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Male Sterility Accompanied with Abnormal Anther Development in Plants – Genes and Environmental Stresses with Special Reference to High Temperature Injury

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ABSTRACT

The development and differentiation of anther cells, including specification of cell lineage and cell fate, are well-regulated programs. Sporogenous cells differentiate into pollen mother cells (PMCs) and enter meiosis. In addition, differentiated anther wall cells degrade sequentially during pollen maturation and their dehiscence excludes mature pollen. This degradation process appears to be controlled by programmed cell death (PCD). Maternally-inherited male sterility is common in various plant species and is referred to as cytoplasmic male sterility (CMS). In some examples of CMS, floral organ identity is unperturbed, but the anther tissues degenerate by processes of PCD or necrotic cell death. In addition, abiotic stresses dominantly affect male reproductive development. In particular, high-temperature stress causes male sterility in many plant species. We use the double-rowed barley (*Hordeum vulgare* L. cv. 'Haruna-nijyo') as a model for male reproductive development and high-temperature injury in plants. This type of injury relates to premature progression of early developmental programs in anthers and includes proliferation arrest, degradation of anther wall cells and progression to meiosis in PMCs, all of which require comprehensive alterations in transcription. Given the involvement of PCD in anther-specific sequential and cooperative programs, as well as in cell fates, these findings suggest that male reproductive development might be more sensitive to environmental stresses than female reproductive development and vegetative growth. We also introduce certain key genes that have been identified recently and relate specifically to male reproductive development and sterility.

Keywords: environmental stress, high temperature injury, male sterility, pollen mother cell, programmed cell death, tapetum
Abbreviations: CMS, cytoplasmic male sterility; **LTP**, lipid transfer protein; **PCD**, programmed cell death; **PMC**, pollen mother cell

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INTRODUCTION

Morphogenesis of male and female sexual organs initiates during the sporophyte stage of the plant life cycle. Sexual organs comprise several types of differentiated cell masses that perform specific functions. One of the diploid cell lineages in these sexual organs produces haploid gametocytes via meiosis. During this reproductive stage, cells communicate closely with each other and as a result, male and female gametes are established independently (for reviews, Ma 2005; Sun *et al.* 2007). In addition, PCD in anther tapetum cells is essential and key processes for normal reproductive development (Wu and Cheun 2000; Varnier *et al.* 2005; Li *et al*. 2006). It is also known that a certain CMS prematurely causes the PCD in tapetum cells (Balk and Leaver 2001) and high temperature induces PCD and oxidative stress response in plant culture cells (Vacca *et al.* 2004, 2006)

Recent molecular genetical analyses using model plants *Arabidopsis* and rice make possible identification of essential genes involved in reproductive development (**Table 1**). In addition, interactions of mitochondrial and nuclear genes that affect CMS are determined in lots of plant species (for reviews, Hanson and Bentolila 2004; Linke and Börner 2005; Chase 2007; Carlsson *et al*. 2008). Moreover, microarray technology established as a post genome project is

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Invited Review

Table 1 Male sterile mutants in *Arabidopsis* and rice plants.

