and tens of micromolars of protective HSP chaperones. Metabolites, such as carotenoids and glutathione, can mitigate harmful heat-induced ROS, whereas others, such as proline, glycine betaine, and trehalose, can protect

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heat-labile proteins and membranes. The massive accumulation of HSP20s by mid-morning may prevent protein misfolding and aggregation occurring by noon, whereas, in the afternoon, heat-accumulated HSP60s, HSP70s, HSP90s, and HSP100s can resolubilize protein aggregates into biologically active proteins [8].

The design of new crops that can withstand more frequent and acute heat waves likely necessitates combined activities of several heat-induced enzymes to produce thermoprotective metabolites, and of chaperones to protect labile proteins during, and repair heat-damaged proteins after, stress exposure [9]. The breeder's challenge is immense, given the expected trade-off between crop yields and the increased expression of expectedly costly molecular defenses.

Heat sensing and signaling

HS is a transient increase in ambient temperature beyond the optimal plant growth temperature, with deleterious effects on the plant physiology. The severity of heat damage is determined by the rate of temperature increase, the intensity of the HS, its duration, and by the presence of other stresses [10,11]. At the molecular level, higher temperatures can damage fragile complexes, mostly proteins and membranes, whereas polysaccharides may be more resistant. Although heat-denatured RNA and DNA may spontaneously revert to their functional native state after heat stress, heat-induced DNA methylations and demethylations may have long-lasting transgenerational phenotypic and epigenetic consequences [12]. It is generally thought, albeit poorly demonstrated experimentally, that severe HS in plants causes the transient unfolding of heat-labile proteins, leading to their aggregation into insoluble inactive species. In metazoans, protein aggregates can be cytotoxic and clog protein quality control machineries, such as **molecular chaperones** and the proteasome [13,14].

By translating an early moderate temperature increment into an effective heat-priming signal, leading to the onset of new molecular defenses (Figure 1, blue), plant cells can prevent heat damage during exposure to a subsequent noxious HS and repair damaged proteins and membranes once the stress has passed [15]. All organisms, including plants, express several conserved families of core chaperones: ATP-independent HSP20s, four ATP-dependent unfoldases, HSP60s, HSP70s, the co-disaggregases HSP100s, and HSP90s. Together with a plethora of co-chaperones, these control the quality of protein structures and cellular functions [8].

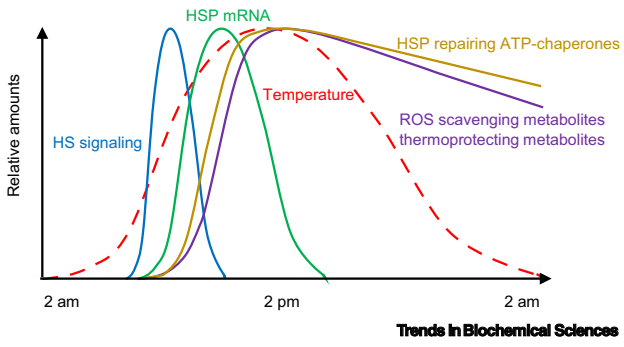

Figure 1. Plant sensing and response to heat stress during a 24-h cycle. Early in the morning of a typical hot summer's day, land plants must sense and evaluate the need for molecular defenses by mid-day to prevent heat damage and repair it by evening. The activity of lipid desaturases at different basal ambient temperatures may set different activation thresholds for the plant heat shock response. By dawn, plants need to detect mild, harmless increments in the ambient temperature in anticipation of an upcoming damaging heat stress by noon (red dashed line). Within minutes, plants need to emit a transient heat shock signal (blue) to produce, within a couple of hours, heat shock protein (HSP) mRNA (green). During the morning, HSP transcription factors and HSP chaperones accumulate (gold) to prevent and repair heat-damaged proteins and produce HSP enzymes accumulating thermoprotecting and reactive oxygen species (ROS)-scavenging metabolites (purple), which can last several days. This thermomemory predisposes plants to better withstand ensuing heat stresses over the following days.

Glossary

Acquired thermotolerance (AT): ability of a plant to accumulate HSPs and thermo- and ROS-protective metabolites, in response to a mild and harmless, prior warming, conferring the ability to survive an upcoming severe harmful HS for a few hours.
Calmodulins (CaMs): calcium-binding proteins that typically bind other proteins, such as cyclic nucleotide gated channels. Upon binding of entrant periplasmic Ca^{+2} ions, CaMs change their conformation and send a specific cellular signal to produce the HSR.
Heat shock proteins (HSPs): proteins that massively accumulate in response to mild HS. Many HSPs are molecular chaperones. Others are heat shock signaling and transcription factors and enzymes that produce thermo- and ROS-protective metabolites. Other HSPs have unknown functions.
Heat shock response (HSR): homeostatic transcriptional program highly conserved in all organisms, where, in response to a mild heat shock, thermoprotective HSPs and metabolites are massively produced, conferring cells acquired thermotolerance.
Heat shock transcription factors (HSFs): family of transcription factors that bind specific sequences in the promoter regions of HSP genes, the concomitant derepression and activation of which lead to the accumulation of HSPs and confer plant AT.
Molecular chaperones: proteins that control the quality of the structure and function of other proteins. Some but not all chaperones are HSPs. Most use ATP to unfold heat-aggregated proteins to be repaired into functional native proteins.
Noxious heat stress (HS): harmful HS causing irreversible damage to the structure and function of thermolabile macromolecules, such as membranes and protein complexes, possibly leading to apoptosis and plant death.
Reactive oxygen species (ROS): highly reactive chemicals or radicals, formed from O_2, such as H_2O_2, ozone, singlet oxygen, superoxide, and hydroxyl radical, that can chemically damage essential lipids, membranes, nucleotides and proteins.
Targetases: substoichiometric co-chaperones that can catalytically recruit an excess of HSP70s onto specific (mostly misfolded) polypeptides

that need to be structurally and functionally modified by the chaperone into differently active proteins.

