

REVIEW: PART OF A SPECIAL ISSUE ON REACTIVE OXYGEN AND NITROGEN SPECIES

Role of redox homeostasis in thermo-tolerance under a climate change scenario

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• **Background** Climate change predictions indicate a progressive increase in average temperatures and an increase in the frequency of heatwaves, which will have a negative impact on crop productivity. Over the last decade, a number of studies have addressed the question of how model plants or specific crops modify their metabolism when exposed to heat stress.

• **Scope** This review provides an overview of the redox pathways that contribute to how plants cope with heat stress. The focus is on the role of reactive oxygen species (ROS), redox metabolites and enzymes in the signalling pathways leading to the activation of defence responses. Additional attention is paid to the regulating mechanisms that lead to an increase in specific ROS-scavenging systems during heat stress, which have been studied in different model systems. Finally, increasing thermo-tolerance in model and crop plants by exposing them to heat acclimation or to exogenous treatments is discussed.

• **Conclusions** Although there is clear evidence that several strategies are specifically activated according to the intensity and the duration of heat stress, as well as the capacity of the different species or genotypes to overcome stress, an alteration in redox homeostasis seems to be a common event. Different mechanisms that act to enhance redox systems enable crops to overcome heat stress more effectively. Knowledge of thermo-tolerance within agronomic biodiversity is thus of key importance to enable researchers to identify new strategies for overcoming the impacts of climate change, and for decision-makers in planning for an uncertain future with new choices and options open to them.

Key words: Antioxidants, biodiversity, climate change, heat stress, reactive oxygen species, ROS, redox homeostasis, thermo-tolerance.

INTRODUCTION

In the near future climate changes will subject plants to more challenging environmental conditions. An increase of 0.2 °C in the average temperature has been predicted to occur over the next decades. Moreover, heatwaves, i.e. an increase of several degrees over the seasonal temperature for a sustained period of days, are projected to become more frequent (Meehl and Tebaldi, 2004; IPCC, 2014). Climate changes have already caused a fall in crop yield, and the expected further temperature alteration will also increase the negative effect on crop productivity by exacerbating other temperature-related stress conditions such as water shortages (Bita and Gerats, 2013).

Different phases of plant development and the timing of exposure to over-optimal temperatures are also critical for the response to heat stress (HS). Complete loss of grain production can be caused by an increase of a few degrees when plants are exposed to HS during flowering (Lobell *et al.*, 2011). An increase in the average seasonal temperature of 1 °C induces a decrease in cereal productivity by 4–10 % (Wang *et al.*, 2012), and similar behaviour has been observed in legumes (Young *et al.*, 2004).

Plants possess several acclimation mechanisms that enable them to alleviate HS damage. Indeed, to cope with HS, resistant plants have evolved a series of strategies to sense temperature changes rapidly and adapt their metabolism, cellular structures

as well as phenology accordingly. Different heat sensors have been suggested to be present in the plasma membrane, nucleus, cytosol and endoplasmic reticulum of plant cells (Sugio *et al.*, 2009; Che *et al.*, 2010; Mittler *et al.*, 2012). However, a Ca²⁺ channel located in the plasma membrane has been indicated as the primary heat sensor in plants. Increments in temperature increase plasma membrane fluidity, activating the Ca²⁺ channel and Ca²⁺ influx to the cytosol (Mittler *et al.*, 2012; Fig. 1). Studies with Ca²⁺ channel blockers suggest that Ca²⁺ accumulation in the cytosol is mainly responsible for the activation of downstream events leading to proteome, transcriptome and metabolome re-programming which results in thermo-tolerance acquisition (Larkindale and Knight, 2002; Mittler *et al.*, 2012).

The induced expression of heat shock proteins (HSPs) has been considered as a major re-programming event required for the acquisition of thermo-tolerance. HSPs recognize and bind unstable proteins, thus preventing their denaturation and misfolding pattern (Schöffl *et al.*, 1998). Once plants have sensed HS, HSP expression is promptly activated by specific transcription factors (HSFs) binding the conserved sequence of the heat shock elements (HSEs) in the promoters of heat-responsive genes (Schöffl *et al.*, 1998). The HSF–HSP network is a highly conserved molecular mechanism protecting organisms from the negative effects of HS on cellular structures and, consequently, functions, but it is not the only defence system operating in HS responses.