Plant species	Mutants	Putative gene function	Phenotype	Reference
Arabidopsis	*defective in anther dehiscence 1 (dad1)*	phospholipase A1	male sterility	Ishiguro *et al*. 2001
Arabidopsis	*extra sporogenous cells (exs)*	LRR receptor kinase	male sterility	Canales *et al*.2002
Arabidopsis	*excess microsporocyto 1 (ems1)*	LRR receptor kinase	male sterility	Zhao *et al*. 2002
Arabidopsis	*tapetum determinant 1 (tpd1)*	unknown protein	male sterility	Yang SL *et al*. 2003
Arabidopsis	*male meiocyto death 1 (mmd1)*	PHD-containing nuclear protein	male sterility	Yang X *et al*. 2003
Arabidopsis	*somatic embryogenesis receptor kinase 1 (serk1, serk2)*	receptor kinase	male sterility	Colcombet *et al*. 2005
Arabidopsis	*ms1*	PHD-finger protein	male sterility	Wilson *et al*. 2001; Vizcay-Barrena and Wilson 2006
Arabidopsis	*dysfunctional tapetum (dyt1)*	transcription factor	male sterility	Zhang *et al*. 2006
Arabidopsis	*At Rad51*	DNA recombinase protein	male and female sterility	Li *et al*. 2005
Arabidopsis	*At Spo11*	DNA topoisomerase	male and female sterility	Stacey *et al*. 2006
Arabidopsis	*myb26/male sterile 35*	transcription factor	male sterility	Yang *et al*. 2007
Arabidopsis	*gne1, gne2*	ND	male sterility	Sorensen *et al*. 2002
rice	*undeveloped tapetum 1 (udt1)*	DNA binding protein	male sterility	Jung *et al*. 2005
rice	*tapetum degeneration retardation (tdr)*	DNA binding protein	male sterility	Li *et al*. 2006
rice	*wax-deficient anther 1 (wda1)*	CER-like protein	male sterility	Jung *et al*. 2006
rice	*msp1*	LRR receptor kinase	male sterility	Nonomura *et al*. 2003

Table 2 The effect of abiotic stresses in plant reproductive development.

Plant species	Abiotic stresses	Abnormal tissues observed	References
Arabidopsis	heat	male organ	Kim *et al*. 2001
barley	high temperature	male organ	Sakata *et al*. 2000; Abiko *et al*. 2005; Oshino *et al*. 2007
canola	high temperature	flower	Polowick and Sawhney 1988
cowpea	high temperature	male organ, flower	Ahmed *et al*. 1992, 1993
Cymbidium	high temperature	male organ	Ohno 1991
maize	high temperature	male organ	Mitchell and Petolino 1988
rapeseed	high temperature	male and female organ	Young *et al*. 2004
rice	heat stress	female organ	Takeoka *et al*. 1991
rice	high temperature	anther	Matsui and Omasa 2002
rice	high temperature	male organ	Mamun *et al*. 2006
rice	high temperature	spikelet	Jagadish *et al*. 2007
rice	cold stress	male organ	Nishiyama 1970; Satake and Hayase 1974; Nishiyama 1976
rice	high temperature	male and female organ	Satake and Yoshida 1978
snap bean	temperature	flower	Konsens *et al*. 1991
sorghum	high temperature	male organ	Jain *et al*. 2007
tomato	temperature	flower	Sawhney 1982
tomato	heat stress	male organ	Peet *et al*. 1998; Pressman *et al*. 2002
wheat	heat stress	male and female organ	Saini *et al*. 1983
wheat	heat, water deficit, abscisic acid	male organ	Saini *et al*. 1984
wheat	water deficit	male organ	Lalonde *et al*. 1997

very useful for accelerating gene discovery and developing working hypotheses for gene regulations of developmental processes and environmental stress responses (Endo *et al.* 2002; Amagami *et al.* 2003; Endo *et al*. 2004; Wang *et al.* 2005; Wellmer *et al.* 2006; Oshino *et al.* 2007).

We also know that the reproductive stage of plant development is much more sensitive to environmental stresses than the vegetative stage in cereals (**Table 2**). For this reason, changes in climate such as lower or higher temperatures, drought and abnormal levels of rain, can cause abortion of plant reproductive development, leading to severe decreases in crop yields. Many researchers have focused on the mechanisms by which environmental stresses inhibit plant reproductive development and these processes have been gradually elucidated with accumulating cytological, biochemical and molecular information.

Since plant male reproductive development and sterility are particularly sensitive to environmental stresses, we have reviewed the information relating to these processes and discussed future issues and perspectives. In particular, we have focused on the serious problem of high temperature injury, since global circulation models predict that increasing greenhouse gasses will elevate the average global temperature between 1.1 and 6.4°C during the 21st century (Lobell and Field 2007).

