Plant, Cell & Environment

Plant, Cell and Environment (2013) doi: 10.1111/pce.12142

The impact of environmental stress on male reproductive development in plants: biological processes and molecular mechanisms

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ABSTRACT

In plants, male reproductive development is extremely sensitive to adverse climatic environments and (a)biotic stress. Upon exposure to stress, male gametophytic organs often show morphological, structural and metabolic alterations that typically lead to meiotic defects or premature spore abortion and male reproductive sterility. Depending on the type of stress involved (e.g. heat, cold, drought) and the duration of stress exposure, the underlying cellular defect is highly variable and either involves cytoskeletal alterations, tapetal irregularities, altered sugar utilization, aberrations in auxin metabolism, accumulation of reactive oxygen species (ROS; oxidative stress) or the ectopic induction of programmed cell death (PCD). In this review, we present the critically stress-sensitive stages of male sporogenesis (meiosis) and male gametogenesis (microspore development), and discuss the corresponding biological processes involved and the resulting alterations in male reproduction. In addition, this review also provides insights into the molecular and/or hormonal regulation of the environmental stress sensitivity of male reproduction and outlines putative interaction(s) between the different processes involved.

Key-words: (a)biotic stress; ABA; invertase; male gametogenesis; male sterility; meiosis; sugar metabolism; tapetum.

Abbreviations: ABA, abscisic acid; ATP, adenosine triphosphate; CO, crossover; DSB, double-strand break; ERAD, ER-associated degradation; GA, gibberellic acid; IAA, indole acetic acid; INV, invertase; MHR, meiotic homologous recombination; MI, meiosis I; MII, meiosis II; PCD, programmed cell death; PMC, pollen mother cell; PMI, pollen mitosis I; PMII, pollen mitosis II; RER, rough endoplasmic reticulum; RMA, radial microtubule array; ROS, reactive oxygen species; SDR, second division restitution; TGMS, thermosensitive genic male sterile; UPR, unfolded protein response.

INTRODUCTION

In plants, the male reproductive programme, including both microsporogenesis and microgametogenesis, is extremely sensitive to adverse environmental conditions. Indeed, for several plant species, abiotic stresses, such as low and high temperatures, salt stress, osmotic shock and water deficit, specifically affect male gamete development, typically resulting in high levels of microspore abortion and associated induction of male sterility (Downes & Marshall 1971; Thakur *et al*. 2010). In a similar way, biotic stress conditions may also affect male reproductive development, thereby conferring substantial reductions in male fertility. Although occasionally used in hybrid breeding (Luo, Qiu & Li 1992; Rerkasem & Jamjod 1997; Li, Yang & Zhu 2007), stress-induced male sterility generally has a negative effect on crop yield and performance, especially in cereals and other crops for which grains are an important yield factor (e.g. breeding populations) (Dolferus, Ji & Richards 2011). Hence, in a global context of instable climate conditions and local weather extremes, the high sensitivity of the male reproductive process to environmental stresses may pose great problems in consolidating the world food yield and feed supply (Parry *et al*. 1999). Due to its socio-economic impact, this issue is one of the most critical points in present-day agronomy, as alleged in recent review papers (Hedhly, Hormaza & Herrero 2009; Singh, Prasad & Reddy 2013).

Stress-induced losses in seed yield can be conferred by different physiological and phenological alterations, including change in flowering initiation, altered pollinator–flower interaction, reduced pollen germination, altered embryo development and reduced pistil acceptance (Cleland *et al*. 2007; Zinn, Tunc-Ozdemir & Harper 2010). However, in plant reproduction and seed formation, the process of male gametogenesis is generally considered the main centre of stress vulnerability. Therefore, in this review, we will focus on the physiological and biological determinants that underlie the stress sensitivity and responsiveness of plant male gametogenesis, covering both meiosis, tapetal behaviour, microspore development and pollen maturation. We thereby mainly focus on abiotic stress conditions, such as heat, cold and drought, but also report on biotic stress responses, for example, pathogen infection. In addition to the physiological response, this review also provides insights into the molecular mechanisms underlying stress-induced male reproductive alterations and outlines new routes for the design of potential applications to alleviate associated male sterility. The impact of stress on later processes in plant reproduction, such as pollen germination, fertilization and embryo formation, and the stress sensitivity of female gametogenesis has been

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addressed extensively in other reviews (Hedhly *et al*. 2009; Zinn *et al*. 2010) and is therefore not in the scope of this paper.

IMPACT OF ENVIRONMENTAL STRESS ON MEIOTIC CELL DIVISION

Influence of environmental stress on meiotic recombination

In higher plants, like in all sexual eukaryotic species, the process of male spore formation starts with a specialized cell division, namely meiosis. The meiotic cell division is typically constituted by a single round of DNA replication followed by two rounds of chromosome segregation (MI and MII) (Villeneuve & Hillers 2001), and serves to halve the genomic DNA content and to reshuffle (recombination) the genetic variation obtained by both parental genomes. As an essential part of meiotic cell division, the process of crossover (CO) formation and recombination is tightly controlled and molecularly regulated by a complex network of interacting factors (Harrison, Alvey & Henderson 2010). However, in several organisms, including plants, the frequency of meiotic CO formation is not only determined genetically but is also under the influence of environmental conditions (Baker *et al*. 1976). Indeed, in several plant species, exposure to environmental stress, for example, both biotic and abiotic, has been found to increase the overall rate of recombination (Wagner & Yanowitz 2005). In maize, for example, both low temperature and water deficit significantly enhance the frequency of meiotic CO formation (Verde 2003). Similarly, in tobacco, pathogenic infection with the tobacco mosaic virus (TMV) not only enhances somatic recombination but also significantly increases the frequency of meiotic homologous recombination (MHR) (Kovalchuk *et al*. 2003). In addition, studies in Arabidopsis revealed a positive correlation between male meiotic CO formation and temperature environment (in the 19–28 °C range) (Francis *et al*. 2007), similar as has been observed in other plants (*Hordeum vulgare*) and animals (*Drosophila melanogaster* and *Caenorhabditis elegans*) (Stern 1926; Powell & Nilan 1963; Grell 1966; Rose & Baillie 1979; Lim, Stine & Yanowitz 2008).

The sensitivity of meiotic recombination to environmental stress may represent a regulatory connection between stress sensing and CO control, or could be attributed to a direct mechanical effect in the process of CO formation. The molecular mechanism underlying stress-enhanced MHR is yet poorly understood; however, based on recent findings in somatic homologous recombination (HR), speculations can be made. Indeed, in plants, like in other organisms, an increased frequency of somatic HR was observed upon exposure to various environmental stresses, including UV, ionizing radiation (Lebel *et al*. 1993), heat shock (50 °C) (Lebel *et al*. 1993), salt stress (Boyko *et al*. 2006), pathogen infection (Lucht *et al*. 2002; Kovalchuk *et al*. 2003), heavy metals (Rahavi *et al*. 2011) and water stress (Yao & Kovalchuk 2011). Preliminary studies hereby provided evidence for the involvement of abscisic acid (ABA) as a putative physiological stress-responsive signal, modulating HR in response to environmental stress. Indeed, studies in tobacco revealed that pathogen-driven increases in HR (TMV and oilseed rape mosaic virus) not only occur in infected but also in non-infected tissues, including meiocytes, indicating the existence of a systemic mobile signal that promotes somatic and meiotic HR in response to biotic stress (Kovalchuk *et al*. 2003). The enhanced somatic HR frequency, together with the increased ABA sensitivity in the Arabidopsis *abo4-1* (*ABA overly sensitive*) mutant, which has a mutation in *POL2a/TILTED1* (*TIL1*), suggests a close link between ABA and HR regulation (Yin *et al*. 2009). Moreover, because exogenous application of ABA increases somatic HR in wild-type Arabidopsis, ABA has been found to serve as a mobile signal that promotes HR in response to plant stress (Yin *et al*. 2009). In search for a putative control mechanism, gene expression studies revealed that ABA positively regulates the expression of MEIOTIC RECOMBINATION 11 (MRE11), a member of the MRN complex (Mre11, Rad50 and Nbs1) that plays a central role in early strand break repair (Borde 2007; Buis *et al*. 2008) and in meiotic synapsis and cross-over formation (Cherry *et al*. 2007). More specifically, MRE11 is an exo-/endonuclease that mediates 5′ end single-strand resection and hence enables DNA break repair through homologous recombination (Bressan, Baxter & Petrini 1999). The enhanced MRE11 transcript level could therefore provide the basis for the promotive role of ABA in HR. Whether this ABA-enhanced expression of MRE11 also leads to an increased level of meiotic crossing-over is questionable as it is generally assumed that extensive DNA resection favours gene conversion (e.g. synthesis-dependent strand annealing) at the expense of crossing-over. Indeed, in reactions with short regions of homology, resection beyond the homologous sequence typically impairs proper Holliday junction formation and impedes subsequent events of crossing-over (Prado & Aguilera 2003). Alternatively, ABA was also found to down-regulate the expression of RAD51 – a homolog of bacterial RecA recombinase that is involved in both somatic and meiotic double-strand break (DSB) repair (Shinohara, Ogawa & Ogawa 1992; Doutriaux *et al*. 1998). Upon DNA strand break formation and processing, RAD51 binds to the 3′ end resected end, forming a helical nucleoprotein filament that recognizes homologous DNA sequences (sister chromatid or homologous chromosome) and hence generates homo- or heteroduplexes as the basis for mitotic DNA repair or meiotic CO formation, respectively (Forget & Kowalczykowski 2010; Osman *et al*. 2011). Studies using AtRAD51 knockdown mutants revealed that a reduced level of RAD51 causes alterations in meiotic prophase I with partial synapsis, the occurrence of chromosome fragmentation and multivalent chromosome associations (Pradillo *et al*. 2011). More importantly, depletion of RAD51 causes the formation of univalents, indicating that ABA-induced reductions in RAD51 transcript level would reduce MHR and CO formation rather than enhance it. Based on this reasoning, the promotive effect of ABA on meiotic recombination is thought not to act through RAD51.