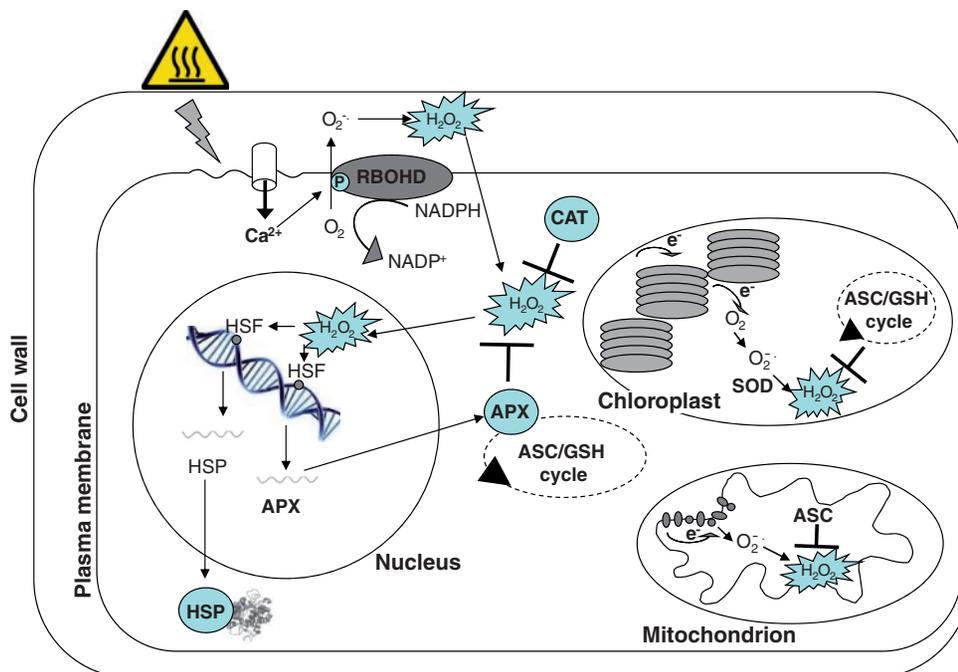


Fig. 1. Redox systems in heat stress response. Schematic representation of the main redox events occurring in plant cells as a consequence of heat stress. Heat perception at the plasma membrane activates a Ca^{2+} channel with a consequent increase in cytosolic levels of Ca^{2+} . Ca^{2+} influx activates RBOHD by promoting its phosphorylation, leading to the increase of reactive oxygen species (ROS). Under heat stress, ROS production also occurs in chloroplasts and mitochondria. Hydrogen peroxide (H_2O_2) works as a second messenger and in the nucleus activates the expression of genes with heat shock elements in their promoters, such as heat shock proteins (HSPs) and cytosolic ascorbate peroxidase (APX). Other redox enzymes and metabolites, such as superoxide dismutase (SOD), catalase (CAT) and the ascorbate–glutathione (ASC–GSH) cycle, work in different cell compartments to maintain appropriate ROS levels.

Oxidative stress emerging as a consequence of HS also seems to play a role in triggering defence mechanisms. Indeed, reactive oxygen species (ROS) and ROS-scavenging systems are part of signalling pathways leading to the activation of defence responses against HS.

This review discusses the recent findings underlying the role of plant redox systems in HS responses and their relevance in thermo-tolerance acquisition.

REACTIVE OXYGEN SPECIES DURING HEAT STRESS

One of the major consequences of HS is the impairment of redox homeostasis. ROS levels are altered and this activates the defence responses in resistant plants or induces oxidative structural or functional damage in sensitive plants. ROS levels and types, as well as endogenous ROS-scavenging systems or the presence of other reactive species in the sites of ROS production/accumulation, are key factors for organism/cell fate (de Pinto *et al.*, 2006).

Several HS-dependent ROS production sites have been described. It has been suggested that the respiratory burst oxidase homologue D (RBOHD), a ROS-generating NADPH oxidase located in the plasma membrane, could have a key role in the oxidative burst occurring during HS (Suzuki *et al.*, 2011; Fig. 1). In arabidopsis and tobacco cell cultures, the increase in H_2O_2 production, occurring during the early phase of HS, can be blocked by the application of an inhibitor of the enzyme

NADPH oxidase (Volkov *et al.*, 2006; Konigshofer *et al.*, 2008). It has also been shown that RBOHD has a central role in HS survival and HS signal transduction (Larkindale *et al.*, 2005; Miller *et al.*, 2009). The activity of this protein is regulated by phosphorylation via protein kinases such as cyclin-dependent protein kinases (CDPKs) and by the direct binding of calcium. Therefore, the cytosolic Ca^{2+} influx, which occurs as a consequence of heat perception, could activate RBOHD and result in the accumulation of ROS (Miller *et al.*, 2009; Fig. 1).

Production of ROS during HS can also occur in mitochondria (Fig. 1). An impaired mitochondrial metabolism seems to be responsible for oxidative bursts occurring in tobacco cells undergoing heat-induced programmed cell death (PCD) (Vacca *et al.*, 2004; Valenti *et al.*, 2007). During HS, calcium uptake by mitochondria and ROS generation occur (Rhoads *et al.*, 2006; Rikhvanov *et al.*, 2007). It has been suggested that the source of ROS resides mainly in the inner membrane of mitochondria where hyperpolarization occurs. Agents able to prevent an increase in electrochemical potential of the inner mitochondrial membrane have also been shown to inhibit thermo-tolerance (Rikhvanov *et al.*, 2007).

In green tissues, chloroplasts probably act as the main ROS-producing site, since photosynthesis is very sensitive to temperature (Crafts-Brandner and Salvucci, 2002; Foyer and Noctor, 2003; Yamamoto *et al.*, 2008; Zinta *et al.*, 2014; Fig. 1). Apart from the direct effect on photochemical reactions in thylakoid membranes, the increase in temperature induces stomatal closure, particularly when water is scarce. The consequent

alterations in CO₂ and O₂ concentrations within mesophyll cells affect the Calvin cycle, thus increasing the impairment in photosynthetic electron flux and leading to a further increase in ROS production. The aim of the water–water cycle is to cope with the ROS overproduction of chloroplasts. However, when HS is persistent or of a particular intensity, such a cycle cannot maintain ROS under control, and oxidative damage occurs (Asada 1999).

