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Pollen Development at High Temperature: From Acclimation to Collapse

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The seeds and fruits derived from the sexual reproduction of flowering plants constitute the major part of the human diet. Our capacity to generate sufficient crop yield is increasingly compromised by human population expansion, competition for land use, biodiversity loss, and global climate change. Hot days and heat waves are predicted to increase in frequency and intensity in many temperate regions in the coming decades as a consequence of global warming (Pachauri et al., 2014). Exposure to high temperature episodes often coincides with the reproductive phase of the plant life cycle. As pollen development and functioning are among the most heat-sensitive processes that impact upon plant fertility, it is crucial to understand the mechanisms and processes underlying heat-related male sterility in order to maintain food security.

Sexual plant reproduction in flowering plants involves two central processes: meiosis, which rearranges the genes and reduces the number of chromosomes; and fertilization, which restores the diploid chromosome number. In between these two, haploid spores develop into multicellular gametophytes, which produce the male or female gametes. Development of the male gametophyte (pollen) has been shown to be sensitive to environmental fluctuations and suboptimal conditions, thereby limiting sexual reproduction (Iwahori, 1965; Schoper et al., 1987; Peet et al., 1998; Dupuis and Dumas, 1990; Ahmed et al., 1992; Kim et al., 2001). Pollen is formed inside the anther locules from diploid pollen mother cells that undergo meiosis to give rise to a tetrad of four haploid microspores surrounding by locular fluid. After release from the tetrad, the free microspores enlarge and divide asymmetrically (pollen mitosis I) to form a larger vegetative cell and a smaller generative cell. The generative cell is then engulfed by the vegetative cell and undergoes a second mitosis (pollen mitosis II), either before pollen is released from the anther or during pollen tube growth, to form two sperm cells (McCormick, 2004). During the differentiation of the pollen mother cells, the innermost anther wall layer forms the tapetum (Goldberg et al., 1993). This tissue is metabolically active, especially at early microspore stage, providing the developing microspores with carbohydrates, nutrients, enzymes, and compounds required for the synthesis of the outer pollen wall (exine). Development of the tapetum is tightly coordinated with microspore development and its degeneration begins shortly after microspores are acclimated and acquired thermotolerance.

Heat acclimation is manifest in the response of anthers and pollen at transcriptome, proteome, and metabolome levels towards maintaining physiological homeostasis.

There is experimental evidence for a role of both the HSR (i.e. HSFA2) and UPR (IRE1) in heat acclimation of anthers and pollen.

Differentiating between heat acclimation responses and injury effects is difficult, but many gene expression responses might be adaptive based on the presence of heat shock elements in the promoters of a significant proportion of heat-affected genes.
released from the tetrad. The correct functioning of the tapetum and its timely degradation are essential for pollen development (Parish and Li, 2010).

One of the abiotic conditions with the most impact on pollen development is high temperature. Plants are sessile organisms, inevitably exposed to ambient temperatures throughout their life cycle and can overheat relative to their environment due to direct absorbance of solar radiation. High temperatures can change the structure of biomolecules, such as DNA, RNA, proteins, and lipids, which in turn can affect basic metabolic processes like translation, photosynthesis, respiration, and redox regulation (Bokszczanin et al., 2013). At organismal level, this leads to disturbance of growth and development, with harmful effects often manifesting themselves only after transition to the reproductive phase. Pollen heat sensitivity is a conserved feature among diverse plant species, including monocots and dicots, and occurs under various high temperature regimes, for example, short heat shock or prolonged growth under mildly elevated day and/or night temperatures (Mesihovic et al., 2016). The sensitivity varies over the course of pollen development, with later stages, after pollen mitosis, being relatively heat tolerant. Medium high-temperature stress may disturb meiosis, but peak sensitivity occurs from meiosis to pollen mitosis I (i.e. at microspore stage), which is the focus of this work.