Although the underlying molecular target is as yet unknown, previous mentioned findings support the hypothesis that ABA constitutes a mobile signal that regulates both mitotic and meiotic HR in response to environmental stress. Whether this regulatory mechanism operates through transcriptional control of genes involved in (meiotic) DSB repair and CO formation is still an issue of debate and needs to be addressed in future studies.

Alternatively to stress-induced ABA signalling, environment-dependent alterations in MHR may also result from direct changes in the expression of meiosis-related genes. In barley (*H. vulgare*), for example, high temperatures (30 °C day and 25 °C night) induce a premature up-regulation of the meiosis-specific *ASY1* (*ASYNAPSIS1*) gene in an early stage of pollen mother cell (PMC) development, leading to a premature initiation of meiotic prophase I (Oshino *et al*. 2007). ASY1 is a chromatin-associated protein that is required for the morphogenesis of the chromosomal axis and the stability of the synaptonemal complex in meiosis I (MI) (Armstrong *et al*. 2002). In addition, ASY1 regulates meiotic CO establishment by coordinating the activity of the DMC1 RecA homolog in favour of interhomolog sequence exchange (Sanchez-Moran *et al*. 2007; Kurzbauer *et al*. 2012). Premature up-regulation of ASY1 upon exposure to stress (e.g. heat) may therefore enhance MHR and hence constitute the molecular basis for stress-enhanced meiotic recombination. In contrast, studies in *Allium ursinum* and *Locusta migratoria* revealed that extreme temperatures (35 and 40 °C, respectively) interfere with meiotic chromosome mechanics, such as synapsis, and hence decrease CO frequency (Buss & Henderson 1988; Loidl 1989). These findings suggest that stress-dependent changes in MHR are transcriptionally controlled within certain environmental limits, but largely suffer from structural aberrations when thresholds are surpassed.

A more comprehensive regulatory mechanism underlying stress-enhanced MHR comes from yeast. In yeast, the frequency of MHR is constituted by a specific amount of basal recombination events, essential to maintain proper meiotic chromosome segregation, and a variable number of additional CO events occurring at meiotic hotspots [e.g. CRE-like (M26) DNA sites]. Typically, upon exposure to stress (osmotic, heat or oxidative stress), an increased level of recombination is observed at the meiotic hotspots (Fox & Smith 1998; Koren, Ben-Aroya & Kupiec 2002). In yeast, this stress-induced up-regulation of MHR is controlled by the conserved, stress-responsive p-38 family kinase Spc1 (Sty1, Phh1), which activates Rec12(Spo11)-dependent recombination at meiotic hotspots through a phosphorylation-independent interaction with the Atf1 (Mts1, Gad7) ATF/CREB transcription factor (Kon *et al*. 1998; Gao, Davidson & Wahls 2009). As the Atf1 recombination–activation domain is well conserved among other eukaryotes, including plants (Gao, Davidson & Wahls 2008), this mechanism could constitute a universal pathway for stress-induced increases in MHR. However, in plants, the putative role of SPO11 kinases and ATF1 homologs in stress-dependent meiotic recombination has not been studied yet.

Environmental stress affects meiotic cell division

Besides the effects on meiotic prophase I, recent studies have demonstrated that environmental stress, and more specifically temperature stress, has also an impact on the subsequent processes in meiotic cell division.

In rose (Rosa), for example, short periods of heat stress (e.g. 48 h at 36 °C) lead to specific alterations in male meiotic chromosome behaviour, resulting in meiotically restituted dyads and triads that contain diploid gametes instead of the normal haploid ones (Pecrix *et al*. 2011). Cytological examination revealed that the restituted products originate from the ectopic induction of parallel and tripolar spindles at metaphase II. In wild-type male meiosis, chromosome segregation in meiosis II (MII) is spatially organized by two physically separated, perpendicularly orientated spindles, forming the structural basis for the tetrahedral arrangement of haploid nuclei at the end of MII (Fig. 1). Under heat stress, alterations in MII spindle orientation lead to bipolar or tripolar configurations that physically (re-)join segregated chromatids in restituted MII dyads or triads (Fig. 1). Based on their altered orientation, these figures are termed parallel (ps) and tripolar (tps) spindles, respectively. In the plant kingdom, alterations in MII spindle orientation are considered the predominant mechanism underlying 2n gamete formation and sexual polyploidization. This is exemplified by the large number of plant species (e.g. alfalfa, red clover, sweet potato) that generate 2n gametes through this mechanism (Vorsa & Bingham 1979; Parrott & Smith 1984; Tavoletti, Mariani & Veronesi 1991; Lopez-Lavalle & Orjeda 2002). Although in some studies a genetic factor was found to underlie ps/tps formation (Mok & Peloquin 1975; Iwanaga & Peloquin 1982; d'Erfurth *et al*. 2008; De Storme & Geelen 2011), the study by Pecrix *et al*. (2011) demonstrated that environmental factors, particularly heat stress, also play an important role in ps-induced 2n gamete formation.

Heat-induced alterations in MII spindle orientation may be attributed to direct mechanical aberrations in the dividing meiocyte. In plants, little is known about the structural elements that define male meiocyte polarity. In yeast and animal oocytes, spindle polarity in MI and MII is not established by centrosomes, like in somatic cells, but instead is constituted by the dynamic localization and polar allocation of γ-tubulin as microtubule-organizing centre, often assisted by other polarity proteins (Tavosanis *et al*. 1997; Lee, Miyano & Moor 2000; Combelles & Albertini 2001; Jang, Rahman & McKim 2005). In these systems, the negative impact of temperature stress on γ- and β-tubulin stability has been documented repeatedly (Ju *et al*. 2005; Rienzi *et al*. 2005). In plants, a similar γ-tubulin sorting mechanism is thought to control male meiocyte polarity (Shimamura *et al*. 2004; Brown & Lemmon 2008); however, structural determinants of MII spindle orientation have not been identified yet, impeding the assessment of the impact of temperature and other stresses.

Alternatively, heat-induced alterations in MII spindle polarity may be caused by regulatory elements that link temperature sensing and MII meiocyte polarity. Genetic studies