Peroxisomes are another site of ROS production, in particular under conditions that increase photorespiration, such as stomatal closure (Vanderauwera *et al.*, 2011).

In HS conditions, ROS play an important role as molecular signals by activating downstream pathways leading to protective effects. There are several redox-sensitive transcriptional factors whose activities rely on redox changes (Tron *et al.*, 2002; Heine *et al.*, 2004; Shaikhali *et al.*, 2008, 2012). The heat shock factor HSFA4a, which rapidly responds to H₂O₂, can function as a direct ROS sensor and rely on a transcription factor function acting upstream of *ZAT12* and *APX1*, two genes with an HSE in their promoters (Miller and Mittler, 2006; Fig. 1).

In relation to the capacity of ROS to act as signals that activate metabolic responses, a temporal–spatial concept of the ROS wave has been proposed that describes ROS signalling as a dynamic process occurring within cells between different organelles, as well as between cells over long distances (Mittler *et al.*, 2011). Starting in the tissues subjected to HS or other stresses and propagating to distal parts of plants, ROS waves induce systemic acquired acclimation, enabling tissues that have not been exposed to HS to increase thermo-tolerance in arabidopsis (Suzuki *et al.*, 2013a).

Under certain conditions, an increase in HS-dependent ROS induces an increase in the cellular systems aimed at restoring redox balance (Fig. 1). This strategy seems to be commonly correlated to thermo-tolerance, as discussed in the next section.

INVOLVEMENT OF REDOX SYSTEMS IN HEAT STRESS RESPONSES

Changes in redox regulation allow resistant plants to survive or to maintain their productivity under HS. These thermo-tolerance properties are based on changes in the levels of redox metabolites, and on the expression/activities of enzymes able to scavenge ROS or to maintain their substrates in the reduced state, even under oxidative conditions. A set of reactions involved in thermo-tolerance is the ascorbate–glutathione (ASC–GSH) cycle. ASC and GSH are the major soluble redox metabolites in plant cells. They act as ROS scavengers directly or as substrates of redox enzymes (Foyer *et al.*, 2009). In the cycle, ASC is used as an electron donor by ascorbate peroxidase (APX), a typical plant enzyme that controls H₂O₂ levels. Oxidation of ASC generates monodehydroascorbate (MDHA), a radical molecule unable to react with oxygen, which spontaneously undergoes dismutation, thus producing ASC and dehydroascorbic acid (DHA). MDHA and DHA are reduced to ASC by two specific reductases using pyridine nucleotides and GSH as electron donors, respectively. In the same cycle, the oxidized form of glutathione, GSSG, is reduced to GSH by GSSG reductase (GR), another enzyme using a pyridine nucleotide as an

electron donor. Interestingly, the ASC–GSH cycle is enhanced under HS in almost all organelles producing ROS (Locato *et al.*, 2009; Fig. 1). Other peroxidases (PODs), catalases (CATs) and superoxide dismutases (SODs) are present, with several isoenzymes acting in different cellular compartments and further regulating ROS levels (De Gara *et al.*, 2010).

Relevance of biodiversity

The HS tolerance of plants seems to be strictly linked to their redox homeostatic capacity, and species-specific differences in antioxidant regulation could play an adaptive role to cope with elevated temperatures. A comparative study performed in plants species with contrasting life forms, such as woody perennial *Populus trichocarpa*, annual legume *Glycine max* or annual forb *Arabidopsis thaliana*, suggests a relationship between heat shock-induced transcriptional networks, ROS-scavenging systems and photosynthesis efficiency (Weston *et al.*, 2011). Weston's study shows that soybean has a higher temperature optimum than arabidopsis and poplar. Transcriptomic analyses demonstrate that ROS scavenging and HSP network transcripts are clustered into the same module for arabidopsis and poplar, and in different modules for soybean, thus suggesting a divergence in ROS regulation among these species. The species-specific variation in the antioxidant regulation in response to heat is confirmed by the enzyme activities of SOD, GR and POD. In arabidopsis and poplar, enzyme activities increase gradually with an increase in temperature, whereas in soybean a rapid but only transient increase in POD and GR activities occurs (Weston *et al.*, 2011).

Maize genotypes are more able than rice genotypes to maintain their growth under HS conditions due to their greater capacity to manage oxidative damage. Indeed, the expression of CAT, APX and GR, as well as ASC and GSH contents, are higher in maize plants compared with rice plants during HS. The different antioxidant capacities of maize and rice could reflect the relative sensitivity of C₄ and C₃ plants to HS (Kumar *et al.*, 2012a).