The development of pollen after exposure to heat stress at the microspore stage has not been described in detail but includes abortion of microspores as well as failure at later stages, leading to a reduction in the number of pollen grains at anthesis and the proportion of mature pollen grains that is viable and able to germinate (Mesihovic et al., 2016). The fact that high-temperature stress may result in a mixed population of both dead and perfectly viable pollen within the same anther locule has recently been explained with a model where initially small differences between microspores in metabolic performance or developmental progression are amplified by competition for nutrients in the locular fluid (Carrizo García et al., 2017). Notably, pollen injury is often accompanied by aberrations in tapetum development (hypertrophy) or morphology and alterations in the timing of tapetum degeneration (Iwahori, 1965; Saini et al., 1984; Ahmed et al., 1992; Kim et al., 2001; Suzuki et al., 2001; Abiko et al., 2005; Oshino et al., 2007; Djanaguiraman et al., 2013; Harsant et al., 2013).

While elevated temperatures may elicit acclimation responses that permit pollen development under restricted heat stress conditions, physiological injury leading to failure of pollen development and functioning occurs at higher temperature stress. The balance between acclimation and collapse thus depends on the heat regime experienced as well as the levels of basal and acquired thermotolerance. Here, we discuss the current understanding of heat acclimation responses and heat injury during microspore development. Thermotolerance mechanisms of mature and germinating pollen as well as vegetative tissues have been discussed elsewhere (Mittler et al., 2012; Bokszczanin et al., 2013; Tunc-Ozdemir et al., 2013; Burke and Chen, 2015).

ACCLIMATION

Data accumulated over the past few decades indicate that pollen and the surrounding anther tissues respond to an increase in temperature at the transcriptome, proteome, and metabolome levels, similar to other plant cell types. Several heat responses that have been characterized as adaptive in vegetative tissues were also found to occur in these reproductive tissues. In recent years, experimental evidence for a role of these responses in maintaining physiological homeostasis during pollen development at high temperature has emerged.

Protein Homeostasis in the Cytosol: The Heat Shock Response

One of the main damaging effects of high temperature results from changes in protein structure. This may interfere with protein function, and if more hydrophobic regions are exposed, proteins can aggregate and become cytotoxic. Failure to prevent the accumulation and aggregation of misfolded proteins may eventually lead to cell death. To counteract these effects, the expression of heat shock protein (HSP) chaperones is induced at high temperature in a process known as the heat shock response (HSR). HSPs accumulate in the cytoplasm and organelles to stabilize, resolubilize, and refold proteins (Vierling, 1991; Hartl et al., 2011). Recently, it was demonstrated that small HSPs are important for heat tolerance in Arabidopsis. Two of the most abundant classes of sHSPs (C1 and CII) were shown to interact with and to protect an overlapping set of heat sensitive proteins involved in translation initiation (eIF4A) and elongation (eEF1B), together with HSP101 (McLoughlin et al., 2016). Underlying the HSR is a network of heat stress transcription factors (HSFs) that bind to a palindromic DNA sequence, the heat shock element, to induce the expression of heat-responsive genes (Scharf et al., 2012; Ohama et al., 2017).

High-temperature induction of HSF and HSP genes in developing anthers, microspores, and pollen has been reported for different species (Frank et al., 2009; Giorno et al., 2010; Bita et al., 2011; Zhang et al., 2014; Li et al., 2015). Furthermore, proteins of different types of HSPs (i.e. belonging to diverse families, like sHSP, HSP70, HSP90, and HSP100) accumulate in developing anthers and pollen grains after a short period of high-temperature stress (Frova et al., 1989, 1991; Jagadish et al., 2010; Chaturvedi et al., 2015), which points to a capacity for both tissues to activate “classical” thermotolerance mechanisms. A recent study in tomato (Solanum lycopersicum) has shown that induction of HSR protects microspores from high temperature (Fragkostefanakis et al., 2016b). One of the main HSPs
regulating the HSR is HSFA2, which forms a “super-activator complex” with HSFA1 proteins (Scharf et al., 2012). Knockdown of HSFA2 resulted in increased sensitivity of developing tomato pollen to a short period of high temperature (Fragkostefanakis et al., 2016b). The effect of reduced levels of HSPs on pollen thermotolerance has not been tested, but members of the Bcl-2-associated anathogene (BAG) family have been shown to be involved in pollen thermotolerance (Doukhanina et al., 2006). BAG proteins are co-chaperones involved in recruiting HSPs to client proteins; they are expressed in developing tomato pollen under heat stress, and their expression may be under control of HSFA2 (Frank et al., 2009; Fragkostefanakis et al., 2015).