MALE REPRODUCTIVE CELLS

Anthers comprise a series of differentiated cells

Plant reproductive development initiates from an apical meristem via differentiation of a reproductive primordium. This primordium then proliferates through several cell divisions, concomitantly differentiating into the appropriate cells for each position. Coordination between cell proliferation and differentiation brings about normal morphogenesis of functional reproductive organs, such as the stamen and pistil. In particular, the anther develops in a sequential manner and it is composed of several types of cell including the epidermis, stomium, endothecium, middle layer, tapetum, connective, vascular bundle and microspore (**Fig. 1**) (Goldberg *et al.* 1993; Canales *et al.* 2002; Ma 2005). In the mature cylindrical anther, microspores are enveloped by four layers of cell, i.e. the tapetum, middle layer, endothecium and epidermis, in order from the center outward, respectively.

Many mutants that exhibit male sterility have now been isolated (Chaudhury *et al.* 1994; Taylor *et al.* 1998; Sanders *et al.* 1999) and well characterized using *Arabidopsis* and rice plants as listed in **Table 1**, and careful cytological analyses of these mutants has begun to elucidate the roles played by different cell layers in anther development (for reviews, Ma 2005; Sun *et al.* 2007).

The epidermis represents the outermost anther layer (fourth layer from the center), in which the gene encoding

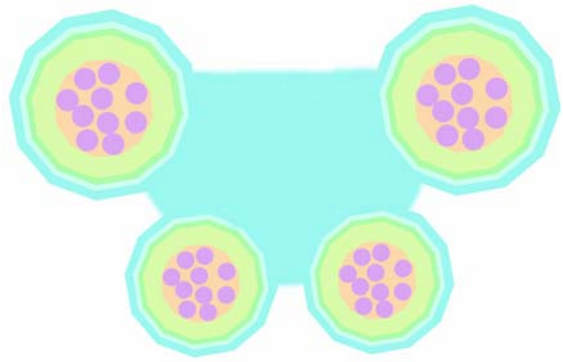

Fig. 1 Schematic illustration of structure of anther cells. Differentiated anther cell layers are composed of several types of cell including the epidermis (blue), endothecium (light blue), middle layer (green), tapetum (light green), and microspore (purple). These cell layers communicate with each other, and developmental program and fate, such as progression to meiosis of PMCs, cell-proliferation arrest and degradation by PCD in anther wall cells, are well organized.

the transcription factor Wda1 is strongly expressed in rice (Jung *et al.* 2006). In the mutant *wda1*, the anther wall develops without cuticles and in addition to this wall irregularity, the microsporocytes do not become coated with pollen-wall exine at the tetrad stage, resulting in abortion. These results suggest that in the anther epidermal cell layer, Wda1 functions directly in wax synthesis and indirectly in pollen coat formation (Jung *et al.* 2006).

The anther's endothecium is the third layer from center and it is here that fibrous deposition occurs and specifically, there is a rich secondary thickening comprised of cellulose and lignin. Consequently, endothecium cells form stronger cell walls and elongate longitudinally (Turner and Hall 2000; Turner *et al.* 2007). The gene encoding transcription factor Myb26/MS35 is expressed preferentially in endothecium cells and in a mutant of this gene male sterility occurs via abortion during secondary thickening (Mitsuda *et al.* 2007; Yang *et al.* 2007). This terminal failure causes arrested dehiscence of the anther wall and the mature pollen is not released. However, since this mutant does not exhibit other pollen development abnormalities such as differentiation of the middle layer or tapetum cells, this finding suggests that the endothecium might only be essential for anther dehiscence.

There are a few sterile male mutants that demonstrate abnormalities of the middle layer cells. In a sterile male mutant *Gus-negative1* (*gne1*) of *Arabidopsis*, both middle layer and tapetum cells are vacuolized and enlarged during late meiosis, which leads to flattened sporogenous cells (Sorensen *et al.* 2002). Identification of a gene such as *gne1* has long been expected, and this gene is important to the discussion regarding interactions between middle layer and tapetum cells.

Many sterile male mutants show abnormal phenotypes in both microspores and neighboring tapetum cells (**Table 1**). Since tapetum cells provide microspores with several enzymes and nutrients that are required for organization of pollen coat exine, these mutants actually illustrate the close relationship between microspores and the tapetum. Although tapetum cells and microspores differentiate from different cell lineages (**Fig. 2**), the differentiation and subsequent disintegration of the former coincides with the post-meiotic program of PMCs. Thus, male sterility is associated with both premature and delayed degradation of tapetum cells.