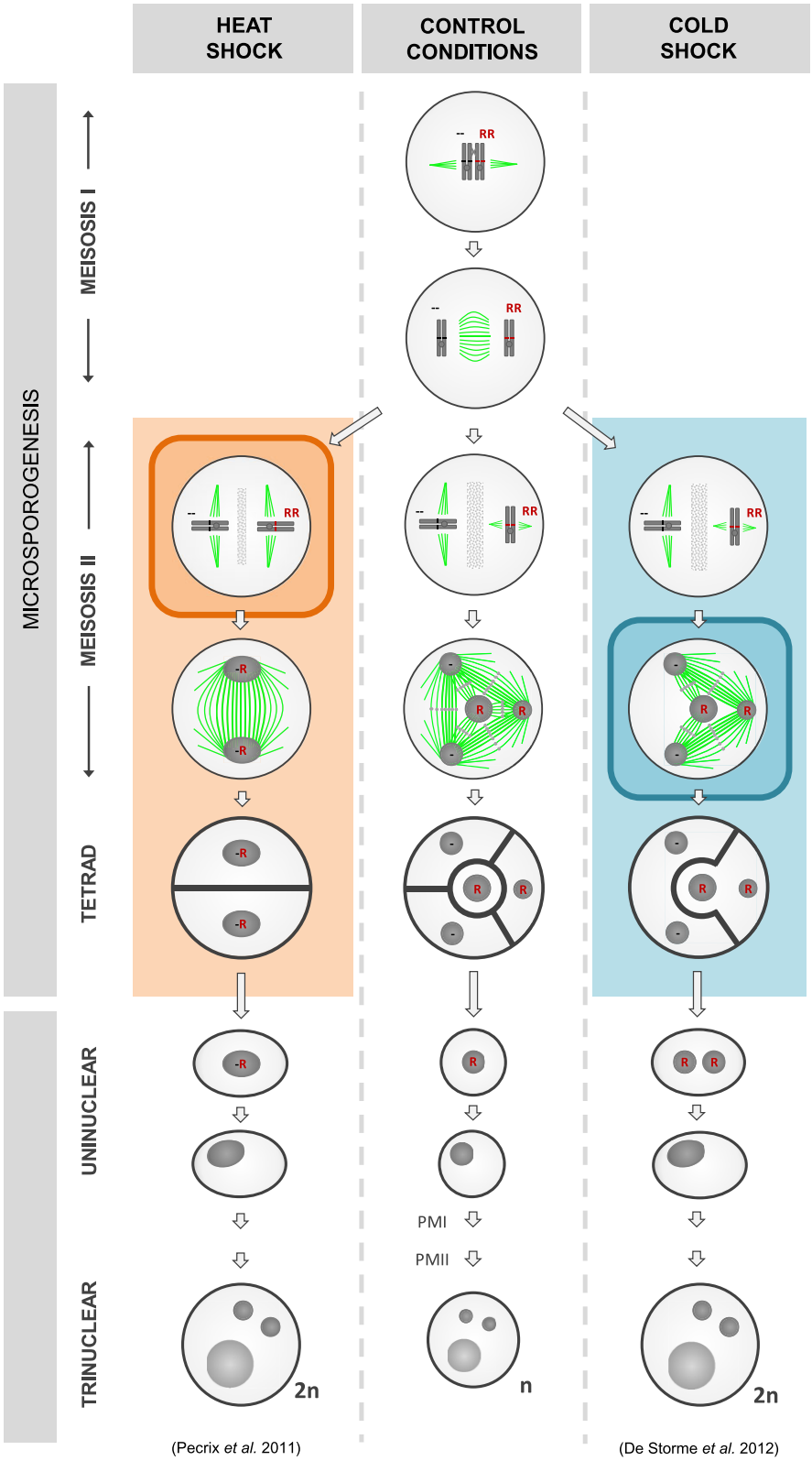

Figure 1. Schematic overview of male meiosis in simultaneous type of PMCs and cytological alterations imposed by temperature stress, as observed in cold-stressed Arabidopsis (De Storme *et al.*, 2012) and heat-stressed rose meiocytes (Pecrix *et al.*, 2011).

in Arabidopsis have identified three proteins that regulate the orientation of the MII spindle apparatus in male meiosis, namely Arabidopsis FORMIN14 (AFH14), JASON (JAS) and *Arabidopsis thaliana* PARALLEL SPINDLES1 (AtPS1). For all three proteins, respective loss-of-function mutants show the formation of diploid male gametes through the ectopic induction of parallel and/or tripolar spindles in MII (d'Erfurth *et al*. 2008; Li *et al*. 2010; De Storme & Geelen 2011). Hence, based on the phenotypic similarity with *atps1*, *jason* and *afh14*, heat stress-induced alterations in MII spindle organization in rose male meiosis could be attributed to an altered functionality of one of the corresponding proteins. However, because little is known about the impact of temperature or other stresses on the transcription profile and post-transcriptional processing of these genes, more extensive studies are needed to test this hypothesis.

Similar to heat stress, short periods of cold also have a restitutional effect on the meiotic cell cycle, but the underlying cellular mechanism strongly differs from the MII spindle alterations observed under high-temperature stress. Indeed, a recent study in Arabidopsis demonstrated that short periods of cold (1–40 h at 4–5 °C) do not interfere with meiotic chromosome segregation, but instead significantly alter post-meiotic cell plate formation and cell wall establishment (De Storme, Copenhaver & Geelen 2012). As a result, cold-stressed PMCs generate syncytial microspores that contain two or more haploid nuclei (Fig. 1). These nuclei subsequently fuse before pollen mitosis I (PMI) to generate normally configured diploid and polyploid pollen. Cytological examination revealed that cold stress specifically affects the internuclear radial microtubule arrays (RMAs), for example, phragmoplast-like structures that are required for de novo cell wall formation, at telophase II and interferes with the subcellular localization of organelles and the deposition of callose at developing MII cell plates, indicating that the process of meiotic cytokinesis is extremely sensitive to low temperatures (Fig. 1; indicated by blue box). Strikingly, the study also revealed that cold stress-induced dyads and triads predominantly contain highly homozygous second division restitution (SDR)-type 2n pollen (genetically corresponding to a loss of MII), indicating that cold-induced defects in meiotic cell plate formation are not random, but instead more frequently occur between chromosome sets separated in MII and less between MI-separated chromosome groups (De Storme *et al*. 2012). At one side, this could mean that the establishment of the internuclear organelle band at the end of MI is less sensitive to adverse temperatures and guarantees proper cell wall formation, even under destabilizing conditions. On the other hand, it could be assumed that lower temperature environments specifically affect microtubular dynamics (e.g. processing of spindle into RMA) between chromosome sets that have separated in MII.

Similarly, in a thermosensitive genic male sterile (TGMS) wheat variant, low temperatures specifically affect the formation of the MT phragmoplast at the end of MI, leading to alterations in meiotic cell plate establishment and eventually causing PMC abortion (Tang *et al*. 2011). Transcriptome analysis hereby revealed that cold stress affects the expression of key actin regulators and several other genes required for actin dynamics (e.g. actin-depolymerisation factor, profiling, formin, villin, LIM domain protein and Arp2/3), suggesting that cold-induced defects in MI cell plate formation originate from alterations in actin cytoskeletal organization. In support of this, Xu *et al*. (2013) found that cold-stressed TGMS wheat PMCs exhibit structural aberrations in the MI actin phragmoplast array. Hence, based on the intimate reaction between microtubuli and microfilaments in cell plate orientation and establishment, it is now thought that transcriptional changes in actin dynamics form the primary cause for cold-induced alterations in meiotic cell plate formation. Moreover, because formins are transcriptionally repressed by cold and loss of *Arabidopsis FORMIN14* (AFH14) causes defects in meiotic RMA formation, formins are speculated to play a prominent role in the cold sensitivity of meiotic cell plate formation in plants. This is in agreement with the proposed role of formins as important players in the signal-transduction cascade that regulates actin cytoskeletal dynamics in response to developmental and environmental stimuli (Deeks, Hussey & Davies 2002). However, because no direct link between formins and cold-defected meiotic cytokinesis has been demonstrated, more detailed analyses are needed to determine their role in PMC stress sensitivity.

In Arabidopsis, the formation of RMAs at the end of male meiosis is regulated by a distinct MAPK signalling pathway. More specifically, TES/STUD/AtNACK2, AtANP3, AtMKK6/ANQ1 and AtMPK4 act together in a cascade that modulates the downstream activity of MT binding proteins (e.g. MAP65s) (Soyano *et al*. 2003; Takahashi *et al*. 2010). Functional loss of one of these proteins leads to a complete or partial loss of male cytokinesis, generating restituted meiocytes that contain binuclear and polynuclear microspores (Melissa Spielman *et al*. 1997; Kosetsu *et al*. 2010). Based on the phenotypic similarity with cold-induced defects in Arabidopsis male meiosis, a putative role for TES or one of the downstream MAPK signalling components in the cold stress sensitivity of meiotic RMA formation is hypothesized. Our own work showed that cold-induced defects in post-meiotic cytokinesis are not mediated by the cold-responsive, MPK4-phosphorylating kinase MKK2 and that expression of main MAPK components is not altered upon cold stress. However, as no extensive molecular studies have been performed, more spatial and temporal analyses are needed to elucidate the molecular mechanism underlying cold-induced loss of (post-)meiotic cell plate formation.

In conclusion, it could be stated that sensitivity of developing meiocytes to environmental stress, particularly temperature stress, is centred on MT–MF cytoskeletal dynamics, with high and low temperatures specifically affecting MII spindle orientation and cytokinetic RMA formation, respectively (Fig. 1). Although these defects may occasionally lead to PMC lethality, in most cases, they induce a restitution of male meiosis, generating diploid and polyploid pollen grains that enable events of sexual polyploidization. Stress-induced alterations in meiotic cytoskeletal biogenesis and dynamics may therefore form a developmental mechanism to modulate

the genomic composition, load and stability (e.g. induction of polyploidy) of plants in response to adverse climatic conditions, forming a basis for an enhanced evolutionary adaptiveness and competitiveness.

EFFECT OF ABIOTIC STRESS ON MICROSPORE DEVELOPMENT

The early microspore stage is critically sensitive to abiotic stress

Abiotic stress during the reproductive process of microgametogenesis generally leads to an abortion of microspore development and the associated induction of male sterility. In most species, the critical male gametophytic stage that is most sensitive to abiotic stress often coincides with the meiosis-to-microspore transition stage (Bingham 1966; Saini & Aspinall 1981; Namuco & Otoole 1986). In Arabidopsis, for example, short periods of heat stress (4 h, 42 °C at 85% humidity) specifically affect pollen development at flower stage 9, for example, when PMCs finalize meiosis and enter into gametogenesis (Kim, Hong & Lee 2001). In rice, male gametophyte development is most sensitive to chilling (2–4 d, 12 °C) during the meiotic tetrad stage and in subsequent transition to the microspore phase (Ito *et al.* 1970; Satake & Hayase 1970; Oliver *et al.* 2005). In addition, the detrimental impact of heat stress (39/30 °C day/night, 2–4 d) on rice pollen performance is attributed to physiological defects specifically occurring in early microsporogenesis (Endo *et al.* 2009). Similarly, in cowpea (*Vigna unguiculata*) and tomato (*Lycopersicon esculentum*), the critical temperature-sensitive phase of male sporogenesis occurs 7–9 and 8–13 d, respectively, before anthesis, closely corresponding to the meiotic–microspore transition phase (Ahmed, Hall & Demason 1992; Sato, Peet & Thomas 2002). Thus, although also other stages in male reproduction (e.g. pollen release, adhesion, pollen tube formation) occasionally show a negative response on abiotic stress (Shivanna, Linskens & Cresti 1991), the early microspore stage appears to be the most critical developmental stage underlying abiotic stress sensitivity.