Several studies performed with sensitive and thermo-tolerant genotypes within the same species underline a clear correlation between thermo-tolerance and the ability to increase one or more ROS-scavenging enzymes. In four different wheat genotypes, heat tolerance, estimated by the capacity to maintain photosynthetic efficiency in seedlings exposed to HS, is correlated with the capability of APX and CAT to dissipate H₂O₂ co-operatively (Dash and Mohanty, 2002). Similarly, studies conducted by exposing different wheat genotypes to HS show that ROS-scavenging enzymes have a positive correlation with chlorophyll content and a negative correlation with membrane damage and heat susceptibility index (Almeselmani *et al.*, 2006; Hameed *et al.*, 2012). Analogous results have been reported in cotton, where genotypic differences in thermo-tolerance have been linked to antioxidant protection of the photosynthetic machinery (Snider *et al.*, 2010).

The association between antioxidant enzymes and heat tolerance has also been shown in Kentucky bluegrass using two different genotypes. During HS, the heat-tolerant genotype 'Midnight' maintained significantly higher activities of SOD, CAT, POD, APX and GR than the heat-sensitive genotype 'Brilliant' (Du *et al.*, 2013).

Relevance of heat stress characteristics

Changes in the activities of antioxidant enzymes under HS also strongly depend on stress duration and intensity.

Generally, a short period of HS enhances ROS-scavenging systems, which enable plants to survive by containing oxidative damage. However, when the HS is prolonged, the proteins and metabolites involved in redox control are overwhelmed, with an irreversible compromise of plant growth and survival. A study on tobacco BY-2 cell culture, a well-known plant model system, shows that short-term HS at 35 °C causes a transient increase in ASC content and in enzyme activity in terms of H₂O₂ removal and in terms of recycling the oxidized forms of ASC and GSH.

The rapid enrichment of antioxidant systems enables tobacco BY-2 cells to avoid oxidizing conditions and to maintain a normal growth rate. On the other hand, under prolonged HS at the same temperature, the redox systems involved in defence mechanisms, in particular enzymatic ones, are initially induced but subsequently suppressed. The decline of these enzymes causes H₂O₂ to increase within cells and leads to the onset of oxidative stress, and consequently cell death (Sgobba *et al.*, 2015). In apple leaves, the ASC–GSH cycle is upregulated in response to high temperatures, but, after reaching the maximum, it declines with an increase in the duration of treatment (Ma *et al.*, 2008).

In wheat, a transcriptomic study has shown that most of the genes involved in the ROS-scavenging pathway, including alternative oxidases, APX3, glutathione peroxidases and thioredoxin, have higher expression levels during short-term heat shock than during long-term heat treatments. The data suggest that plants scavenge ROS generated by heat shock, while the oxidative stress induced by long-term HS impairs a plant's capacity to increase these salvage mechanisms (Qin *et al.*, 2008). A study conducted on alfalfa showed that the APX transcript increases after 24 and 48 h of exposure at 48 °C, but drastically decreases after 72 h of HS. Interestingly, no phenotypic effects were observed after 24 h of heat treatment, whereas leaves were scorched after 72 h of exposure to HS (Li *et al.*, 2013). An increase in genes coding for redox enzymes, in particular APX and DHA reductase, has also been reported in grapevine leaves exposed to 45 °C for a short period (Liu *et al.*, 2012). In lily plants, the high capacity to overcome short periods of exposure to high temperatures seems to be due to the increase in antioxidant molecules and enzymes, which prevent oxidative damage (Yin *et al.*, 2008).

Long-term HS is probably critical for thermo-tolerance since it negatively affects the scavenger capacity of ROS of the exposed tissues. The decrease in SOD and CAT in the leaves of two creeping bentgrass genotypes after long-term HS is responsible for the damage to cell membranes and leaf senescence (Liu and Huang, 2000). The decline in SOD and CAT activities with increasing root-zone temperatures in creeping bentgrass negatively affects turf quality (Wang *et al.*, 2003). The reduction in SOD activity after long-term HS is responsible for the heat sensitivity of the 'Brilliant' Kentucky bluegrass cultivar (He and Huang, 2010), and causes membrane damage and decreases the photosynthetic rate in sorghum leaves (Djanaguiraman *et al.*, 2010), as well as oxidative damage and growth reduction in cucumber plants (Zhang *et al.*, 2012).

The intensity of HS is another pivotal parameter for thermo-tolerance. The increase in temperature stimulates the activity and the expression of antioxidant enzymes until a threshold temperature is reached. If the temperature is higher than the threshold, which changes considerably among species and cultivars, it causes a decrease in redox metabolites and proteins and a consequent decrease in growth (Locato *et al.*, 2008; Chakraborty and Pradhan, 2011; Kumar *et al.*, 2012a).

MECHANISMS REGULATING ROS-SCAVENGING SYSTEMS DURING HEAT STRESS

In model systems, such as arabidopsis plants or cultured cells, the regulatory mechanisms that lead to an increase in specific ROS-scavenging systems during HS have been studied in depth. In this section the mechanisms regulating ASC biosynthesis, APX and thiol-containing proteins are analysed.

Ascorbate biosynthesis

The ASC level influences plant responses to heat stress. The arabidopsis mutants *vtc1* and *vtc2*, with a low ASC content (Conklin *et al.*, 2000), have a lower level of basal thermo-tolerance than the wild type. The low levels of ASC increase the heat-dependent oxidative damage found in the *vtc1* and *vtc2* mutants, also in HS conditions where there is a small decrease in survival (Larkindale *et al.*, 2005).