The plant heat shock response

Within minutes of a mild heat-priming treatment (several degrees above ambient temperature without reaching noxious HS) [16,17], ~1% of plant genes, including some encoding chaperones, are massively transcribed (Figure 1, green). The ability of plants to respond to abrupt changes in conditions was recently reviewed by Kollist *et al.* [18]. In the case of HS, genes encoding molecular chaperones, such as HSP101, HSA32, some HSP70s and HSP90s, possibly acting as repressors of HSFA1 activity at ground temperatures [19], and many cytosolic HSP20s, possibly blocking heat-induced apoptosis, are overexpressed and accumulate predominantly in the cytosol [16,20]. Within hours, various HSPs involved in HS defenses accumulate (Figure 1, gold). In animal and moss cells, 100 or so newly heat-accumulated HSPs represent a net 2% mass gain in total cellular proteomes at the expense of an across-the-board net 2% protein mass loss of thousands of proteins, mostly with house-keeping functions, which are outcompeted on the ribosomes by the abundant new HSP mRNAs [16,21].

When a promoter is tuned by evolution to constantly create just enough new mRNA to replace a degraded polypeptide with a new one, terminally differentiated cells do not necessitate sensors and signaling pathways to maintain a constant cellular level of that protein. By contrast, to express HSPs only on a need-to-defend basis, cells may require thermosensors connected to a specific signaling pathway. Both in animals and plants, the transcription of HSP genes at non-HS temperatures is tightly repressed by bound HSP70-HSP90 chaperones, which maintain HS transcription factors (i.e., HSFA1) inactive in the cytosol, and by histones enwrapping HSP genes in the nucleus [22] (Figure 2, Key figure).

Upon mild warming (priming), thermosensors must first send a signal to derepress transcription of HSP genes by dissociating the inhibitory HSP70-HSP90 chaperones from HSFA1. In addition, a specific heat-induced Ca^{2+}-entry signal from the plasma membrane must activate a kinase, which in turn not only activates the chaperone-liberated HSFA1 to transcribe new HSP genes [23] (Figure 2, Box 1), but also acts on the chromatin remodeling machinery to evict the bound histones from HSP genes [24]. Priming is a required step in **acquired thermotolerance** (AT), which involves molecular modifications that may be maintained longer than the initial heat priming, resulting in thermomemory lasting several hours to a few days. Hence, before this memory vanishes, plants can 'learn' to respond more readily to a new HS (Figure 1). Most of the heat-induced mRNA produced during the first hour of HS is degraded in less than 24 h [21,25]. By contrast, the degradation of heat-induced chaperones, transcription factors, and thermoprotective metabolites may take a few days [12]. Thermomemory can also involve chromatin remodeling, which can last up to a week [12,26]. Experimental data suggest that transgenerational heat stress memory can be inherited via an HSFA2-activated H3K27me3 demethylase [27,28]. Consequently, on subsequent hot days, plants are better prepared to withstand a noxious HS [12].

From the plasma membrane to the production of thermoprotective compounds

During the first minutes of an HS, the phosphoinositide-specific phospholipases PLC3 and PLC9 are rapidly activated, with the concurrent accumulation of cytosolic Ca^{2+} [29,30] (Figure 2). PLC3 and PLC9 hydrolyze phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) [30], respectively, which was suggested to activate Ca^{2+} release from intracellular stores [29] and trigger a signaling cascade involving **calmodulins** (CaMs) and kinases [31]. Whereas a chloroplast-specific calcium signal was identified in response to heat, without evidence for an increase in cytosolic calcium [32], ratiometric Ca^{2+} reporter-based analyses provided strong evidence that HS induces cytosolic Ca^{2+} signals in plant leaves [33]. Moreover, electrophysiology showed specific heat-induced transient entry of external Ca^{2+} into the cytosol of protoplasts [34].