Defects in tapetal development form the basis for abiotic stress-induced male sterility

In several plant species, stress-induced spore abortion and male sterility is associated with alterations in tapetal development and occasional defects in the surrounding cell layers, such as the middle layer and the endothecium (Parish *et al.* 2012). As an essential part of the male sporangium (e.g. anthers), the tapetum is constituted by a single layer of endopolyploid cells that surrounds the locules of developing microspores and pollen grains (Ma 2005). During normal sporogenesis, the tapetum is strongly metabolically active and serves as a nutritive source by providing essential elements and energy to the neighbouring microspores (Pacini, Franchi & Hesse 1985; Scott, Spielman & Dickinson 2004). In addition, as a secretory cell layer, the tapetum provides enzymes for the release of microspores out of the meiotic tetrad (Goldberg, Beals & Sanders 1993) and supplies cell wall components for the construction of the pollen exine layer, such as sporopollenin (Fig. 2) (Shivanna & Johri 1985; Ariizumi & Toriyama 2011). In a later stage of male gametogenesis, at PMI, the

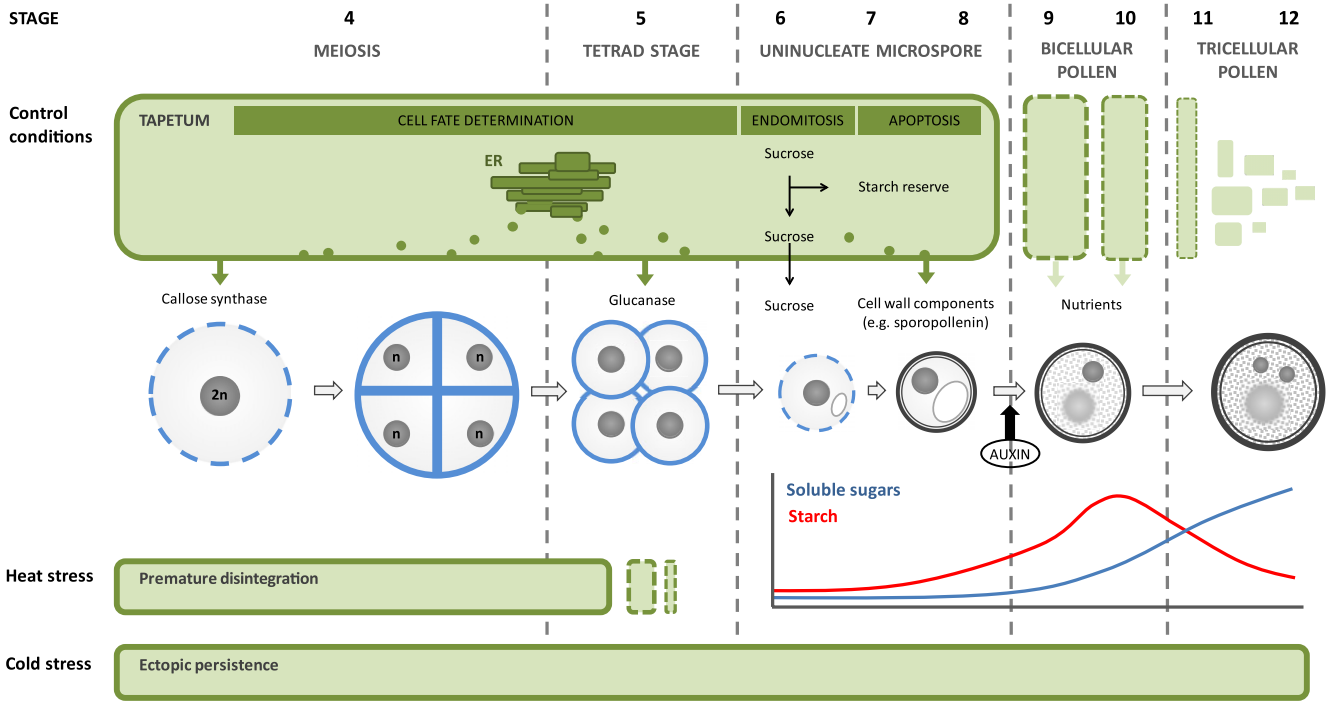

Figure 2. Multiple functions of the tapetum in male sporogenesis and gametogenesis, and the correlated impact of environmental stress on tapetal cell disintegration.

tapetal cells undergo a programmed cell death (PCD) and subsequently disintegrate (Papini, Mosti & Brighigna 1999; Wu & Cheung 2000; Varnier *et al*. 2005; Parish & Li 2010). This programmed tapetum-specific PCD and disintegration is essential for proper microspore development and pollen maturation and fertility, as indicated by several reports that show an induction of male sterility upon premature or delayed degradation of the tapetum (e.g. in rice *ms1*, *tdr* and *ptc1* mutants) (Ku *et al*. 2003; Li *et al*. 2006, 2011; Vizcay-Barrena & Wilson 2006).

Upon exposure to heat stress, anthers typically show a premature disappearance of the tapetal cell layer together with severe alterations in microspore development (Fig. 2). In wheat, for example, short periods of high temperature (3 d at 30 °C) lead to a premature degeneration of the tapetum and the associated invasion of the periplasmodial cell layers at meiosis (Saini, Sedgley & Aspinall 1984). Due to loss of nutritive supply, these tapetal defects strongly affect the progression of male gametogenesis and preclude microspores to complete PMI (Fig. 2). Similarly, in barley, high temperature-induced male sterility (at 30/25 °C day/night, 1–5 d) is associated with a premature degeneration of the tapetal cell layer (Abiko *et al*. 2005). Cytological analysis hereby demonstrated that the tapetum and the outer three anther wall layers (epidermis, endothecium and middle layer) display severe subcellular alterations, such as increased vacuolization (hypertrophy), chloroplast overdevelopment and excessive mitochondrial swelling (Oshino *et al*. 2007). In addition, high temperatures cause a severe transcriptional inhibition and hence drastically reduce the dividing capacity of the tapetum and the surrounding anther layers. Similarly, in TGMS rice, high temperatures (32/26 °C day/night) induce a premature degradation of the tapetum at the early uninuclear microspore stage, with typical apoptotic symptoms such as membrane blebbing, ruptured vacuoles, irregularly shaped mitochondria and cytoplasmic degradation (Ku *et al*. 2003).

Under cold stress conditions, the tapetum does not show a premature abortion but instead abnormally expands and persists up until the mature pollen stage (Fig. 2) (Oda *et al*. 2010). In rice, ectopic persistence of the tapetal cell layer is thought to be the major factor underlying cold-induced pollen abortion and male sterility (at 19 °C), similarly as observed in the tapetal PCD-defective *tdr* mutant (Li *et al*. 2006). Tapetal rice cells that have been exposed to chilling (22/12 °C day/night for 4 d) show morphological aberrations, such as abnormal vacuolization, reduced dividing capacity and hypertrophy (Mamun *et al*. 2006). Moreover, the ectopically persisting tapetal cells exhibit an increased peroxidase activity and a substantial accumulation of reducing substances, suggesting a putative involvement of oxidative stress responses.

Developmental abnormalities in anthers exposed to water stress have been extensively studied in wheat. Similar to temperature stress, short periods of water deficit (4 d without watering) during meiosis do not affect the meiotic cell division, but instead alter subsequent microspore development, typically showing premature spore degeneration and loss of reproductive cell orientation (Lalonde, Beebe & Saini 1997). At the same moment, drought-stressed tapetal cells (leaf water potential –2.54 MPa) display an abnormal vacuolization, and microspores ectopically separate from the inner anther wall (Saini *et al*. 1984). Moreover, in contrast to the normal tapetum degradation at the mid-vacuolar microspore stage (2–3 d post-meiosis), drought-stressed tapetal cells ectopically persist up to 8 d after meiosis (Fig. 2). Because tapetal degeneration provides nutrients and other signalling components essential for microspore development, its ectopic persistence is suggested to be the primary cause for cellular defects in pollen maturation. Together with tapetum-derived nutrition, maintenance of microspore polarity is also essential for proper microspore development (Christensen & Horner 1974). Hence, defects in microspore polarity may also constitute the basis for water stress-associated male sterility. However, as spore orientation is typically maintained by the physical contact with the surrounding tapetal cell layer, altered microspore polarity in water-stressed anthers could be attributed to alterations in tapetum development.

Besides water deficit, nutrient deficiency has also been found to lead to male sterility through defects in tapetal development. In copper-deficient barley (*H. vulgare* L.), for example, microscopic analysis revealed that the tapetum becomes highly expanded at the uninuclear microspore stage and invades into the anther locule, severely hindering proper microspore development (Jewell, Murray & Alloway 1988). In addition, major irregularities in microspore exine deposition, tapetal endoploidy and organelle composition were observed, indicating that copper-deprived male sterility in barley is caused by defects in tapetal development.