The role of AtDjB1, a member of the arabidopsis J-protein family, in the acquisition of thermo-tolerance seems to be related to the control of the ASC concentration (Zhou *et al.*, 2012). In the AtDjB1 knockout plants *atj1-1*, the ASC content is lower than in the wild type, which leads to a high accumulation of oxidative products after HS. Interestingly, the exogenous addition of ASC confers thermo-tolerance to *atj1-1* plants. AtDjB1 is localized in mitochondria and, interacting with the mitochondrial HSP70 mtHSC70-1, acts as an ATPase. As a consequence, the accumulation of ATP, which occurs in *atj1-1* plants, inhibits the electron transport chain (Zhou *et al.*, 2012).

Interestingly, the last step of ASC synthesis depends on the electron transport chain (Bartoli *et al.*, 2000) since, L-galactone- γ -lactone dehydrogenase (GLDH), the last enzyme of ASC biosynthesis, is an integral part of plant mitochondrial complex I (Millar *et al.*, 2003). Therefore, AtDjB1 could act by modulating ASC concentrations through an indirect effect on GLDH activity. The accumulation of H₂O₂, occurring as a consequence of an ASC decrease and the impairment of the electron transport chain, could be responsible for decreased thermo-tolerance in *atj1-1* plants (Zhou *et al.*, 2012).

The importance of ASC in the HS response has also been shown in tobacco BY-2 cells. During the HS-induced PCD, a decrease in ASC content occurs (Vacca *et al.*, 2004; Locato *et al.*, 2008). The decrease in ASC observed in tobacco BY-2 cells undergoing HS-induced PCD could be due, at least in part, to an impairment of GLDH (Valenti *et al.*, 2007). Moreover, the exposure of tobacco BY-2 cells to a long moderate HS determines an inhibition of cell growth and a decrease in cell viability. The exogenous addition of GL, the last

precursor of ASC, increases the ASC content and enhances basal thermo-tolerance (Sgobba *et al.*, 2015).

Ascorbate peroxidase

Ascorbate peroxidase plays a key role in heat shock response. *Arabidopsis thaliana* possess eight APX isoenzymes with different localizations: APX1, APX2 and APX6 are soluble cytosolic isoenzymes; APX3, APX4 and APX5 are microsomal isoenzymes, and sAPX and tAPX are localized in the chloroplast (Panchuk *et al.*, 2005).

Treatments inducing heat shock responses (1–2 h at 37 °C) increase the mRNA levels of all APX genes; however, the effects change qualitatively and quantitatively for different genes (Panchuk *et al.*, 2002; Koussevitzky *et al.*, 2008).

The APX1 and APX2 promoters contain an HSE, binding the HSF, characteristic for heat shock genes (Storozhenko *et al.*, 1998; Panchuk *et al.*, 2002; Fig. 1). APX3 and APX5 possess a similar sequence to HSE but are unable to bind HSF (Panchuk *et al.*, 2002). However, microsomal APXs are involved in the response to HS (Shi *et al.*, 2001).

In arabidopsis, APX1 is regulated by HS in an HSF-dependent mode, and APX1 may link the antioxidant pathway to HS-induced protection of general cellular functions (Storozhenko *et al.*, 1998). HS is also able to trigger APX2 gene expression at the mRNA level, and this correlates with the appearance of a new thermo-stable APX isozyme, APX^S. The contribution of HSF to the induction of APX2 is supported by the constitutive levels of APX2 mRNA and APX^S activity in HSF3 transgenic plants under non-stress conditions. APX2/APX^S is probably required to compensate for the HS-dependent decline of APX1 activity in the cytosol (Panchuk *et al.*, 2002). AtHSA2 is also a key regulator of APX2 (Nishizawa *et al.*, 2006). A tomato cytosolic APX, homologous to arabidopsis APX2, is also considerably upregulated in pollen during HS (Frank *et al.*, 2009).

In arabidopsis subjected to HS, a deficiency in APX2 may induce signals with a greater role than signals activated by a deficiency in APX1 (Suzuki *et al.*, 2013b). Consistently, in arabidopsis, HS affects root growth more severely in the double mutants *apx1/apx2* and in the mutant *apx2* than in wild-type or *apx1* plants. It has also been proposed that the signals related to APX2 deficiency play a key role in the regulation of plant productivity during HS (Suzuki *et al.*, 2013b).

On the other hand, cytosolic APX1 seems to play a key role in the acclimation of plants to a combination of drought and HS. During drought, ROS are thought to be produced in the chloroplast and peroxisomes (Asada *et al.*, 2006; Van Breusegem and Dat, 2006), whereas, during HS, ROS are mainly produced in chloroplasts and mitochondria (Suzuki and Mittler, 2006). In plants subjected to a combination of drought and HS, ROS can also be produced in the apoplast. In the drought and HS combination, APX1 could affect the diffusion of ROS from the various compartments to the nuclei. Consequently, during the depletion of APX1 caused by multiple stresses, more ROS could reach the nuclei and negatively affect cellular survival (Koussevitzky *et al.*, 2008) or modify the pattern of gene expression (Miller and Mittler, 2006).