Taken together, various HSFs and HSPs are induced in anthers and microspores by high temperature, and genetic studies confirm that they play an active role in protecting developing pollen against heat stress.

Protein Homeostasis in the ER: The Unfolded Protein Response

Upon exposure to elevated temperatures, a second set of genes, including ones that encode chaperones, is induced to protect cells against toxic levels of unfolded proteins in the endoplasmic reticulum (ER) (Howell, 2013). Protein folding is an essential function of the ER, involving the guidance of polypeptides through several modification steps to reach their desired conformation. The folded protein may remain in the endomembrane system, be targeted to the cell membrane, or be secreted. A polypeptide that is not glycosylated is folded by the luminal binding protein (BiP)-Hsp70/DnaJ and Hsp90 chaperone machineries, which are favored by the highly oxidizing environment of the ER. Protein disulfide isomerases facilitate formation of disulfide bonds, which confer structural stability to the protein. Alternatively, polypeptides are glycosylated at ER entry through N-linked glycosylation, followed by disulfide bond formation. N-linked glycosylation provides the sugar molecules that form the key ligand for the lectin-like chaperones calreticulin and calnexin. An excess of misfolded proteins in the ER leads to enhanced expression of a number of the components of the ER protein folding machinery, in a process known as the unfolded protein response (UPR). Two pathways are involved in eliciting this response; one is dependent on release of bZIP28/bZIP17 from the ER membrane and the other on alternative splicing of bZIP60 by IRE1 (Deng et al., 2011; Srivastava et al., 2013). However, bZIP60 is able to heterodimerize with bZIP28 and bZIP17, indicating that the two arms of the UPR signaling pathways merge. Several of the UPR genes are induced upon heat and seedlings of a bZIP28 knockout mutant were shown to be sensitive to high temperatures, suggesting an essential role of the UPR in general heat stress response and thermotolerance (Fragkostefanakis et al., 2016a).

The main components of the ER protein folding machinery, such as calreticulin, calnexin, and BiP, are present throughout microspore and pollen development (Honys and Twell, 2004; Sheoran et al., 2006; Chaturvedi et al., 2013). Moreover, the UPR is essential for regular pollen development, given that several mutants in the pathway have male gametophyte defective or lethal phenotypes (Fragkostefanakis et al., 2016a). During or shortly after a heat shock or long-term exposure to mild heat, the UPR is up-regulated in male reproductive tissues at transcript and protein level (Frank et al., 2009; Biti et al., 2011; Chaturvedi et al., 2015; Li et al., 2015; Fragkostefanakis et al., 2016b), suggesting the UPR has a function in acclimation of developing pollen to heat. Experimental support for this hypothesis was recently delivered, through the analysis of an ire1a ire1b double knockout mutant, which inactivates the RNA-splicing arm of the UPR signaling pathway. The mutant was found to be fertile at room temperature, but male sterile at slightly elevated temperatures, showing reduced viability of mature pollen and altered pollen coat composition (Deng et al., 2016). Interestingly, conditional male sterility in the mutant was a sporophytic trait and when the double mutant was grown at elevated temperature, defects appeared in the structure of the tapetum. It is conceivable that increased protein folding capacity of the tapetal ER is essential for functioning at high temperature, given the high secretory activity of the tapetum.

Reactive Oxygen Species Scavenging

Part of the cellular damage by high temperature is ascribed to accumulation of reactive oxygen species (ROS). ROS are produced during aerobic metabolism, deriving from different cellular compartments, including mitochondria, chloroplasts, peroxisomes, and the apoplast (Mittler et al., 2004). In addition to being toxic metabolic by-products, ROS also act as signaling molecules mediating stress responses (Baxter et al., 2014). Cells possess extensive ROS scavenging and detoxification machinery, which consists of enzymes such as catalase, ascorbate peroxidase (APX), and superoxide dismutase, as well as antioxidant substances like ascorbic acid and flavonoids (Mittler et al., 2004). Many of the ROS scavenging-related genes are responsive to ROS levels, which results in a regulated balance between production and scavenging under steady state conditions. Exposure to high temperature can disturb this balance, and a number of studies in vegetative tissues and cell types show that heat rapidly leads to accumulation of ROS, resulting in a secondary, oxidative stress. To cope with the excess amount of ROS upon heat, the expression of ROS scavengers and levels of antioxidants are rapidly up-regulated by heat (Driedonks et al., 2015). The fact that increased antioxidative activity increases vegetative tissue/organ thermotolerance in different plant species indicates...
that this response is adaptive (Gupta et al., 1993; Singh and Grover, 2008; Chen et al., 2013).