Tapetal RER as a centre of male gametophytic stress sensitivity

In search for the cellular process underlying stress-induced tapetal alterations, Ku *et al*. (2003) found that heat-stressed tapetal cells in TGMS rice (32/26 °C day/night) display a precocious fragmentation of DNA, suggesting that male sterility is caused by a premature induction of tapetal PCD. In agreement with this, heat-induced PCD has also been frequently documented in somatic cells. Indeed, in Arabidopsis vegetative cells, cultured tobacco cells and many other plant cell types, short periods of high temperature typically induce PCD and consequently cause an arrest or abortion of the mitotic cell cycle (Fan & Xing 2004; Vacca *et al*. 2004, 2007; Egorova, Lo & Dai 2011). Similarly, pollen sterility in heat-stressed snap bean (*Phaseolus vulgaris* L.; 28.6 °C versus 26.2 °C) is also caused by the precocious induction of tapetal cell death (Ahmed *et al*. 1992; Suzuki *et al*. 2001). Cytohistological studies hereby demonstrated that the tapetum displays apoptotic alterations (e.g. vacuolization) starting from the meiotic tetrad stage and prematurely disintegrates in the early vacuolated microspore stage (before PMI). In addition, Suzuki *et al*. (2001) found that heat-stressed tapetal cells exhibit clear changes in the ultrastructural morphology of the rough endoplasmic reticulum (RER), displaying linear, wavy, looped or circular structures instead of the normally stacked ones (Fig. 2). A similar defect in tapetal ER was also

observed under low-temperature stress. Indeed, in cold-stressed rice (continuous 16–20 °C), the tapetum does not show any structural change in any organelle, except for the ER, which typically displays altered configurations together with the abundant presence of oval-shaped ER-derived compartments (Gothandam, Kim & Chung 2007).

In plants, like in all other eukaryotic cell systems, the subcellular ER mediates post-translationally processing of newly formed secreted and membrane-specific polypeptides (e.g. glycosylation and protein folding). In addition, the ER also operates as a quality control system that eliminates misfolded proteins through the ER-associated degradation (ERAD) machinery (Hurtley & Helenius 1989; Helenius *et al*. 1993). Under environmental stress, the ER typically accumulates a high abundance of improperly folded proteins, which are then either directed through the protein folding process or degraded through ERAD (Liu & Howell 2010). When the ER processing machinery reaches its maximum capacity limit, an auto recovery system is automatically induced, for example, the unfolded protein response (UPR) (Malhotra & Kaufman 2007; Liu & Howell 2010). In the UPR reaction, both the protein folding and the ERAD machinery are transcriptionally up-regulated to enhance processing capacity (Martinez & Chrispeels 2003; Kamauchi *et al*. 2005). However, under conditions of severe stress or prolonged ER dysfunctioning, the UPR may also induce an apoptotic PCD response (Xu, Bailly-Maitre & Reed 2005; Liu & Howell 2010). Hence, excessive overload of ERAD and the UPR-associated induction of PCD in early tapetal cells may constitute an important mechanism underlying stress-induced male sterility in plants (Fig. 2).

Besides protein processing, tapetal RER also plays an important role in the degradation of the callosic cell wall that surrounds developing microspores (Fei & Sawhney 1999), most presumably through the synthesis and secretion of callose-degrading enzymes (β-1,3-glucanases) (Stieglitz 1977; Bucciaglia & Smith 1994; Wu & Yang 2005). Indeed, a recent study in maize demonstrated that the transport of β-1,3-glucanase from the tapetal cytoplasm to the wall facing the anther locule is mediated by ER-derived vesicles (Li *et al*. 2012). Studies in Petunia, Arabidopsis and other plants have demonstrated that callose processing is highly essential for spore development as it constitutes a basic framework for the establishment of the future pollen wall (Izhar & Frankel 1971; Worrall *et al*. 1992; Tsuchiya *et al*. 1995; Jin, Horner & Palmer 1997). Hence, premature dissolution or non-degradation of the callosic cell wall in respectively PMCs and developing microspores typically leads to spore abortion. In line with this, Suzuki *et al*. (2001) suggested that heat-induced male sterility in snap bean is caused by a reduced degradation of PMC callose, more specifically by the altered tapetal RER-mediated secretion of callase. In addition, several authors have observed clamped, tetrad-shaped spores in heat-stressed male gametogenesis (in Arabidopsis; 4 h at 42 °C), indicating that high temperatures indeed cause a loss of PMC callose degradation (Kim *et al*. 2001). In contrast, under cold stress (in rice; 22/12 °C day/night for 4 d), developing meiocytes typically exhibit a premature degradation of the surrounding callosic cell layer, leading to defects in microspore wall formation and pollen sterility (Mamun *et al*. 2006).

Although these data suggest that alterations in PMC callose biogenesis/degradation are the primary cause for temperature stress-induced male sterility, also other gametophytic processes that depend on tapetal ER functionality may constitute the basis for environmental-induced pollen abortion. Indeed, the tapetum ER not only secretes enzymes involved in callose metabolism but also produces enzymes required for PMC wall degradation and proteins involved in pollen development and exine formation (Bih *et al*. 1999; Ariizumi & Toriyama 2011; Zhou *et al*. 2012). As such, stress-induced defects in one of these processes through tapetal ER modulation may also lead to pollen abortion. In rice, for example, Ku *et al*. (2003) demonstrated that heat-induced pollen lethality is caused by structural alterations in the pollen exine layer, most presumably originating from a reduced supply of tapetal-derived pollen wall components. Hence, it is now generally believed that stress-induced male sterility is conferred by the combinatorial effect of multiple cellular defects that all originate from alterations in tapetal RER. We therefore postulate that the tapetal RER constitutes an important centre of stress sensitivity and mediates stress-induced male sterility through a variety of biological processes.

Abiotic stress induces alterations in anther sugar content and carbohydrate profile

Developing microspores constitute a strong photosynthetic sink and accumulate photoassimilates, such as starch and other carbohydrates. Initially, at the uninuclear microspore stage, the carbohydrate reserve is quite low, but upon PMI, developing spores typically display a rapid phase of starch biosynthesis and quickly accumulate high amounts of starch (Fig. 2) (Datta, Chamusco & Chourey 2002). At this stage, for example, the engorged pollen stage, the vacuole disappears and the cytoplasm of the vegetative cell becomes filled with starch (Christensen, Lersten & Horner 1972). Next, during final pollen maturation, the level of starch progressively decreases through degradation into soluble sugars (Fig. 2). As such, in most plant species, the maximum peak of starch is reached at the young bicellular pollen stage, whereas the total soluble sugar content (glucose, fructose and sucrose) gradually increases in developing anther walls and spores to reach a maximum at anthesis (Aloni *et al*. 2001; Pressman, Peet & Pharr 2002). Accumulated anther sugars not only serve as an energy source to fuel microsporogenesis and pollen maturation but also later on provide energy for pollen tube formation and serve as an osmolyte in conferring pollen tolerance to desiccation and other abiotic stresses (e.g. sucrose protects membrane integrity). As such, carbohydrates are not only essential for pollen development but also constitute a major determinant for pollen viability and germination capacity (Hoekstra & Vanroekel 1988; Hoekstra, Crowe & Crowe 1989; Speranza, Calzoni & Pacini 1997).

Plants undergoing abiotic stress during male sporogenesis typically show a reduced level of soluble sugar and starch in their anthers. As this reduction in carbohydrate reserves is often associated with defects in pollen development, stress-induced reductions in the anther's sugar reserve are suggested to be a predominant factor causing plant male sterility. In wheat, for example, water stress-induced male sterility (4 d of water depletion at meiosis) is associated with a strong reduction of the spore's soluble sugar content and alterations in starch distribution in the different anther layers (Dorion, Lalonde & Saini 1996). Similarly, in tomato, the decreased pollen viability under continuous high-temperature stress (32/26 °C day/night) strongly correlates with a reduced accumulation of sugars in the anther walls and developing pollen, but with an increased sugar level in the locular fluid (Pressman *et al*. 2002). In sorghum (*Sorghum bicolor* L. Moench), heat-stressed microspores (36/26 °C day/night) exhibit an altered carbohydrate profile, with a complete deficiency of sucrose, accumulation of hexoses and a reduced level of starch (Jain *et al*. 2007). In line with this, Fu *et al*. (2011) found that drought-tolerant rice shows an increased sugar content in its anthers, compared with the relatively low levels in a susceptible line. In rice and wheat, water stress-induced male sterility strongly correlates with a significant reduction in starch accumulation. However, as in these cases an enhanced accumulation of soluble sugars is observed (Sheoran & Saini 1996; Nguyen *et al*. 2010), stress-induced male sterility is not caused by a reduced amount or availability of carbohydrates. Thus, although deprivation of the anther sugar content is often considered one of the predominant factors underlying stress-induced male sterility, it is most likely not the primary reason for associated microspore abortion.