Key figure

Heat shock sensing, signaling, and responses in plant

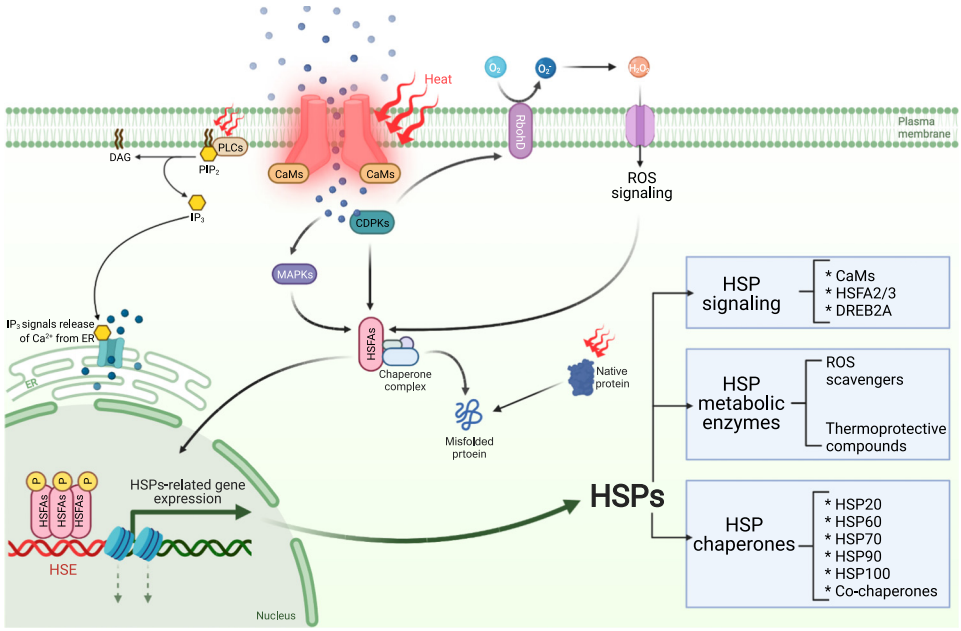

Figure 2. The plant heat shock response (HSR) is initiated by an increase in the fluidity of the plasma membrane (PM) and the possible activation of phospholipases (PLCs) hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), leading to the controlled entry of Ca^{2+} from the endoplasmic reticulum (ER) and activation of calmodulins (CaMs) and the heat shock signaling pathway. The cyclic nucleotide-gated ion channels (CNGCs) embedded in the PM (red) respond to the temperature-increased fluidity of the PM and mediate the controlled entry of periplasmic Ca^{2+} into the cytosol. Ca^{2+} binding to CNGC-bound CaMs initiates a specific signaling cascade that activates kinases, which then phosphorylate and activate heat shock transcription factor A (HSFA). Reactive oxygen species (ROS) production by NADPH oxidase RbohD at the PM may also lead to co-activation of some heat shock factors (HSFs). The Ca^{2+} entry-dependent heat shock signal phosphorylates HSFA1 and evicts the bound HSP70–HSP90, thereby activating HSFA1 to translocate to the nucleus and bind heat shock elements (HSEs). This leads to the eviction of bound histones from HSP genes and the recruitment of RNA polymerase. The consequent massive synthesis, in minutes, of HSP mRNA and the accumulation, in hours, of heat shock signaling proteins, enzymes producing thermo- and ROS-protective metabolites and of molecular chaperones, ultimately confers acquired thermotolerance to the plant cell, which can last for days. Abbreviation: MAPK, mitogen-activated protein kinase.

Land plants contain ~20 different cyclic nucleotide-gated ion channels (CNGCs) [6,34,35]. In particular, CNGC2/4, which, similar to their distant animal relatives, the heat- and nociceptive TRPV1 channels [36], form homo- and heterotetrameric transmembrane ion channels, with their cytosolic parts interacting with CaMs and cyclic nucleotides (Box 2). In animals and plants, the **heat shock response** (HSR) depends on the degree of fluidity of the plasma membrane in which the thermosensory channels are embedded [37,38]. At low temperatures, CNGC2/4 channels are closed and poised to readily respond to a heat-induced increase in the fluidity of the surrounding plasma membrane. Under HS, the channels quickly open, allowing periplasmic Ca^{2+} to enter and bind CaMs associated with the cytosolic C-terminal domain of the CNGCs [39] (Figure 2). Consequently, kinases phosphorylate HSFs, which translocate to the nucleus and

Box 1. Feel the heat (sensing)

The conductor of an orchestra must first produce a gesture to signal musicians when to execute a musical movement. Similarly, plant cells must sense a mild temperature rise to signal to the protein synthesis machinery when to execute a HSR and build up effective molecular defenses in anticipation of damage from an upcoming noxious HS (see Figure 1 in the main text). Whereas sending a signal from cellular thermosensors to the nucleus may take seconds, and mRNA synthesis minutes, the accumulation of HSPs and thermo- and ROS-protective metabolites, takes hours (see Figure 1 in the main text). Thus, to induce an effective HSR, land plants need thermosensors to detect mild warming, which is unlikely to cause protein aggregation, and yet enables them to respond fully to HS.

The accepted model is that as-yet unidentified thermolabile proteins must first respond to warming upon undergoing thermal unfolding and recruiting HSP70 and HSP90 chaperones from inactive HSF1, thereby initiating a specific HS signal [31,47]. Indeed, an 'unfolded protein response' (UPR) has been identified in the ER and cytosol, suggesting that thermolabile proteins act as cellular thermosensors [113]. A putative heat-responsive retrograde pathway was also reported in chloroplasts, in which the photosynthetic apparatus is a primary target for heat damage. HS in the chloroplast triggers a signal leading to the transcription of HSPs in the nucleus [114]. The dissociation of histone variant H2A.Z at high temperatures from HSP genes has also been suggested to serve as a direct thermosensory mechanism in the nucleus, inducing the HSR in higher plants [50]. Yet, because H2A.Z is associated with many promoters of genes that are not activated by heat, this histone is unlikely to serve as the primary heat sensor of plant cells. It rather stands at the very end of the HS signaling pathway, receiving eviction orders from heat-activated histone-remodeling complexes, such as ARP6 [50], which in turn receive orders from the plasma membrane via a calcium entry-dependent signal [34,35,42].

Moreover, light and temperature signals are intertwined. The photoreceptor phytochrome B and transcription factors called 'phytochrome interaction factors' (PIFs) change conformation and activity under warming [115,116]. When the activity of phytochrome B is reduced by far-red light or by mildly elevated temperatures, PIFs accumulate and promote hypocotyl elongation. Indicative of a crosstalk between light signaling, the circadian clock, and temperature signaling [53,117], plants in the dark may be less responsive to HS than in the light, possibly due to thermosensitive photosensors [25,118,119].

bind conserved *cis*-elements called heat shock elements (HSE) in the promoters of HSP genes [40,41] (Figure 2).