Male sterility has been reported with some meiotic defects in PMCs. In all eukaryotes, Rad51 is essential for DNA recombination during meiosis and the defect *At-rad51C* in *Arabidopsis* causes abnormal microspores that vary in size after the tetrad stage. However, these microspores can continue to differentiate and eventually become irregular pollen grains of varying sizes (Li *et al.* 2005). Similar irregular pollen grains are found in the *Arabidopsis* mutant *spo11*, which encodes a meiosis-specific endonuclease that is required for initiation of meiotic recombination; this gene is also conserved widely among eukaryotes (Stacey *et al.* 2006). These reports indicate that although the tapetum cells may not show abnormalities, failure in meiotic recombination affects the size of microspores and resulting pollen grains. Thus, it appears that microspores do not positively control function and differentiation of tapetum cells.

Cell lineage and cell-cell communication during anther development

Cell lineage and cell-cell communication play important roles in the processes of cell differentiation and proliferation during the development of multicellular organisms (Dahmann and Basler 1999). In the structure enveloping microspores, each cell layer differentiates from primary parietal cells (**Fig. 2**) and since the number of cells in each layer is under strict control, we cannot find certain abnormalities, such as loss of the middle layer on one side or direct contact between the tapetum and endothecium via patches. What is the control mechanism underlying cell proliferation and differentiation during anther development? One part of this control is regulated by cell lineage, as indicated from cytological analyses of tobacco and *Arabidopsis* plants (Goldberg *et al.* 1993; Canales *et al.* 2002). Further elucidation of the influence of cell lineage is expected to arise with the increasing availability of new genetic methods such as clonal analysis.

Cell-cell communication provides a means of control that is dependent upon positional information. In *Arabidopsis*, a mutant in the leucine-rich repeat (LRR) receptor-like protein kinase gene *EXCESS MOCROSPOROCYTES1* (*EMS1*)*/EXTRA SPOROGENOUS CELLS* (*EXS*) exhibits an increased number of microspores that lack a tapetum, resulting in a sterile male phenotype (Canales *et al.* 2002; Zhao *et al.* 2002; Sun *et al.* 2007). Interestingly, the total cell number is almost identical to the number of microspores and tapetum cells in wild-type plants. *EMS1/EXS* is expressed strongly in normally developing tapetum cells (Canales *et al.* 2002; Zhao *et al.* 2002). The gene *TAPETUM DETERMINANT1* (*TPD1*) encodes a putative small secreted protein (Yang SL *et al.* 2003) that is expressed primarily in developing anther microsporocytes. In the absence of TPD1, the inner secondary parietal cells develop into microsporocytes instead of tapetum cells, with a phenotype that is identical to that of the *ems1/exs* mutant (Yang SL *et al.* 2003). In addition to a complementary expression pattern between *EMS1/EXS* and *TPD1*, Yang *et al.* 2005 showed that ectopic expression of TPD1 activates cell division in the transgenic carpel and delays degeneration of tapetum cells. This finding implies that activation of cell

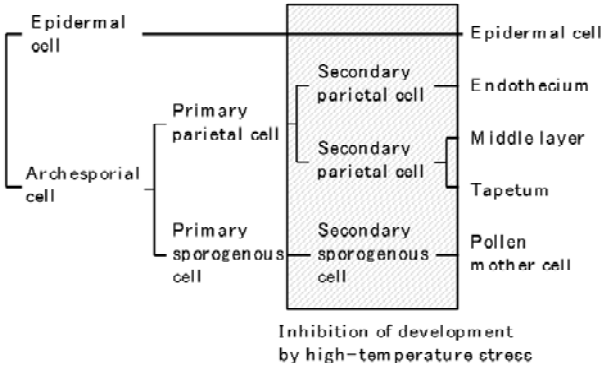

Fig. 2 Cell lineage of male gametophytes and anther wall cells affected by high temperature. The developmental events of anther cells and the sensitive event affected by high temperature stress are illustrated.