Abiotic stress-associated alterations in sugar metabolism: a central role for anther invertase

The general reduced accumulation of starch and other carbohydrate components in abiotically stressed microspores is either caused by alterations in sugar metabolism or in the supply of assimilates. Saini (1997) suggested that the failure of male spore development under abiotic stress is attributed to a reduced sugar delivery to the reproductive tissues. Indeed, several studies have demonstrated that abiotic stress alters photosynthetic activity (e.g. reduced intercellular CO_2 through stomatal closure) and reduces the associated export of photoassimilates to the major sink organs, including anthers (Dinar & Rudich 1985; Harding, Guikema & Paulsen 1990; Tezara *et al*. 1999; Chaves *et al*. 2002; Sudhir & Murthy 2004; Dai *et al*. 2007). However, contrary to this hypothesis, anthers of wheat and rice were found to contain an increased level of sucrose and other soluble sugars upon stress (Dorion *et al*. 1996; Sheoran & Saini 1996). As such, it was suggested that the altered carbohydrate profile in stressed anthers is caused by alterations in sugar utilization and metabolism, rather than by changes in sucrose supply. In agreement with this, many studies have demonstrated that the main enzymes involved in sucrose and starch metabolism show a reduced activity in abiotically stressed anthers. In sorghum, for example, various genes involved in sugar cleavage and utilization, sugar transport and starch synthesis are differentially expressed in heat-stressed microspores (Jain *et al*. 2007). More specifically, season-long heat stress (36/26 °C day/night) significantly reduces the transcript level of cell wall invertase (CWI) in the male gametophyte, as has also been observed in heat-stressed (32/26 °C day/night) tomato anthers (Sato *et al*. 2006). Similarly, in rice anthers, the activity of both acid invertase and soluble starch synthase is significantly lower upon exposure to water stress (Sheoran & Saini 1996), whereas other enzymes involved in carbohydrate synthesis (e.g. ADP-glucose pyrophosphorylase and sucrose synthase) do not show any significant alteration. In contrast, in wheat anthers, water stress not only reduces the activity of soluble acid invertase but also affects the enzymatic activity of ADP-glucose pyrophosphorylase and sucrose synthase (Dorion *et al*. 1996). Koonjul *et al*. (2005) hereby additionally reported that water stress (4 d without watering; –2.3 MPa) specifically impairs the activity of two invertases, namely Ivr5 and Ivr1. Both Ivr enzymes are expressed within the tapetum and developing microspores and show a selective down-regulation at the meiotic PMC stage under water deficiency. Drought-induced male sterility in wheat hence results from the down-regulation of invertases and the associated loss of hexose accumulation in developing microspores (Dorion *et al*. 1996; Koonjul *et al*. 2005).

In line with these observations, Oliver *et al*. (2005) found that the perturbed carbohydrate metabolism in cold-stressed rice anthers (3 d at 12 °C) is triggered by transcriptional repression of *OsINV4* invertase. Moreover, as cold-tolerant rice does not show any reduction in *OsINV4* expression upon cold, *OsINV4* was found to play a central role in stress-induced male sterility. Rice *OsINV4* encodes an anther-specific cell wall acid invertase, which is expressed in the tapetal cell layer at the young microspore stage and in maturing microspores at later stages of pollen development (Oliver *et al*. 2005). Similarly, in several other species, stress-induced male sterility is either directly attributed or indirectly linked to changes in anther invertase activity. For example, in bell pepper (*Capsicum annuum*), heat-induced (8 d at 32/26 °C day/night) alterations in pollen sugar utilization and the associated reduction in pollen germination strongly correlate with reduced activities of acid invertase (Aloni *et al*. 2001).

The absence of starch in abiotically stressed microspores is generally caused by the transcriptional down-regulation of the invertase b-D-fructofuranosidase (EC 3.2.1.26). In plants, invertases are subdivided into three types based on their cellular localization pattern: cell wall specific (CW-INV), vacuolar (V-INV) and cytoplasmic (C-INV) (Ruan *et al*. 2010). Together with sucrose synthase, invertase enzymes are required to cleave the transported sugar module, for example, sucrose, into single hexose units (fructose and glucose) to enable further sugar processing and starch build-up (Fig. 3). In spores and anthers of wheat and several other species, invertase (INV) is by far the most dominant enzyme-regulating cleavage of sucrose (Saini & Westgate 2000; Jain *et al*. 2007). Moreover, because developing microspores are physically

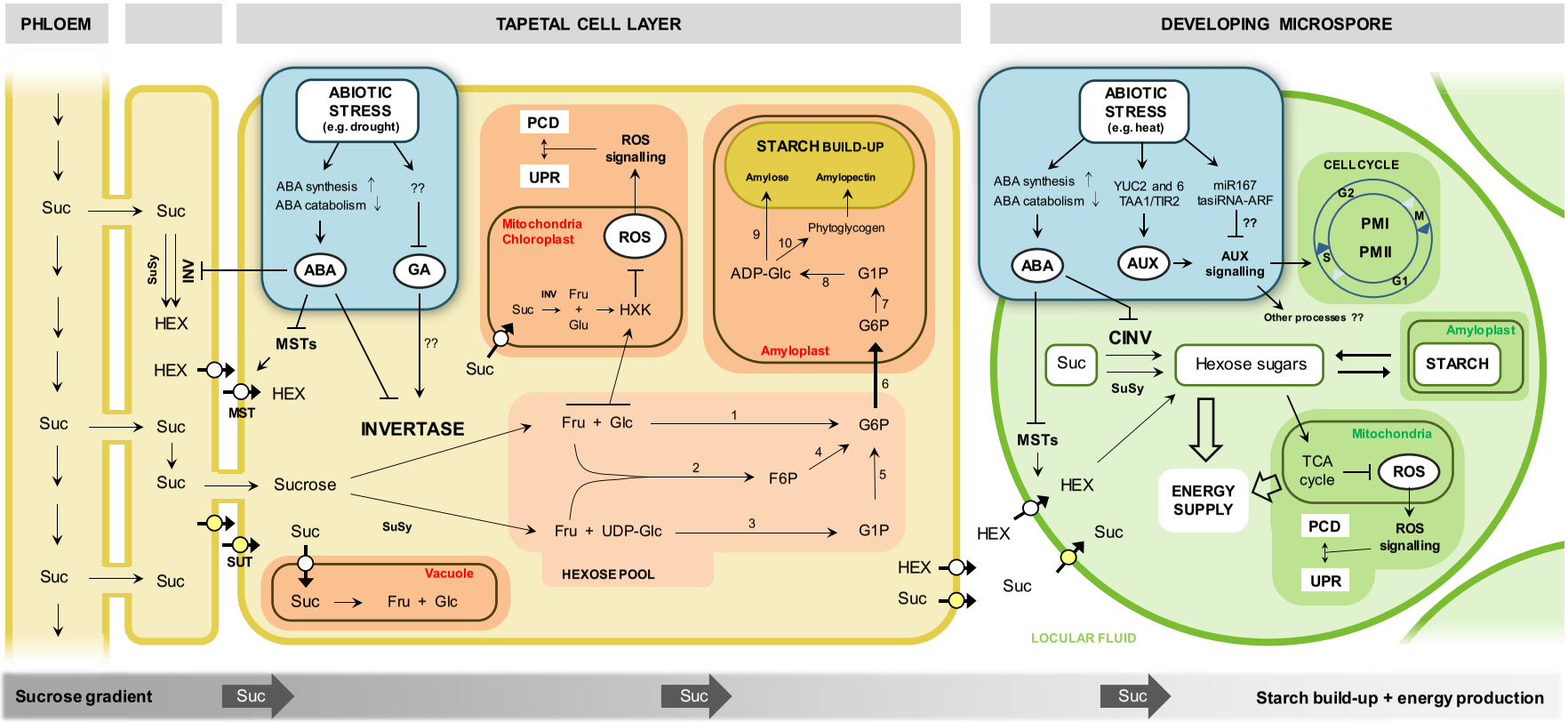

Figure 3. Schematic overview of the impact of abiotic stress on sugar metabolism and transport in the tapetal cell layer, and developing spores and the associated interaction with the oxidative stress response.

Organ-specific accumulation of starch and soluble sugars and the associated carbohydrate metabolism, which is characterized by a dynamic relationship between sugar biosynthesis, degradation and source-to-sink transport, is a complex process that includes many enzymatic factors and transporter proteins. In non-photosynthetic sinks, such as developing microspores, sucrose is the main metabolic substrate. Similar to all other photosynthates, sucrose is transported from source (photosynthetic leaves) to sink through the phloem vascular bundle system. Phloem loading and unloading and the associated entrance of sucrose into adjacent basal cells is mediated by sucrose transporter proteins (SUTs) that are anchored in the plasma membrane (Aoki *et al*. 1999; Braun & Slewinski 2009). In order to generate a carbohydrate sink reserve (e.g. starch), sucrose is cleaved by the cooperative activity of two enzymes: sucrose synthase (SuSy) and sucrose invertase (Inv) (Fernie, Willmitzer & Trethewey 2002; Vandeputte & Delcour 2004). SuSy catalyses the conversion of cytosolic sucrose into fructose and UDP-glucose (Geigenberger & Stitt 1993), which is further converted into glucose-1-phosphate (G1P) by UDP-glucose pyrophosphorylase (UGPase). The generated G1P then serves as a substrate for cytosolic phosphoglucomutase (PGM) that further catalyses it into glucose-6-phosphate (G6P). Sucrose invertase, on the other hand, mediates the hydrolysis of sucrose into fructose and glucose (in cytosol and apoplast), which is converted into G6P by the activity of hexokinase. After the formation of G1P or G6P, both types of hexoses are translocated into the amyloplast by phosphate translocators (Tetlow, Bowsher & Emes 1996; Kammerer *et al*. 1998).