HSFs are classified into three classes with different functions: HSFA, B, and C [42]. HSFA1A is a master regulator of plant AT that triggers the HSR through the induction of HSFA1b and the overexpression of new HSFA2; HSFA2 thus becomes a major heat stress transcription factor amplifying HSPA1a's activatory effect, leading to the HSR and AT [43,44] and, together with HSFA3, is prolonging thermomemory, [45]. HSFB1 acts as a co-regulator enhancing the activity of both HSFA1A and HSFA2 [40,42,46]. The so-called 'titration model' for HSF activation was proposed based on the observation that, at non-HS temperatures, hypophosphorylated HSFAs are maintained inactive in the cytosol by bound HSP70 and HSP90 [47] (Figure 2). Under HS, HSFAs are

Box 2. The role of CNGCs

Growing evidence points at the plasma membrane as a central thermosensing component in land plants [31,120]. Hence, moss plants constantly grown at 22°C, with plasma membranes naturally enriched with unsaturated lipids, produced more HSPs in response to 1 h of HS at 38°C than when constantly grown at 28°C with plasma membranes enriched in saturated lipids [37]. This, together with evidence that a membrane fluidizer can artificially decrease the threshold temperatures at which a strong HSR is produced, suggests that plant thermosensors do not directly respond to a given elevated temperature: it is the basal temperature of growth that dictates the ratio between fluidizing unsaturated fatty acids and rigidifying saturated fatty acids. Thus, plant thermosensors embedded in the plasma membrane can react to a specific net gain of membrane fluidity, enacted by a given ratio of saturated and unsaturated lipids set by the ground temperature of growth [37].

Further demonstrating the key role of the plasma membrane in thermosensing, depletion or chelation of external Ca^{+2} ions completely, albeit reversibly, blocked the heat-induced HSR in both plants and human cells [48], pointing at members of the plasma membrane-embedded CNGCs, as potential membrane fluidity-responsive protein thermosensors in plants. Indeed, disruption of *CNGC2* or *CNGC4* resulted in a hyperthermosensitive phenotype with a HSR occurring at heat-priming temperatures lower by ~4°C. The hyperthermosensitive mutants unnecessarily accumulated HSPs at nonstressful temperatures and grew extremely slowly, but were less in need of heat priming to effectively resist a noxious HS, indicating their increased thermotolerance [34].

found to dissociate from the chaperones and become functional trimers in the nucleus. Although evidence for protein aggregation in the cytosol of plant cells under mild heat-priming temperatures is lacking, it is generally believed that chaperone binding to these heat-aggregated proteins causes chaperone dissociation from the inactive HSFA1 [40]. Noticeably, during HS, when the entry of external Ca^{2+} is prevented by EGTA, the plant HSR does not occur [35]. This implies that, without a Ca^{2+}-entry dependent signal from the plasma membrane, chaperone titration by putative heat-labile protein aggregates does not suffice to elicit full activation of HSFA1. Similarly, blockage by a specific inhibitor, capsazepine, of Ca^{2+} entry under HS through the TRPV1 thermosensory channel in the plasma membrane of vertebrates does not suffice to activate the accumulation of HSPs [48]. By analogy, releasing the handbrake is not enough to start moving a car uphill: one must concomitantly press on the gas.

Upon binding to HSE, the activated phosphorylated HSFAs may also need to instruct the chromatin remodeling complexes to evict bound histones that would otherwise repress the transcription of HSP genes [49,50]. Alongside HSFs, other transcription factors control HSP gene expression, such as dehydration-responsive element-binding protein (DREB 2A), some NAC family transcription factors [51], multiprotein-bridging factor 1c (MBF1c), and some members of the Ethylene-responsive transcription factor (ERF) family [52]. Moreover, the time of the day impacts the translation of HSP transcripts in response to HS [25] and an HSF-independent pathway involving circadian-regulated genes has been described [53]. Thus, evolution may have favored that, for the same extent of HS, a land plant may need to produce a costly optimal HSR at noon, while the high cost of a full HSR may be spared at night, when there is less light and fewer ROS-generating stresses. Whereas an excess of ROS has detrimental effects on plant cells, low ROS can mediate stress responses and affect plant development [54]. In addition to Ca^{2+}, H_2O_2 levels at the plasma membrane also increase quickly in response to severe HS. Suggesting a crosstalk between the ROS and Ca^{2+} signaling cascade, this process, which is catalyzed by the NADPH oxidase RbohD, is activated by Ca^{2+} binding to EF-hand motives [55,56] (Figure 2). H_2O_2 was also shown to activate particular HSFs [57] (Figure 2).

Surviving the heat: thermoprotective metabolite production

Abiotic stresses have a wealth of cumulative adverse effects on plant fitness and survival. Plants can accumulate organic compounds in the tens of millimolar range with thermo- and ROS-protective properties that can mitigate some of the heat-damaging effects [58]. Hydrophobicity is central to the maintaining of membranes and proteins in the native state. However, as the temperature increases, hydrophobicity is not strong enough to compensate for the low entropy of the single native state. Whereas membranes may undergo hyperfluidization [59], proteins may transiently unfold and readily acquire different, more compact misfolded and aggregated conformations that are more entropic than the native state, while still satisfying the requirement of sequestering most of their hydrophobic parts [60].

From a thermodynamic point of view, many protective metabolites, often called osmolytes, can lower the osmotic potential, thereby acting as thermoprotectants of native proteins and membranes. Thermoprotective metabolites can be amino acids (proline), polyamines, quaternary ammonium compounds (glycine-betaine), sugars (trehalose), polyols, sugar alcohols (mannitol, galactinol), or tertiary sulfonium compounds [61]. Amino acids, such as tyrosine, valine, proline, tryptophan, and glutamine, accumulate during plant responses to various abiotic stresses, including HS [62]. Amino acids likely have an essential role in the regulation of osmotic adjustment to keep plant turgor pressure under heat-accelerated evaporation. However, various metabolites do not equally protect plants from HS damage. Whereas proline accumulates in some drought-stressed plants, it can inhibit growth of *Arabidopsis* seedlings upon HS [63]. Under a combination

of drought and HS, proline is replaced by sucrose [64]. Glycine-betaine is known to stabilize macromolecular structures in response to dehydration and HS and protect the cytoplasm and photosystem II in chloroplasts from heat and ion toxicity [65]. It was suggested that, during HS, increased levels of trehalose, *myo*-inositol, and galactinol, which are precursors of oligosaccharides, stabilized membranes by interacting with the phosphates of the phospholipids [66]. Moreover, HSFs also control the increased levels of essential metabolites, such as galactinol and its derivatives, with potential thermoprotective effects [67]. The accumulation and production of thermoprotective metabolites is regulated by several pathways [68], including ROS-mediated mitogen-activated protein kinases (MAPKs), salt overly sensitive (SOS), abscisic acid and calcium signaling, via CaMs.