ROS play an important role in the formation of viable pollen. The programmed cell death of tapetal cells during microspore development involves ROS action. ROS levels in anthers were shown to peak during tapetum degeneration and at pollen maturity in Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa; Hu et al., 2011; Xie et al., 2014; Yi et al., 2016). Accordingly, a proteomic study detected catalase (CAT3) and glutathione reductase (GRI) in developing pollen (Chaturvedi et al., 2013). Pollen and tapetum cells have also been shown to accumulate large numbers of mitochondria and show high rates of respiration (Lee and Warmke, 1979; Selinski and Scheibe, 2014). Under high temperatures, this might be expected to cause a dramatic increase in ROS. Indeed, long-term mild heat has been shown to increase ROS levels in sorghum pollen (Djanaguiraman et al., 2014). In rice, heat induced the expression of several ROS-related genes in florets (Zhang et al., 2012; Zhang et al., 2014), and GST and APX genes were upregulated in developing tomato anthers and pollen (Frank et al., 2009; Bita et al., 2011; Fragkostefanakis et al., 2016b). The up-regulation of GST and APX genes in response to heat is reflected by increased levels of the corresponding proteins (Chaturvedi et al., 2015). These data point toward the accumulation of ROS in anthers and pollen upon heat, although APX genes are also responsive to heat in a ROS-independent manner, due to the presence of heat shock elements in their promoters (Driedonks et al., 2015). There is no direct evidence that enhanced ROS scavenging activity supports pollen development under high temperature conditions. However, excessive ROS at the late microspore stage in rice mads3 led to tapetal dysfunction and pollen abortion (Hu et al., 2011; Luo et al., 2013). Conversely, Arabidopsis and rice mutants with reduced amounts of ROS fail to activate the timely onset of tapetum programmed cell death, leading to pollen failure (Xie et al., 2014; Yi et al., 2016). Thus, the tight regulation of ROS content is essential for the production of viable pollen, making it likely that increased scavenging activity contributes to heat acclimation. Further support for this hypothesis is provided by experiments in wheat and rice, which suggest increased pollen viability upon heat treatment after exogenous application of antioxidants (Kumar et al., 2014; Fahad et al., 2016).

Distinguishing Acclimation from Injury

Untargeted “omics” studies have shown that high temperature elicits a suite of transcriptomic, proteomic, and metabolomic changes in developing anthers and pollen, many of which are not directly associated with the well-characterized heat acclimation responses described above (transcriptomic: Abiko et al., 2005; Oshino et al., 2007; Endo et al., 2009; Frank et al., 2009; Bita et al., 2011; Zhang et al., 2012; Min et al., 2014; Zhang et al., 2014; Li et al., 2015; Fragkostefanakis et al., 2016b; proteomic: Jagadish et al., 2010; Chaturvedi et al., 2015; metabolomic: Li et al., 2015; Fragkostefanakis et al., 2016b). Although some of these changes may be passive consequences of heat injury and play no role in acclimation, it seems likely that others have adaptive value.

Functional data would allow differentiation of the two categories. For example, transcripts related to ethylene and abscisic acid (ABA) signaling accumulate in developing tomato pollen and in rice florets after a short heat episode (Frank et al., 2009; Bita et al., 2011; Zhang et al., 2012; Zhang et al., 2014). Pollen of an ethylene insensitive tomato mutant was found to be more sensitive to chronic mild heat stress, while chemical induction of ethylene production prior to a short heat stress treatment improved pollen thermotolerance and application of an ethylene inhibitor reduced it (Firon et al., 2012). Together, these results make a strong case for ethylene as an acclimation factor. For ABA, the relationship is less clear. In rice florets exposed to reoccurring heat stress for 5 d, ABA concentrations were higher than under control conditions (Tang et al., 2008). Although it has been shown that ABA contributes to heat acclimation of vegetative organs (Larkindale and Huang 2005), this has not been shown for reproductive tissue. In fact, ABA accumulation seems to negatively affect pollen development (Parish et al., 2012). Several other studies have noted the down-regulation of ribosomal proteins and transcripts (Abiko et al., 2005; Oshino et al., 2007; Jagadish et al., 2010; Bita et al., 2011). It has been suggested that a reduction in protein synthesis rate with heat might act to alleviate the stress caused by misfolded proteins in the cytoplasm and ER (Ruberti and Brandizzi, 2014), but this has not been tested.