In the amyloplasts, G6P is converted into G1P through the activity of apoplastic PGM and the resulting G1P is metabolized into ADP-glucose by ADP-glucose pyrophosphorylase (AGPase) (Vandeputte & Delcour 2004). In the final step, glucosyl units provided by ADP-glucose are progressively introduced in the growing starch molecule by the synthetic activity of starch synthase. In microspores, as in all other photoassimilative sinks (e.g. seeds, fruits, tubers and roots), precursors for starch biosynthesis are generated in the apoplast and the cytosol, whereas the actual biosynthesis of starch polymers occurs in the amyloplast (Vandeputte & Delcour 2004).

isolated from the surrounding tapetal cell layer (no symplastic PD channels), extracellular CW invertases play a key role in the unloading of sucrose from the tapetum into the apoplastic space and in the establishment of a soluble sugar gradient along the different anther fractions (anther wall > locular fluid > microspore) (Fig. 3) (Clement & Audran 1995; Goetz *et al*. 2001; Castro & Clement 2007). Specific down-regulation of CW invertase in abiotically stressed anthers hence blocks the import of hexose sugar units into developing microspores and impedes the accumulation of starch in maturing pollen, leading to severe aberrations in pollen development and viability (Dorion *et al*. 1996; Koonjul *et al*. 2005; Oliver *et al*. 2005). In support of this, RNAi-mediated silencing of the *Lycopersicon* CW-INV5 (LIN5) was found to significantly reduce pollen viability (Zanor *et al*. 2009), indicating that INV-mediated hydrolysis of sucrose is essential for pollen development and pollen viability.

Together with a reduced starch accumulation in spores, stress-induced down-regulation of INV also alters sucrose utilization in other anther tissues, and therefore often leads to the ectopic accumulation of sugars in non-microspore organ types, such as the connective tissue and the endothecium (Lalonde *et al*. 1997; Oliver *et al*. 2005). These changes in anther sugar partitioning may lie at the basis of the morphological defects in abiotically stressed anthers. For example, in cold-stressed rice (4 d at 22/12 °C day/night), the ectopic accumulation of starch in the endothecium and in other anther layers typically induces structural aberrations, such as abnormal vacuolization and poor cell wall formation, leading to aberrations in spore development (Mamun *et al*. 2006). Moreover, because the role of INV in sugar partitioning is co-regulated with the expression of sugar transporter proteins (Ehness & Roitsch 1997), stress-induced changes in INV expression are often associated with a reduced expression of transporter genes and thus with a reduced apoplastic sucrose loading (Jain *et al*. 2007). In line with this, Zhao *et al*. (2000) demonstrated that the co-suppression of plasma membrane H^+-ATPase results in a reduced pollen sugar uptake and consequently leads to an impaired male fertility. Similarly, in rice anthers, chilling stress not only reduces expression of *OsINV4* but also significantly affects transcription of the anther-specific monosaccharide transporter *OsMST8* (Mamun *et al*. 2006). The vacuolization and hypertrophy of water-stressed tapetum cells therefore most presumably originates from osmotic imbalances in the tapetum, triggered by the reabsorption of callose breakdown products in the absence of *OsMST8* activity.

Based on these findings, we conclude that anther-specific alterations in sugar content and the associated induction of male sterility under abiotic stress are caused by the combinatorial effect of two processes involved in sugar metabolism, for example, reduced sucrose transport and altered sucrose metabolism (Fig. 3).

Hormonal control of stress-induced alterations in sucrose invertase activity

Due to the central role of INV in stress-induced male sterility, research is now focused on the elucidation of putative underlying regulatory mechanism(s). Both the plant hormones ABA and gibberellic acid (GA) are relevant candidates because they are both involved in regulating carbohydrate supply to the tapetum and developing microspores.

ABA has been found to accumulate in plants on several types of abiotic stress, including heat, cold, salt stress and water deficit (Thomashow 1999; Jia *et al*. 2002; Zhang *et al*. 2006). ABA regulates the osmotic stress signal transduction response and confers plant stress tolerance through the up-regulation of a large set of stress-responsive genes (Xiong, Schumaker & Zhu 2002; Fujita *et al*. 2011). More importantly, there is accumulating evidence that ABA interacts with the sugar signalling pathway to activate the plant's stress response (Arenas-Huertero *et al*. 2000; Rook *et al*. 2001, 2006; Eckardt 2002; Gibson 2004; Dekkers, Schuurmans & Smeekens 2008). This interaction is not only retrieved in somatic tissues, but occurs in male reproduction as well. In rice, for example, accumulation of ABA in cold-stressed anthers (3 d at 12 °C) specifically interferes with the tapetal apoplastic sugar transport and consequently induces a high level of pollen abortion (Oliver, Dennis & Dolferus 2007). ABA thereby specifically reduces the expression of *OsINV4* and the monosaccharide transporter genes *OsMST7* and *OsMST8*. In line with this, anthers of the cold-tolerant rice variety *R31* show a reduced level of ABA compared with the cold-sensitive *Doongara* line. Because these differences in ABA accumulation strongly correlate with a differential expression of ABA metabolic genes, ABA is believed to act as a stress-responsive signal that induces pollen abortion by specifically repressing apoplastic sugar transport in the anther. Similarly, in a drought-sensitive wheat variety, drought-induced pollen sterility is associated with a severe accumulation of ABA, achieved by an altered expression of metabolic genes, whereas drought-tolerant anthers contain significant lower levels of ABA (Ji *et al*. 2011). Moreover, endogenous increases of ABA in wheat spikes through exogenous application or reduced catabolic activity (ABA 8′-OH deletion lines) substantially increase male gametophytic sensitivity to drought. Similar as in rice, ABA hereby specifically represses the expression of the anther CW-INV, for example, TaIVR1, leading to alterations in sugar utilization and a reduced level of hexoses in developing spores. In line with this, transgene-based reduction of ABA in rice anthers (by a tapetum-driven wheat TaABA 8′hydroxylase 1) results in the stabilization of *OsINV4* activity under cold stress conditions, significantly improving the tolerance to cold. Collectively, these findings support a pivotal role for ABA in controlling sugar metabolism, for example, by altering INV activity, in the male reproductive organs under stress.

Although ABA seems to be the major signalling component suppressing INV activity under adverse environmental conditions, GA has also been found to play a role in the transcriptional control of anther INV. In tomato, Proels *et al*. (2003) found that the anther-specific extracellular invertase *Lin7* gene contains gibberellin-responsive cis-acting elements in its promotor and additionally demonstrated that GA is required for the expression of *Lin7* in developing anthers (Proels *et al*. 2003; Proels, Gonzalez & Roitsch 2006).

Similarly, transcriptional control of INV through GA has also been observed in other tissues, including seeds (Mitsuhashi *et al*. 2004), shoots (Wu *et al*. 1993), petioles (Gonzalez & Cejudo 2007) and internodes (Jones & Kaufman 1971). Through the control of INV and its regulatory role in phloem unloading, carbohydrate partitioning and growth of sink tissues (Tymowska-Lalanne & Kreis 1998), GA is considered a major player in the process of source and sink formation and photosynthetic assimilate distribution (Iqbal *et al*. 2011).

GAs typically regulate plant growth and development, and are therefore not classified as 'stress hormone'. However, similar to ABA, accumulation of GA in plants has been found to occur in response to environmental cues, such as light, temperature and other abiotic stresses (Hedden & Thomas 2012). GA may therefore constitute an (additional) stress-signalling factor conferring stress-induced male sterility through alterations in anther INV activity and sugar utilization. However, despite its presumed role in reproductive stress, no studies have yet been performed to assess the involvement of GA in abiotic stress-induced male sterility.

Abiotic stress induces oxidative damage and PCD in developing microspores

One of the major mechanisms underlying stress-induced damage in plant cells is through the accumulation of reactive oxygen species (ROS). In plants, ROS, such as hydroxyl radicals, superoxide anion and hydrogen peroxide, have been found to accumulate upon exposure to environmental stress, including high and low temperatures, high light intensities (photo-oxidative stress), drought, air pollution, UV, pathogen invasion (hypersensitive reaction) and herbicides (Foyer, Lelandais & Kunert 1994; Larkindale & Knight 2002; Mittler *et al*. 2004). As ROS typically constitute highly unstable molecules that induce lipid peroxidation and oxidation of DNA, sugars and proteins, stress-induced accumulation of ROS generates a wide range of intercellular biochemical damages, such as membrane degradation, reduced translation and transcription, and eventually apoptotic cell death (Tiwari, Belenghi & Levine 2002; Blokhina, Virolainen & Fagerstedt 2003; Li *et al*. 2004; Van Breusegem & Dat 2006; Ryter *et al*. 2007). In plants, the accumulation of ROS is prevented by the activation of an antioxidant system that implies the production of antioxidants (e.g. ascorbic acid, glutathione, tocopherols) and enzymes that regenerate reduced antioxidant forms and the activation of ROS-interacting enzymes such as superoxide dismutase (SOD), peroxidases (PODs) [e.g. ascorbate peroxidase (APX)] and catalases (CATs). In many plant organs, including anthers, the protective ROS-scavenging response is up-regulated upon exposure to environmental stress. In tomato, for example, heat-stressed microspores (2 h at 43–45 °C) typically display an enhanced expression of genes that encode ROS-scavenging enzymes (e.g. SlAPX3), allowing spores to reduce the detrimental accumulation of ROS under abiotic stress (Frank *et al*. 2009). In contrast, reduced scavenging capacities typically result in an accumulation of ROS in the male reproductive organs that eventually lead to pollen abortion. In rice, for example, reduced expression of MT-1-4B, a type 1 small Cys-rich metal binding protein that has ROS-scavenging activity (in *mads3* anthers or through amiRNA) leads to the accumulation of ROS in tapetal cells and developing microspores and hence causes premature pollen abortion (Hu *et al*. 2011). Similarly, in many cytoplasmic male sterile crop varieties (e.g. cotton, rice, pepper), pollen PCD is caused by an excessive mitochondrial accumulation of ROS together with a decreased scavenging capacity in developing spores (Li *et al*. 2004; Jiang *et al*. 2007; Wan *et al*. 2007; Wang *et al*. 2009; Huang *et al*. 2011; Deng *et al*. 2012). Hence, ROS scavenging in the anther is extremely important to maintain pollen viability under abiotic stress.