The term 'chemical chaperones' frequently used to describe these metabolites is misleading. These thermoprotective metabolites act by stabilizing labile macromolecular structures, such as native proteins and membranes, under otherwise heat-denaturing conditions. By contrast, HSP60, HSP70, and HSP100 are sophisticated ATP-fueled molecular machines with strong affinities for already-formed misfolded and aggregated proteins. Thus, chaperones may 'repair' proteins that have been heat damaged into their native, functional, state. By accumulating protective metabolites typically during the morning of a summer's day, heat-primed plants may stabilize thermolabile macromolecules in their native state, despite the denaturing temperatures at noon (Figure 1, purple). Priming leads to a transcriptional upshift and subsequent accumulation of heat-induced proteins, including enzymes that accumulate thermo- and ROS-protecting metabolites, allowing plants to withstand repeated HS [69], and ATPase chaperones to repair heat-damaged proteins during and after HS.

Surviving the heat: heat-accumulated molecular chaperones

The term 'molecular chaperones' was reinstated by John Ellis [70] to describe proteins assisting the native (re)folding and assembly of various proteins complexes, without being part of the final assembled oligomers. Experimental data have since shown that many chaperones act as polypeptide unfolding catalysts, and are major components of the cellular protein homeostasis network [13], involved in controlling both physiological processes and repairing stress-damaged proteins during and following HS. Noticeably, the general designation of chaperone proteins as being HSPs is misleading; although about one-third of chaperones and co-chaperone genes are overexpressed under HS, the remaining two-thirds are not. Nevertheless, chaperone and co-chaperone genes are ~15 times more likely to be heat inducible compared with other gene categories, confirming that heat-induced chaperones are key to the prevention and repair of structural heat damage in labile proteins [16,21]. Chaperones are found in all cellular compartments (Table 1). Whereas protein crowding is generally thought to aggravate protein aggregation, heat-induced plant chaperones mostly accumulate, counter-intuitively, in the cytosol, where protein crowding is significantly lower than in the endoplasmic reticulum (ER), mitochondria and chloroplasts [16] (Figure 3A). In general, viable single T-DNA insertion lines have no detectable HS or AT phenotypes, likely because of the high redundancy of orthologous genes in the various chaperone families (except HSP101) [71].

Interestingly, HS generates stress granules [72–74] containing mRNAs, elongation initiation factors, and several chaperones ,such as HSP20s and HSP101. HSP20s are unable to actively promote the solubilization of already-formed stable aggregates, but can prevent protein aggregation. HSP101 is a co-chaperone that increases the stand-alone disaggregase activity of HSP70s and mediates protein hydrolysis by the 26S proteasome [75,76]. In heat-stressed cells, HSP chaperone levels can persist from hours to several days and, therefore, are central to the onset of plant AT [12,28] (Figure 1, gold).

Table 1. Chaperone families and their representative members in four plant species

Class	Representative members				Subcellular localization	Refs
	Arabidopsis thaliana	*Physcomitrium patens*	*Oryza sativa*	*Populus trichocarpa*		
HSP20s	18	12	23	29		[77]
Subfamily						
I	6	9	9	16	Cytosol	
II	2	2	2	1	Cytosol	
III	1		1	1	Cytosol	
IV	1		1	1	Cytosol	
V	1		1	2	Cytosol	
VI	1			2	Cytosol	
ER	1		2	1	ER	
Mitochondria/plastids	5	1	7	5	Mitochondria/plastids	
HSP60s	17	14	20	28		[82,121]
Subfamily						
Group 1: CPN60	9	6	11	10	Mitochondria/plastids	
Group 2: CCTs	8	8	9	18	Cytosol	
HSP70-110	18	17	32	20		[82,121,122]
Subfamily						
Hsp/Hsc70	11	11	14	13	Cytosol/Mitochondria, plastids	
Bip	3	2	6	4	ER	
Hsp110	4	4	8	3	Cytosol/ER	
HSP90	7	6	9	10	Cytosol/Mitochondria, plastids/ER	[82,100,121]
HSP100-ClpB	4	4	5	5	Cytosol/mitochondria, plastids	[82,121]
HSP40-JDPs	99	100	95	163	Cytosol/mitochondria, plastids	[92]
Subfamily						
Class A	8	14	13	11		
Class B	9	9	7	11		
Class C	82	77	75	141		

HSP20s: the most heat-responsive plant chaperone

HSP20s are a family of ancient ubiquitous proteins with a conserved α-crystallin domain suggested to bind denatured proteins. They are divided into about a dozen subclasses (Table 1) (Figure 3A,B), with diverse N-terminal domains responsible for their assembly into oligomers of 12 or more subunits [77]. It is not known what the state of the HSP20-bound polypeptides is in the cell: unfolded, misfolded, aggregated, or native. Following the suggestion that HSP20s bind misfolded species, they were shown to collaborate *in vitro* with the ATPase unfoldases HSP70-40 and HSP60, and also indirectly with HSP100 in the refolding of heat-predenatured proteins [78].