As an alternative approach to identifying adaptive heat responses, dependency of heat-induced changes on signaling pathways associated with acclimation might be taken as an indication. HSF transcription factors are the main regulators of the HSR, acting upstream of many HSP responses (Scharf et al., 2012). Fragkostefanakis et al. (2016b) found that most heat-responsive genes in anthers carried the HSF targeted stream of many HSP responses (Scharf et al., 2012). Despite this, HSF42 activity, and interestingly, the same was true for some metabolomic changes, such as accumulation of the nonprotein amino acid GABA, which is thought to have a protective role under oxidative stress (Kinnnersley and Turano, 2000). Thus, a significant number of responses may contribute to heat acclimation, even though their specific roles are largely unknown, requiring further research as discussed in the “Perspective” section.

COLLAPSE

Despite the acclimation responses, pollen development is adversely affected at temperatures at which most other plant tissues and processes show limited
effects. In general, developing pollen and ovules experience high temperature simultaneously, but the former is more sensitive to heat (Gross and Kigel, 1994; Peet et al., 1998; Oshino et al., 2007). The sequence of events that lead to pollen failure under high temperature remains to be determined, and the tissue(s) that are primarily affected have not been unequivocally established; in principle, high temperature might directly affect developing microspores, the supporting sporophytic tissues, or both.

Carbon Starvation

The importance of carbohydrate partitioning under stress conditions has been widely documented (Ruan et al., 2010). However, as photosynthesis and overall plant growth are not significantly affected by relatively short or mild high temperature regimes that impair pollen development (Sharkey, 2005; Mathur et al., 2014), carbohydrate supply at source tissues is not likely to be limiting in this case. On the other hand, there are indications that carbohydrate metabolism and unloading in the anther and pollen play a role. Starch and soluble sugar levels are finely regulated during pollen development. Under normal conditions, Suc concentrations remain fairly stable, but starch accumulates to reach a peak after pollen mitosis I, often followed by gradual breakdown into soluble sugars at anthesis (Facini et al., 2006; Pressman et al., 2012). Suc and hexoses serve as energy sources for development and pollen germination and are also thought to act as osmolytes (Pressman et al., 2012). Under mild heat stress, Suc content is reduced in young microspores and starch buildup in binucleate pollen is lower. Consequently, soluble sugar content is also lower at anthesis (Pressman et al., 2002; Firon et al., 2006; Sato et al., 2006; Jain et al., 2007). A relationship between carbohydrate content and pollen viability is supported by findings that more tolerant genotypes were better able to maintain pollen starch and sugar levels than sensitive genotypes (Pressman et al., 2002; Firon et al., 2006).

Suc is the main form of photosynthetic assimilate exported from source tissue. As with the microspores, its unloading and uptake in symplastically isolated cells depends largely on the activity of cell wall acid invertase (CWIN; De Storme and Geelen, 2014). CWIN activity is maintained at high levels in tapetal cells and developing pollen (Goetz et al., 2001; Pressman et al., 2012), and in several studies, heat stress was shown to lower CWIN transcript levels and enzyme activity in developing microspores and anthers (Pressman et al., 2006; Sato et al., 2006; Jain et al., 2007; Kaur et al., 2015). In accordance with the potential role of CWIN, a heat tolerant rice variety had relatively high CWIN expression under mild heat (Li et al., 2013), while the silencing of CWIN genes in tomato and tobacco (Nicotiana tabacum) significantly reduced pollen viability (Goetz et al., 2001; Li et al., 2015; Zanor et al., 2009). Similarly, the expression of vacuolar invertase was down-regulated by continuous mild heat in meiotic and microspore stage anthers (Sato et al., 2006), and it has been shown that silencing of vacuolar invertase in reproductive organs can lead to reduced pollen viability (Wang and Ruan, 2016).