Cytosolic APX6, an APX isoenzyme replacing APX1 in developing and germinating seeds, may play a role in the heat

tolerance of arabidopsis seeds (Chen *et al.*, 2014). Indeed seeds lacking APX6 accumulate high levels of ROS, exhibit increased oxidative damage, and show reduced germination under control conditions. These effects increase under HS. In addition, ripening APX6-deficient seeds exposed to HS display reduced germination vigour (Chen *et al.*, 2014).

The involvement of APX in HS responses has also been extensively studied in tobacco BY-2 cells. A short-term exposure (10 min) of tobacco BY-2 cells at 35 or 55 °C causes an increase or decrease, respectively, in total APX activity (Locato *et al.*, 2008). The increase in APX activity induced by exposure at 35 °C is only due to an increase in cytosolic isoenzymes. On the other hand, the short exposure at 55 °C determines the decline of cytosolic, plastidial and mitochondrial isoenzymes (Locato *et al.*, 2009). The changes in cytosolic APX activity are also accompanied by changes in gene expression (Locato *et al.*, 2008, 2009). However, the regulation of the cytosolic APX activity under HS inducing PCD (10 min at 55 °C) occurs more precociously than the regulation of its gene expression, and seems to be due to nitrosylation, ubiquitination and consequent proteasome-dependent degradation of the protein (Vacca *et al.*, 2004; de Pinto *et al.*, 2013).

In tobacco BY-2 cells, APX also plays a role in response to a moderate HS of different durations. Cytosolic APX activity and expression increase in the first 3 d of exposure at 35 °C. However, when the HS is prolonged, there is a decline in cytosolic APX, which is responsible for the consequent increase in oxidative damage leading to cell death (Sgobba *et al.*, 2015).

Thiol-containing proteins

Several thiol-containing proteins are involved in redox homeostasis and ROS scavenging, and their levels and activities are regulated by HS-dependent mechanisms.

Glutaredoxins (GRXs) are small ubiquitous proteins of the thioredoxin (TRX) family which mediate a reversible reduction in the disulphide bonds of their substrate proteins in the presence of GSH via a dithiol or monothiol mechanism (Rouhier *et al.*, 2006). GRXs could play a key role in oxidative stress responses since they are able to regulate the cellular redox state. Various studies have reported that GRXs are involved in the HS response. For instance, the expression of PvGRX5, a GRX of the fern *Pteris vittata*, in arabidopsis increases the thermo-tolerance (Sundaram and Rathinasabapathi, 2010). AtGRXS17, a monothiol GRX of arabidopsis, is involved in the control of temperature-dependent post-embryonic growth. AtGRXS17 expression is induced by elevated temperatures, and an alteration in AtGRXS17 expression leads to hypersensitivity to high temperatures. In *atgrxs17* mutants, HS induces higher levels of ROS and membrane damage than in the wild type.

The sensitivity of *atgrxs17* mutants to high temperatures is dependent on both the duration and degree of the temperature treatment. At 22 °C, *atgrxs17* mutants display minor growth defects, at 25 °C more severe phenotypes, and at 28 °C the growth of *atgrxs17* mutants is severely repressed. These different effects are linked to the different levels of accumulation of ROS in the growing tissues (Cheng *et al.*, 2011). Experiments conducted in tobacco and tomato expressing a green fluorescent protein (GFP)–AtGRXS17 fused protein show that this GRX is

localized in the cytoplasm and the nuclear envelope under optimal temperature, and migrates into the nucleus during HS. In addition, during HS, AtGRXS17-expressing tomato plants show an upregulation of HSF and HSPs, suggesting that the increase in the nuclear pool of AtGRXS17 increases the expression of the HS-related transcription factor (Wu *et al.*, 2012).

The expression of AtGRXS17 in tomato plants also reduces oxidative damage, and the increased thermo-tolerance is linked with increased CAT activity and reduced H₂O₂ accumulation. AtGRXS17 may even protect CAT activity directly, by physically interacting with this protein, or indirectly, by increasing HSP transcription (Wu *et al.*, 2012).

Peroxiredoxins (PRXs) are a family of thiol-based peroxidases that can be divided into three classes: 1Cys-PRX, typical 2Cys-PRX and atypical 2Cys-PRX (Rhee *et al.*, 2005).

Peroxiredoxins are able to enhance plant tolerance to HS (Kim *et al.*, 2010). In *A. thaliana*, induction of the *PER1* gene, which encodes 1Cys-PRX, is mediated by the interaction of FCA, a plant-specific RNA-binding protein of the autonomous flowering pathway, with ABA-INSENSITIVE 5 (Lee *et al.*, 2015). FCA could be involved in environmental adaptation responses through RNA processing and chromatin modification (Kumar *et al.*, 2011b). FCA could play a role in the stimulation of thermo-tolerance by enhancing antioxidant activity. The increase in antioxidant capacity during HS in arabidopsis plants overexpressing FCA could be explained, at least in part, by the transcriptional regulation of the *PER1* gene by FCA. Indeed, the *PER1* gene is induced by heat and FCA overexpression, whereas in *fca* mutants, the observed decrease in total antioxidant activity may be due to the failure of the heat induction of the *PER1* gene (Lee *et al.*, 2015).