In plants, HSP20s are the most heat responsive of all chaperone classes (see Outstanding questions). Remarkably, the basal expression of HSP20s at non-HS temperatures is generally null, implying a strong repressive mechanism [15,16] (Figure 3B) and suggesting that their overexpression has a high cost to the plant fitness. Heat-accumulated HSP20s account for ~30% of the overall net mass gain of heat-induced proteins. Although being the most dramatically heat-induced chaperones in the cytosol of higher plants, current knowledge is limited on the

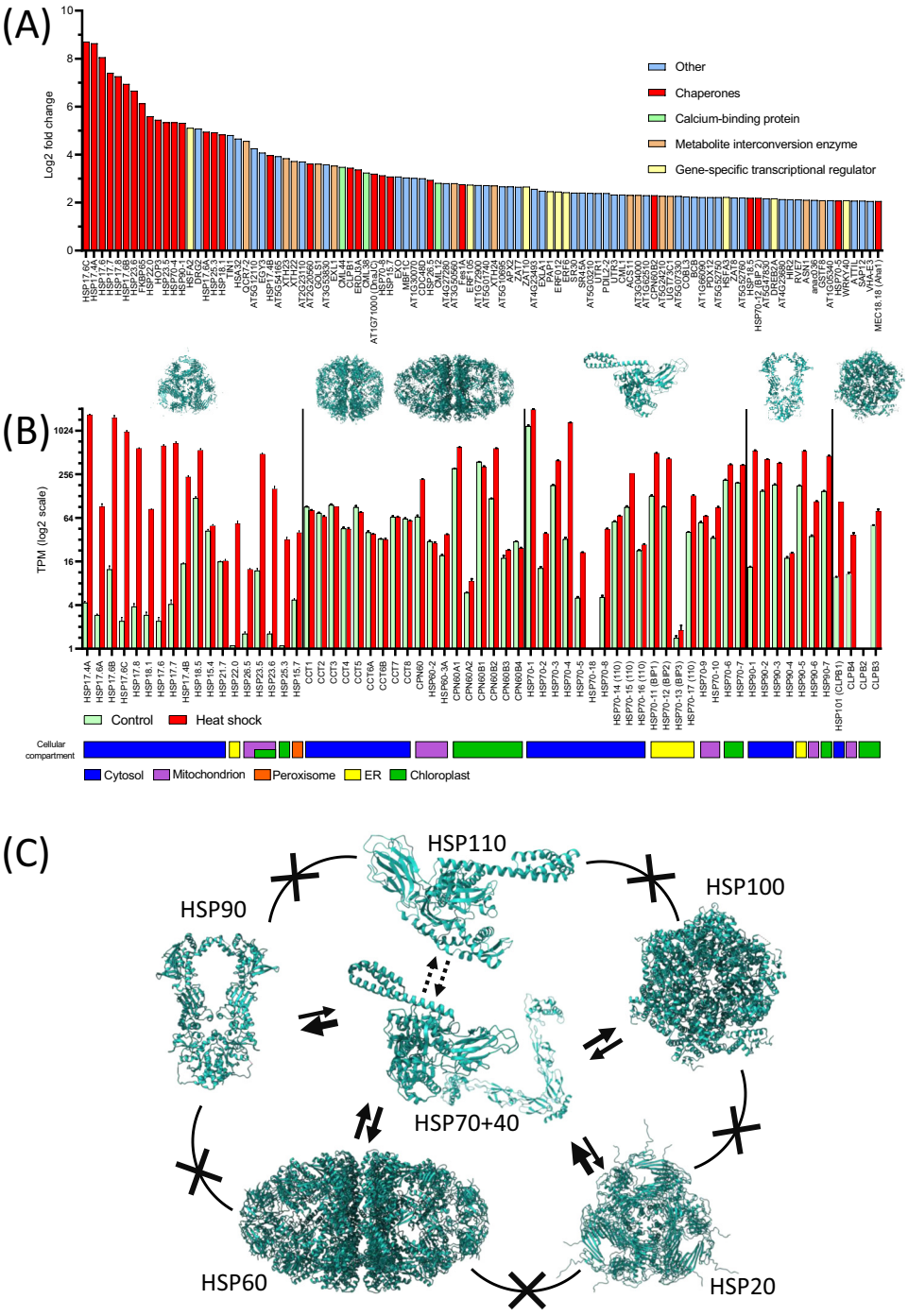

Figure 3. RNA sequencing (RNA-seq) of untreated and mild heat-treated *Arabidopsis thaliana* seedlings and the chaperone collaboration network. (A) Log_2 fold-change of the 100 most heat-induced genes in 10-day-old *A. thaliana* seedlings, treated for 90 min at a priming temperature of 33°C. Chaperones genes are in red, calcium-binding genes are in green, metabolic genes are in orange, transcriptional regulators are in yellow and others are in blue. (B) mRNA transcripts per million (TPM) of mRNAs of the core-chaperone families in untreated (green) and warmed (33°C) (red) *A. thaliana* seedlings. From left to right: HSP20s, HSP60s, HSP70s, HSP90s, and HSP100s. Insert above the

(Figure legend continued at the bottom of the next page.)

mechanism by which HSP20s effectively contribute to plant AT. During HS, HSP20s have been shown to stabilize lipid bilayers and possibly protect membranes from hyperfluidization [79,80]. In addition to their ability to passively prevent aggregation, HSP20s may also carry out specific physiological functions, such as repressing heat-induced apoptosis [16]. The plastid metalloprotease FtsH6 and HSP21 jointly regulate thermomemory in *Arabidopsis* [81]. Noticeably, higher plants are the only eukaryotes that express a HSP20 in the ER [82] (Figure 3A,B).

HSP60s: cage unfolding chaperonins

Together with HSP20s, HSP60s [83] belong to an ancient chaperone family that was already present in the last common ancestor to all organisms [82]. Group 1 HSP60s (CPN60 and GroEL) are found in bacteria, chloroplasts, and mitochondria, and were initially found to mediate ribulose-1,5-bisphosphate carboxylase oxygenase assembly in chloroplasts [70]. Group 2 HSP60s (CCT/TRiC) are found in archaea and the cytosol of eukaryotes, including plants. Remarkably, CCTs, which predominantly fold actin and tubulin, are poorly heat induced in differentiated plant cells [10] (Figure 3B). Yet, enhanced expression of Group 2 HSP60s in root stem cell may maintain proteome integrity and suppress protein aggregation [14].