Thus, it could be speculated that carbohydrate depletion in developing pollen may be the result of decreased hexose supply by the tapetum or reduced uptake by the pollen at high temperatures. Developing pollen and tapetum cells seem to have unusually high energy demands as indicated by their high numbers of mitochondria (Lee and Warmke, 1979; Selinski and Scheibe, 2014); depletion in carbohydrate reserves might thus affect tapetum and pollen more than other cells. However, experimental proof for the carbohydrate deficiency hypothesis is still lacking: Studies have not yet clarified whether reduced carbohydrate levels at elevated temperatures cause pollen abortion or merely reflect the consequence of reduced pollen functioning.

Other Types of Heat Injury

Other putative physiological injuries have been suggested to play an intermediary role in causing pollen sterility in response to heat. Increased protein misfolding and ROS accumulation are described above and there are reasons to believe that these might reach levels beyond protection capacity faster in anthers and pollen than elsewhere in the plant. Heat leads to irregular ER structure in tapetal cells and in developing pollen (Suzuki et al., 2001; Oshino et al., 2007), which might point to an overload of the UPR. Classical sets of HSPs were found to be hardly induced in mature or germinating pollen upon heat shock (Müller and Rieu, 2016). At earlier stages of pollen development, HSFs and HSPs are induced by heat, but to a lesser extent than in vegetative tissue (Frova et al., 1989; Gagliardi et al., 1995; Volkov et al., 2006; Fraggostefanakis et al., 2016b), which may contribute to higher heat stress sensitivity of developing pollen. It has been suggested that microspores compete for nutrients in the anther locule, especially under suboptimal growth conditions (Carrizzo García et al., 2017). While in vegetative plant tissues, HSPs accumulate to become among the most abundant proteins upon high-temperature exposure (Vierling, 1991), resource scarcity might prevent microspores from investing heavily in protective measures. Furthermore, the relatively high numbers of mitochondria in developing pollen and tapetal cells might produce disproportionate levels of reactive oxygen species in response to heat, as observed in sorghum pollen (Djanaguiraman et al., 2014). Interestingly, high-temperature defects in developing pollen and tapetum share some similarities with those observed in plants showing cytoplastic male sterility, a phenomenon thought to be linked to mitochondrial dysfunction and ROS activity (Müller and Rieu, 2016).
Other molecules are also affected by high temperature. Pro acts as a compatible solute in osmoprotection and accumulates in response to different abiotic stresses (Krasensky and Jonak, 2012). Pro is a key factor for pollen viability (Lansac et al., 1996), and in several species, levels decrease in pollen under high temperature regimes that disturb pollen development (Mutters et al., 1989; Tang et al., 2008). Interestingly, the expression of Pro transporter 1 mRNA was reduced under these conditions. This might suggest that Pro is incorporated into pollen grains from the locular fluid rather than being produced by pollen itself and may be reduced at high temperature (Sato et al., 2006). Lipids are also affected by heat. The type of lipids in cellular membranes and their saturation level are important determinants of membrane fluidity and functioning. In barley (Hordeum vulgare), long-term growth at mildly elevated temperature led to alterations in phospholipid saturation in pollen (Prasad and Djanaguiraman, 2011). This in turn might make the membranes more susceptible to ROS damage. Finally, the levels of two phytohormones important for pollen development seem to be affected by high temperature. Auxin levels in anthers are reduced by high temperature in Arabidopsis, rice,

![Diagram](image.png)

**Figure 1.** Effects of heat related to acclimation or collapse of developing microspores. Genes involved are indicated between brackets. Resource limitation is hypothesized to impose a trade-off between acclimation and development (see “Perspective” section).
and barley, in contrast to the response of vegetative tissues (Tang et al., 2008; Sakata et al., 2010). Interestingly, exogenous application of auxin improved tolerance of developing pollen to continuous mild heat stress in barley (Sakata et al., 2010). Tang et al. (2008) found that bioactive gibberellin (GA) content also decreased in mature anthers under heat stress in rice. Furthermore, in an independent study of rice, the set of tapetum-specific genes that were downregulated under continuous mild heat stress was enriched for GA-responsive genes (Endo et al., 2009). The GA deficiency/insensitivity phenotype is notably similar to the heat phenotype, sharing features such as abnormal tapetal development, delayed or inhibited programmed cell death, and developmental arrest at microspore stage (Jacobsen and Olszewski, 1991; Aya et al., 2009). Moreover, one class of GA target genes expressed in the tapetum are the invertases described above (Proels et al., 2006). Putatively related to changes in GA signal, it was recently found that mild heat reduces the expression of B-class MADS box genes and that partial down-regulation of these genes mimics the heat phenotype, including reduced pollen viability (Müller et al., 2016).