A C-type NADPH-dependent thioredoxin reductase (NTRC) has been proposed as an efficient electron donor to 2Cys-PRX (Moon *et al.*, 2006; Pérez-Ruiz *et al.*, 2006). NTRC is induced in response to environmental stress (Serrato *et al.*, 2004). Overexpression of NTRC in arabidopsis determines enhanced thermo-resistance against heat shock. In contrast, in NTRC-deficient arabidopsis (*ntrc1*), growth inhibition and plant death occur during HS. NTRC can act as a disulphide reductase, with both foldase and holdase activities. These multiple functions are heat shock regulated. HS induces a switch from a low molecular weight to a high molecular weight complex of NTRC. This switch, which is reversible, increases the redox-dependent holdase chaperone function for NTRC, and provides enhanced thermo-tolerance (Chae *et al.*, 2013).

IMPROVEMENT IN PLANT THERMO-TOLERANCE

The involvement of antioxidant systems in the defence response activated by plants against HS has also been proved by studies demonstrating that many treatments able to increase the redox capacity of plants reduce heat-dependent damage by increasing thermo-tolerance.

Heat acclimation

Heat acclimation is obtained by priming, i.e. the exposure to a temperature lower than the threshold temperature or to a

short-term HS, and generally enhances antioxidant systems and improves thermo-tolerance, thus making plants more resistant to a subsequent higher HS.

In Orchardgrass, heat acclimation, achieved by a short treatment at 38/30 °C (day/night) and a subsequent recovery at 25/20 °C, increases thermo-tolerance to a subsequent prolonged treatment at 38/30 °C. Acclimated plants show a higher activity and expression of several ROS-scavenging genes than non-acclimated plants. The improvements in membrane stabilization and in the efficiency of water use and photosynthesis have also been attributed to the enhancement of these antioxidant enzymes (Zhao *et al.*, 2014). Similarly, heat-acclimated turfgrass species with higher ASC and GSH contents are able to overcome a subsequent HS of almost 15–20 °C higher than optimal temperatures by maintaining higher membrane thermo-stability, lower lipid peroxidation and lower chloroplast damage than non-acclimated plants (Xu *et al.*, 2006).

The reproductive phase is particularly sensitive to HS. Pre-anthesis heat acclimation alleviates the photosynthetic and oxidative damage caused by post-anthesis HS in wheat flag leaves. Again, the beneficial effects can in part be attributed to changes in the activity and expression of antioxidant enzymes. The upregulation of mitochondrial Mn-SOD and chloroplastic Cu/Zn-SOD is of particular relevance, which suggests an important role for the organelles in the thermo-tolerance of wheat (X Wang *et al.*, 2011).

The enhancement of antioxidant enzymes in specific organelles in conferring thermo-tolerance after heat acclimation is also important in wheat seedlings. Heat acclimation improves the redox homeostasis of seedlings exposed to a later high temperature stress, by increasing the activities of SOD in chloroplasts and of GR and POD in mitochondria. As a consequence, chloroplasts and mitochondria of acclimated plants show a lower superoxide radical production rate and lipid peroxidation than non-acclimated plants. The improvement in antioxidant capacities of chloroplasts may contribute to the higher photochemical efficiency of the acclimated seedlings (Wang *et al.*, 2014).

Exogenous treatments

Several studies report the capacity of different plant growth regulators to reduce the oxidative damage occurring when plants are exposed to high temperatures. Treatments with abscisic acid (ABA), 1-aminocyclopropane 1-carboxylic acid (ACC) and salicylic acid (SA) reduce HS-dependent ROS accumulation and lipid peroxidation in *Agrostis stolonifera* (Larkindale and Huang, 2004). The use of the arabidopsis insensitive mutants *abi1* and *etr1*, as well as the transgenic line expressing the bacterial gene *nahG*, coding for salicylate hydroxylase, further demonstrates a positive correlation between the impairment in ABA-, ethylene- or SA-dependent signalling pathways and HS susceptibility (Larkindale and Knight, 2002).

Interestingly, all these plant growth regulators are well known for their role in the activation of plant defence responses against different kinds of stress, including ABA in drought, ACC in flooding, and SA in plant–pathogen interactions (Grichko and Glick, 2001; Riera *et al.*, 2005; An and Mou, 2011). The defence mechanism activated by these regulators

seems to involve the enhancement of ROS-scavenging systems, such as an increase in the activities of the redox enzymes APX, POD, CAT and SOD, which improves the thermo-tolerance (Larkindale and Knight, 2002; Larkindale and Huang, 2004; Wang and Li, 2006; Ding *et al.*, 2010; Kumar *et al.*, 2012b). However, there are some contradictions in the literature, possibly depending on the varying sensitivities of different species to these molecules. In soybean leaves, treatments with 1-methyl-cyclopropene, an inhibitor of ethylene biosynthesis, reduced senescence symptoms caused by HS, by decreasing ROS and increasing CAT and SOD activities. This suggests that an inverse correlation exists between ethylene and HS resistance, at least in this species (Djanaguiraman *et al.*, 2011).