HSP70s and HSP40s: the central hub of the chaperone network

HSP70s [84] are one of the most highly conserved class of chaperones, accounting for ~1% of the total protein mass of unstressed eukaryotic cells [85]. HSP70s act as ATP-fueled polypeptide-unfolding enzymes that coordinate the activity of the other families of chaperones in the cellular proteostasis network [82] (Figure 3C). They control protein homeostasis in all ATP-containing compartments of eukaryotic cells, both under physiological and HS conditions. Some HSP70s are constitutively expressed, whereas others, such as HSP70-4, are strongly heat upregulated [86] (Figure 3B). Overexpression of heat-inducible HSP70-1 improves plant thermotolerance, whereas its reduced expression is lethal [87]. HSP70s can use the energy of ATP hydrolysis to apply a pulling and unfolding force that remodels high-affinity (alter)natively folded or stress-misfolded proteins into differently folded native proteins, each with a different structure and carrying a specific biological activity [88,89]. To perform their protein-remodeling action, HSP70s rely on J-domain co-chaperones, JDPs (HSP40s and DNAJs), acting as specific obligate HSP70-**targetases**. JDPs, which are about ten times less abundant than HSP70s, act as catalysts that upload misfolded or alternatively folded protein substrates onto the HSP70 machineries to be structurally modified into low-affinity native products [89–91].

Plants are eukaryotes with the largest number of JDP-encoding genes [92], which are divided in three classes [90]. Classes A and B are conserved JDPs families, also called HSP40s, which preferentially act as 'generalists' bringing misfolded protein substrates onto HSP70s for unfolding [93]. By contrast, class C JDPs are diverse with different conserved domains, mostly with

columns are representative structures of members from each chaperone family [Protein Data Bank (PDB): 3J07, 7LUM, 4PKN, 4B9Q, 2IOP and 5VJH, respectively]. Below, members of each chaperone family grouped according to the cellular compartments in which they occur; cytosol (blue), mitochondria (purple), chloroplasts (green), endoplasmic reticulum (yellow), and peroxisome (orange). (C) Plant chaperone network organization. HSP60s use ATP hydrolysis to unfold misfolded polypeptides. HSP20s bind misfolded polypeptides and prevent their aggregation ('holdase'). HSP70-JDPs use ATP hydrolysis to unfold misfolded polypeptides and solubilize proteins aggregates, which can be partially prevented from forming by HSP20s. Polypeptides that are incompletely unfolded by HSP70-JDPs can be further unfolded by HSP60s or HSP90s. HSP100s act as HSP70-dependant co-disaggregases and HSP90s act as HSP70-dependant co-foldases. HSP110s act as additional HSP70-dependant co-disaggregases. Whereas HSP100s, HSP20s, HSP110s HSP90s, and HSP60s do not directly interact, they closely collaborate and exchange unfolding/misfolding intermediates through HSP70-JDPs, which act as integrative hubs for the entire chaperone network. Arrow directions and widths indicate the preferred flow of protein un/folding intermediates between the different members of the network. RNA-seq data adapted from [123]; adapted from [82] (C).

unknown functions [94]. The few known class C JDPs serve as specialized co-chaperones to target unique, alternatively folded protein complexes onto HSP70, for example to import polypeptides into organelles, [95,96]. Less than a dozen out of 80 bioinformatically identified class C JDPs in the genome of *Arabidopsis* have biological functions assigned. The variety of JDPs in plants likely serves to recruit HSP70s onto proteins that control various plant-specific physiological and stress-related processes. Several plant class A and B JDPs are stress upregulated, indicating a role in protein quality control during and following HS. Deletion of AtADJA1 and A2 (orthologs of yeast Ydj1) in *Arabidopsis thaliana* showed impaired thermotolerance in seedlings [97].

HSP90s: mysterious ATP-consuming co-chaperones of HSP70s

HSP90s [98] are a family of abundant chaperones in prokaryotes and in the cytosol, ER, and organelles of eukaryotes [85], accounting for 1% of total cellular protein. Many HSP90s are induced by various stresses, including HS (Figure 3A,B). HSP90s appear to systematically act in tandem with HSP70s (Figure 3C), particularly in the cytosol, where their ATPase cycle is regulated by several conserved co-chaperones [99]. In *A. thaliana*, seven genes encoding HSP90s are expressed in all ATP- and HSP70-containing cellular compartments (Table 1). Overexpression of HSP90s was shown to confer plant resistance to several biotic and abiotic stresses, such as heavy metal, oxidative, and salt stresses and pathogen infection [100]. Bound HSP90s are thought to repress HSFA1 at ambient temperature, while, under HS, they dissociate, with HSFA1 becoming concomitantly active in the transcription of HSP genes [101]. Remarkably, HSP90 inhibitors can cause abnormal morphological phenotypes in seedlings and affect organ growth, supporting a role of HSP90s in buffering protein evolution [82] and in plant development. Unlike HSP70-JDPs, which show stand-alone autonomous unfolding/refolding chaperone activity *in vitro* and in primitive bacteria naturally lacking Hsp90, the reverse is not true, suggesting that HSP90s act as downstream co-chaperones of HSP70s, facilitating the folding of HSP70 substrates that failed to properly fold by themselves [102]. It is unclear how, in close collaboration with HSP70s, energy from ATP hydrolysis is harnessed by HSP90s to remodel the structure of heat-damaged proteins. Thus, there is a vital need to identify the most heat-labile proteins, the heat denaturation of which would be limiting for plant growth and compromise survival when ineffectively repaired by the combined action of HSP90s and HSP70s.