**PERSPECTIVE**

It is clear that developing anthers and pollen have the capacity for acclimation to high temperature, and further research may reveal many more heat responses to be adaptive than currently thought. Collectively, these responses permit the production of viable pollen at certain levels of heat stress (Fig. 1). What remains unclear is how mild heat stress results in defective pollen development and why developing microspores and pollen are heat sensitive compared to other plant tissues (Box 1; Fig. 1). Does the latter response arise as the lesser of two evils, i.e. does inherent energy or nutrient limitation in the anther locule prevent microspores deviating from a fixed developmental path toward strong acclimation?

Based on the fundamental influence of heat on all molecules, it is likely that pollen failure is not the result of a single primary effect, propagated as a linear series of consequences, but of a combination of effects that behave synergistically. The finding that heat tolerance in vegetative tissues can be improved by targeting different physiological processes supports this hypothesis (Singh and Grover, 2008). But how can we proceed to identify what injuries are causally linked to the pollen phenotype? First, it will be essential to generate more specific hypotheses by applying analyses with increased temporal and spatial resolution. The fact that both tapetum and microspores/pollen constitute only part of the anther reduces the resolving power of many studies that sample whole anthers or even flowers. Furthermore, the rapid and inherently asynchronous development of cells of interest in the anther restricts temporal resolution (Carrizo García et al., 2017). Promising new expression profiling methods include various types of immunopurification-based transcript capturing in combination with a cell- or stage-specific activation (Bailey-Serres, 2013). Similarly, recently developed techniques allow for cell-specific metabolome analyses (Fessenden, 2016). Further
**OUTSTANDING QUESTIONS**

- Why is developing pollen more heat sensitive than other plant tissues?
- Given the resource competition hypothesis, is it possible to increase high temperature protection of pollen without disturbing pollen development?
- What is the effect of heat on the tapetum and the vegetative and/or generative cells and which cell type limits pollen thermotolerance?
- Do ROS accumulate in tapetum and developing pollen under high temperature and do they play a role in heat injury?
- Are carbohydrate availability and metabolism limiting factors for pollen development at high temperature?
- How conserved is the role of auxin deficiency in heat-induced pollen failure? Do changes in gibberellin or abscisic acid accumulation or signalling contribute?
- Is reduced anther identity a cause for heat-induced pollen failure and what is its relation with other injuries?
- What is the genetic and physiological basis of natural variation in pollen thermotolerance between accessions and species? Is it a monogenic/multigenic or highly polygenic trait?
- Is the sensitivity of developing pollen to high temperature an adaptive trait?

opportunities exist in examining the similarities between the effects of heat and other abiotic stresses, such as cold, drought, and high salinity on male gametophyte development (De Storme and Geelen, 2014; Das et al., 2015; Sharma and Nayyar, 2016). To test the (new) hypotheses generated, the phenotypic effect of mimicking the injury could be suggestive, as applied to invertases, B-class MADS box genes, and GA signaling. However, complementation studies, where specific defects are counteracted using pharmacological or genetic approaches, are necessary to establish cause-and-effect relationships. The auxin rescue experiment in barley by Sakata et al. (2010) provides an instructive example, and it will be interesting to see whether their findings will extend to other species. Applying this principle, it would be logical to determine whether, for example, increased levels of invertases in the developing tapetum or pollen are beneficial for thermotolerance. Studies into the genetic basis of natural and artificial variation have also been highly effective in dissecting other plant-environment interactions, so this strategy holds promise for identifying major determinants of pollen thermotolerance. It has been suggested that pollen heat sensitivity could be an adaptation itself, preventing investment in reproduction under adverse conditions (Müller and Rieu, 2016). If true, one could expect less heat sensitivity in dioecious species and, counterintuitively, higher pollen thermotolerance in species or genotypes originating from moderate temperature habitats.

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**LITERATURE CITED**


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