Different signalling molecules have also been tested for their capacity to increase thermo-tolerance in plants. Calcium pretreatment reduced H₂O₂ and superoxide anion levels by increasing the activities of APX and SOD, respectively, in tomato plants exposed to HS (Ding *et al.*, 2012). The involvement of calcium in the activation of defence responses against HS has also been demonstrated by the use of Ca²⁺ chelators and Ca²⁺ channel blockers that are able to inhibit the Ca²⁺ protective effect against HS (Larkindale and Knight, 2002; Ding *et al.*, 2012).

Pharmacological approaches have been used to show that nitric oxide (NO) acts as a Ca²⁺ upstream signal in HS responses (Wang *et al.*, 2014). Treatments with sodium nitroprusside, an NO donor, demonstrate that NO is able to reduce lipid peroxidation in *Chrysanthemum morifolium* plants exposed to HS, by the upregulation of the antioxidant enzymes SOD, CAT, POX and APX (Yang *et al.*, 2011). NO is also involved in the signalling pathway leading to HS-dependent HSP expression, acting upstream of H₂O₂, a key molecule of the HS response (Wang *et al.*, 2014). As previously mentioned, ROS, and in particular H₂O₂, are involved in the activation of HSP expression. In arabidopsis, ROS scavengers inhibit HSP expression, and HSP expression is also strongly reduced in *rho*h mutants. In both cases, the impairment of H₂O₂ production decreases plant thermo-tolerance (Larkindale *et al.*, 2005; Volvok *et al.*, 2006). In line with this, pre-treatments with H₂O₂ protect thylakoidal membranes of *Cucumis sativum* from lipid peroxidation occurring during HS (Gao *et al.*, 2010). This protective mechanism seems to depend on the acclimation induced by H₂O₂, which increases the activities of chloroplastic SOD and ASC–GSH cycle enzymes, thus preparing these organelles to cope with the following HS (Gao *et al.*, 2010).

Treatment with ASC may also reduce H₂O₂ overaccumulation during HS, leading to a reinforcement of the antioxidant systems in mungbean plants (Kumar *et al.*, 2011a). ASC protects plants by stimulating the increase of proline in the tissue (Kumar *et al.*, 2011a). Proline is an osmoprotectant that protects enzymes from inactivation by HS. Treatments with osmoprotectants such as proline, glycine betaine and trehalose have also been shown to increase thermo-tolerance in different plants, by promoting membrane stability and reducing oxidative stress in the HS-exposed tissues (Rasheed *et al.*, 2011; Kaushal *et al.*, 2011; Kumar *et al.*, 2012b).

However, the ability of proline to protect from HS may vary according to the developmental phases in which the stress is imposed. For instance, in arabidopsis seedlings ectopically

expressing the D(1)-pyrroline-5-carboxylate synthetase 1 gene under the control of a heat shock promoter, proline accumulation under HS decreases the thermo-tolerance. The inhibition of thermo-tolerance in these conditions seems to be due to an increased ROS production via the inhibition of ABA and ethylene biosynthesis (Lv *et al.*, 2011).

Polyamines are also metabolites involved in plant responses during environmental stresses. They seem to act as ROS scavengers and promoters of antioxidant defences (Gill and Tuteja, 2010). Spermidine treatment increases the viability of tobacco cells under severe oxidative stress conditions and during lethal HS (Marsoni *et al.*, 2010; Vannini *et al.*, 2012). Putrescine application to *Triticum aestivum* plants exposed to HS reduces lipid peroxidation by increasing the redox shield in grains, roots and shoots (Asthir *et al.*, 2012).

p-Hydroxybenzoic acid has also been reported to induce thermo-tolerance in cucumber plants by increasing redox-dependent defences (Zhang *et al.*, 2012).

PERSPECTIVES AND CONCLUSIONS

An increase in average temperatures and further heatwaves and other extreme events will have a strong impact on food production in the future. A better understanding of the eco-physiological and molecular mechanisms that enable plants to overcome these climate changes is thus fundamental in order to minimize the negative impact on plant yield. The use of model systems, such as cultured cells or arabidopsis plants, and crops has led to the identification of redox systems among the metabolic pathways activated in response to HS (Fig. 1) and more generally to climate change.

However, it is clear that in each species and, within species, different genotypes react to HS by activating the specific redox defences in different ways, thus showing a diverse ability to react to temperature increases. We thus need to improve our knowledge of the capacity of specific crops to activate redox defences in order to overcome climate change, by performing experimental studies in which the putative changes are simulated as realistically as possible. Further studies are also required in order to identify exogenous treatments that are able to alleviate the oxidative damage due to HS in specific species or genotypes.

The complexity of these studies has increased due to the fact that in a naturally changing environment more parameters have to be taken into consideration. For example, heatwaves can be accompanied by either humid or dry conditions, thus with substantially different impacts on plant physiology. Climatic variability also strongly depends on regional differences, and in different regions different plants are more suitable for cultivation.

A better evaluation of agronomic biodiversity is also key in enabling researchers to identify new strategies to overcome climate changes and in assisting decision-makers to plan for an uncertain future with new choices and options.

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