HSP100s and HSP110s: co-disaggregases of HSP70s

The HSP70-JDP chaperone machinery is, by itself, able to target stable preformed protein aggregates and use ATP hydrolysis to unfold, solubilize, and reactivate them back into native proteins [103]. HSP100s [104] are specific co-disaggregases of HSP70s belonging to the AAA+ ATPases superfamily. They form hexameric cylinders in the central cavity from which, once activated by HSP70s, misfolded loops protruding from aggregates are forcefully stretched [105]. The genome of *A. thaliana* encodes four classes of HSP100, which are constitutively expressed at low temperature and may accumulate under HS [106]. In *A. thaliana*, the cytosolic Hot1 HSP101 mutant is more sensitive to HS compared with wild-type, and HSP101 is required for basal thermotolerance, acquired thermotolerance [107–109] and the memory of heat acclimation [110]. Besides their role during HS, HSP100s may perform housekeeping functions in plant growth and chloroplast development.

HSP110s are eukaryote-specific co-disaggregases of HSP70s. Sequence-wise, HSP110s share a common ancestor with HSP70s. Generally, HSP110s are ten times less abundant compared with HSP70s, and are obligate ATP-dependent catalysts that accelerate the exchange of ADP into ATP, thereby ameliorating the basal intrinsic disaggregase activity of cytosolic and ER HSP70s. Similar to fungi, plants co-express both types of HSP70-codisaggregase, HSP101 and HSP110, in their cytosol, suggesting that plants have a powerful HSP70-centrered disaggregation network that is able to process a wide array of protein aggregates. In contrast to aging metazoans that lack

HSP100 co-disaggregases, there is no evidence for the formation of toxic aggregates and amyloid fibrils *in planta*, despite predictions of prions in their proteomes [111]. It has been reported that plant stem cells have an enhanced ability to prevent protein misfolding and aggregation under stress conditions, compared with their differentiated counterparts [14]. It is not clear whether plant cells chose to undergo programed cell death before accumulating toxic aggregates. Possibly owing to their sessile life style, higher plants may have evolved proteomes particularly poor in aggregation-prone proteins compared with metazoans [82].

Concluding remarks

Land plants have evolved powerful but costly protection and repair mechanisms to counteract heat damage, mostly to heat-labile proteins and membranes. A plant cell can be compared to a country, the security of which would necessitate the expensive maintenance, even in times of peace, of a large army in anticipation of rare but possible external aggressions. Plant cells have adopted a cheaper but more risky strategy: to maintain a small core of highly specialized molecular defenses, such as chaperones and enzymes to produce metabolites, that, in the case of an upcoming HS, will benefit within a few hours from the back-up of a large pool of freshly recruited 'militia' in the form of heat-accumulated HSPs, produced in response to a preceding milder heat-priming signal. This strategy demands a sophisticated 'intelligence service' that can identify early warnings and transform a mild harmless warming into a signal to produce high levels of costly defensive HSPs. It also requires that, without HS, the expression of HSP genes be tightly repressed, although they remain poised to be readily de-repressed in response to a signal from the heat sensors in the plasma membrane.

The dramatic increase in atmospheric temperatures due to global warming has become a major concern for human food security. To produce more thermotolerant crops, breeders need detailed understanding of the molecular mechanisms leading to the onset of plant thermotolerance. Some loss-of-function mutants in model plants and crops are more sensitive to various stresses, whereas the overexpression of metabolic enzymes and HSPs result in slightly more heat-resistant crops, albeit mostly under laboratory conditions. Recent transcriptomic, proteomic, and metabolomic studies revealed that temperature tolerance in plants is a polygenic trait. It includes not only HSP chaperones, generally suggested to prevent the heat-induced aggregation of ill-defined thermolabile proteins, but also metabolic enzymes, which can produce ROS-quenching and thermoprotective metabolites, as well as heat-induced signaling proteins and transcription factors. Moreover, plants adapt to warmer temperatures by changing their physiological and morphological characteristics. They may choose to reorient or bluntly shed their leaves and, for plants that can afford it, cause local cooling by increased water evaporation. Many desert plants evade scorching temperatures by choosing to be semi-dry quiescent embryos within dehydrated heat-resistant seeds, deeply buried in the soil, waiting to germinate in a year with a cooler and rainier winter.

Thus, researchers aiming to maintain agricultural productivity in a world undergoing climate change need to investigate the molecular mechanisms that promote stress resilience in model plant species and in crops. Researchers have recently benefited from rapidly accumulating data from various 'omics approaches and from genome editing with CRISPR/Cas9, allowing the engineering of combined new traits in crops [9,112]. Yet, the major challenge remains to produce heat-resistant crops by expressing costly energy-consuming defenses, while maintaining high yields that can still feed the ever-growing human population of the planet.

Outstanding questions

How do HSP20 chaperones prevent the aggregation of heat-labile proteins? What is the state of HSP20-bound polypeptides: unfolded, misfolded, or small aggregates?

Why is the expression of HSP20s so tightly inhibited at low temperatures? Are HSP20s toxic to unstressed plants?

Higher plants are the only eukaryotes that have recently evolved an ER-located HSP20. What is the particular role of this HSP20 in plant thermotolerance ?

Whereas HSP20s can act as passive 'holding chaperones' that prevent protein aggregation during the average lifetime of the lens in a human eye, to what extent do plant HSP20s contribute, mass-wise, to the effective prevention of heat-induced proteins aggregation in plant cells?

What is the mechanism that uses the energy of ATP hydrolysis to enable HSP90 to modify the structure of polypeptides?

Which are the most thermolabile proteins that misfold and aggregate during a heat shock, and are limiting plant growth and survival, and that are repaired by HSP chaperones?

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Declaration of interests

The authors have no conflict of interest.

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