Based on the lethal effect of ROS on pollen development, stress-induced male sterility may be attributed to the accumulation of ROS and the associated PCD response in developing microspores. In rice, for example, water stress-induced (no watering from spike initiation to anthesis) pollen sterility is ascribed to an oxidative stress response that is caused by a reduced level of antioxidant transcripts (CAT, APX and DHAR) in the developing anther (Selote & Khanna-Chopra 2004). In a similar way, Nguyen *et al*. (2009) found that the exposure of rice anthers to short periods of drought stress (3 d without watering) resulted in an increased accumulation of hydrogen peroxide, a reduced level of antioxidant transcripts and the associated depletion of ATP, leading to an enhanced accumulation of ROS. Moreover, the additional observation of DNA fragmentation in the tapetum and other anther tissues suggests that drought-induced oxidative stress eventually causes PCD in developing anthers. Similarly, in a comparative study in rice, drought-stressed anthers of the sensitive Zhenshan 97B line exhibited lower antioxidant enzyme activity (SOD, POD and CAT) and higher malonaldehyde content compared with the drought-tolerant Jin 23B line (Fu *et al*. 2011). In the same report, the enhanced level of oxidative stress in drought-stressed rice anthers was found to appear strongly linked to alterations in sugar metabolism. Stress-induced accumulation of sucrose was hereby postulated to reduce the mitochondrial activity during the tricarboxylic acid cycle and could therefore lead to an excessive production of ROS and an associated depletion of ATP in developing anthers (Fig. 3) (Bolouri-Moghaddam *et al*. 2010; Fu *et al*. 2011). Further evidence for a regulatory function of soluble sugars in the oxidative stress response is provided by several other metabolic processes (oxidative pentose-phosphate pathway, carotenoid biosynthesis and photosynthesis) and is additionally confirmed by transcriptome analyses, indicating that sugar signalling or sugar-mediated gene expression plays an important role in the control of ROS homeostasis (Couee *et al*. 2006). Stress-induced alterations in the anther sugar content (e.g. INV) could therefore lead to an enhanced accumulation of ROS and the associated induction of PCD in developing spores.

Thus, although stress-induced accumulation of ROS has prevalently been documented in vegetative tissues and somatic cell cultures (Vacca *et al*. 2004), evidence is accumulating that oxidative stress also occurs in male reproduction where it constitutes a major factor affecting spore development and pollen viability under adverse abiotic conditions.

The role of auxin in stress-induced male sterility

The plant hormone auxin or indole acetic acid (IAA) plays a central role in cell expansion, division and differentiation, and hence controls different developmental processes, including phototropism, geotropism, embryogenesis and vascular differentiation. Recent studies have demonstrated that auxin also plays a prominent role in the development and functionality of the gametophytic organs. More specifically, in male gametogenesis, auxin regulates two distinct developmental processes: (1) the progression of microspore development through control of the pollen mitotic cell division (Feng *et al*. 2006) and (2) late anther development through regulation of filament elongation, pollen maturation and anther dehiscence (Cecchetti *et al*. 2008).

Although not generally known as a stress hormone, there is accumulating evidence that auxin plays a role in the developmental regulation of plants under (a)biotic stress, particularly under adverse temperatures (Ghanashyam & Jain 2009). Recent findings suggest that cold-induced changes in plant growth and development (roots assessed after 8–12 h at 4 °C) are governed by alterations in the intracellular auxin gradient, which is generally constituted by polar movement and intracellular trafficking through auxin carrier molecules, such as PINs (Shibasaki *et al*. 2009; Rahman 2013). Moreover, in several plant tissues, activation and up-regulation of auxin biosynthesis and the associated accumulation of IAA have been reported upon exposure to high temperatures (Gray *et al*. 1998). In contrast, in developing anthers, heat stress (5 d at 30/25 °C day/night in barley and 31–33 °C in Arabidopsis) leads to a decreased level of endogenous auxin. Moreover, Sakata *et al*. (2010) found that the stress-induced reduction in anther auxin is the primary cause for associated male sterility (Sakata *et al*. 2010). In barley and Arabidopsis anthers, heat stress significantly reduces the transcript level of several auxin biosynthesis proteins, including YUC2, YUC6 and TAA1/TIR2, and by consequence represses endogenous auxin production in developing PMCs and tapetal cells (e.g. particularly at the uninuclear microspore stage), leading to a premature abortion of microspore development (Sakata *et al*. 2010). Because a similar induction of male sterility has been observed in double or triple mutants that include *yuc2* and *yuc6* (Cheng, Dai & Zhao 2006) and exogenous auxin application was found to reverse the heat-induced pollen sterility (Sakata *et al*. 2010), high temperature-induced microspore abortion is directly caused by alterations in endogenous auxin metabolism. Moreover, because male sterility is not observed in auxin transport or perception mutants (Cecchetti *et al*. 2008), heat-induced pollen abortion appears directly caused by a reduced synthesis of endogenous auxin in developing anthers. Based on the finding that free auxin is required to perform mitotic cell division in developing microspores (PMI and PMII) (Feng *et al*. 2006), it could be hypothesized that the reduced accumulation of auxin in heat-stressed anthers specifically blocks the progression of the microspore's mitotic cell cycle, leading to a premature abortion of pollen development. In line with this, Oshino *et al*. (2011) demonstrated that reduced IAA levels in heat-stressed barley panicles induce an arrest of nuclear and organellar DNA proliferation in developing microspores, most presumably through loss of gamete-specific DNA replication.

A similar block of microspore cell cycle progression and the associated induction of male sterility have also been observed in Arabidopsis *pin5* and *pin8* mutants (Ding *et al*. 2012). Both *PIN8* and *PIN5* encode ER-localized PIN auxin efflux proteins that mediate intracellular auxin homeostasis and nuclear-directed auxin transport in developing spores (Mravec *et al*. 2009; Dal Bosco *et al*. 2012). As such, it could be hypothesized that heat stress-induced male sterility is caused by alterations in microspore-specific intracellular auxin distribution and nuclear signalling or, more structurally, through physical alterations in the ER membrane (similar as in the tapetum, see previous paragraph). An alternative hypothesis is based on the suggestion that the tapetal cell layer is a major supplier of IAA to the developing pollen grains (Aloni *et al*. 2006). In this perspective, temperature-induced defects in tapetal development would hinder proper auxin transport to the anther locule, significantly reducing the endogenous auxin level in developing microspores.

A second link between stress-induced male sterility and auxin metabolism has only been provided recently (Tang *et al*. 2012). In spike tissues of a wheat TMGS line, in which the early phase of microspore development is susceptible to cold, deep sequencing of small RNA (smRNA) libraries and associated qPCR analysis identified two cold stress-responsive smRNAs that play an important role in the regulation of auxin signalling, namely miR167 and tasiRNA-ARF (auxin-responsive factor). Accumulation of these smRNA species under low-temperature conditions may invoke an RNAi-based repression of auxin signalling in developing anthers, leading to a premature abortion of microspore development. However, because expression of both cold stress-responsive smRNAs and their target genes showed a low level of correlation (Tang *et al*. 2012), more research is needed to clarify whether smRNA-directed alterations in auxin signalling constitute a direct basis for cold-induced male sterility.

CONCLUSIONS AND FUTURE PERSPECTIVES

The impact of environmental stress on male gametogenesis in plants covers many biological processes and affects several cytological mechanisms that are strongly interrelated. Recent research has revealed an important and often intertwined role for cytoskeletal dynamics, tapetal ER stability, sugar metabolism and oxidative stress, and has additionally demonstrated the putative involvement of several stress-signalling components, including major plant hormones (ABA, GA and auxin) and epigenetic regulators (e.g. smRNAs). Depending on the type of stress involved, the process of male sporogenesis and gametogenesis is affected differently. However, from a more general perspective, stress sensitivity of the male reproductive system appears to be spatially centred to the tapetal cell layer and more specifically to its ER. Notwithstanding the current advances in the

physiological and biochemical processes underlying environmental stress-induced male gametophytic alterations (e.g. male sterility and 2n gamete formation), the molecular factors and regulatory networks involved are still largely unknown. We therefore believe that the molecular unravelling of stress-induced male aberrations should be the focus of future research, not only because it provides additional insights in the underlying biological processes but also because it forms an important incentive for the putative design and implementation of strategies to counter environmental stress-induced spore abortion and seed loss.

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Received 20 December 2012; received in revised form 30 April 2013; accepted for publication 8 May 2013