division is dependent upon normal EMS1/EXS function. Furthermore, these results suggest that EMS1/EXS and TPD1 control tapetal fate via cell-cell signaling between sporogenous and tapetum cells. In rice, the gene *MULTIPLE SPOROCYTE1* encodes a receptor-like protein kinase that exhibits considerable homology and function to EMS1/EXS (Nonomura *et al.* 2003). This finding suggests that at the very least, there may be conservation of cell-cell signaling between the LRR receptor and its ligand within the male reproductive development and differentiation of the *Magnoliophyta*.

Altered reporter gene expression patterns have been observed in anther cell layers from a heterozygotic mutant of a cell cycle-related gene (Inzé and De Veylder 2006) and this finding suggests the presence of an additional relationship between cell fate determination and division. It is difficult to study altered reporter gene expression in homozygotic mutants of cell cycle-related genes, because almost all of these mutations are lethal prior to initiation of sexual organ development (Hemerly *et al.* 2000; Capron *et al.* 2003; Dissmeyer *et al.* 2007). The use of tissue-specific and developmental stage-specific conditional knockout techniques may overcome such lethality problems, leading to elucidation of the regulatory mechanisms between the cell fate determination and division.

MALE STERILITY ACCOMPANIED WITH ANTHER DEGENERATION

Cytoplasmic male sterility with abnormalities of anther or pollen development

Maternally-inherited male sterility is common among several plant species (for reviews, Hanson and Bentolila 2004; Linke and Börner 2005; Chase 2007; Carlsson *et al*. 2008). Sterility caused by failure to produce functional pollen is attributed to mitochondrial mutations called CMS, and the mutations can be suppressed or counteracted by the products of one or more nuclear genes known as *restorer-of-fertility* (*Rf*) genes (Hanson and Bentolila 2004; Linke and Börner 2005; Chase 2007). CMS phenotypes encompass a wide range of male reproductive abnormalities, such as homeotic changes, carpeloid stamen and petaloid stamen, degenerate anther, and abortion of pollen maturation, but not in female or vegetative organs (Hanson and Bentolila 2004; Linke and Börner 2005; Chase 2007; Carlsson *et al.* 2008). The CMS type with homeotic changes has been studied in tobacco cybrids whose plants are regenerated from fused protoplasts with the nuclear genome of *Nicotiana tabacum* and the cytoplasm of *Hyoscyamus niger*, in wheat, carrot, and *Brassica* (Zubko *et al.* 2001; Murai *et al.* 2002; Linke *et al.* 2003; Carlsson *et al.* 2008). These male reproductive organs (stamens) are converted to female reproductive organs (carpels), or to petals, in which early steps of flower formation are impaired.

This section focuses on other CMS types accompanied with abnormalities of anther or pollen developments. In the PET1 CMS of sunflower, premature tapetum degeration with abnormal vacuolation occurs during meiosis of PMCs (Balk and Leaver 2001). In addition, the hallmarks of mitochondria-signaled PCD such as cytochrome *c* release from mitochondria and fragmentation of nuclear DNA are observed in the PET1 CMS (Balk and Leaver 2001). In maize plants with the Texas type CMS, the tapetum and middle layer cells show premature degeneration with features of necrotic cell death after meiosis of PMCs (Warmke and Lee 1977). In PCF CMS of petunia, similar premature tapetum degeneration is observed (Conley and Hanson 1994). These CMS plants reveal aborted pollen, in which phenomena are similar to premature abortion of tapetum cells following high temperature injury of wheat and barley, as described later.

In Owen CMS of sugar-beet plants, the abnormal enlargement of tapetum cells occurs (Majewska-Sawka *et al.* 1993; Matsuhira *et al.* 2007). Strong expression of anther-specific lipid transfer protein (LTP) associated with normal degeneration of tapetum cells is inhibited in the CMS sugar beet (Matsuhira *et al*. 2007). The phenotype of abnormal enlargement of tapetum cells is similar to that of chilling injury in rice plants (Nishiyama 1970, 1976).

CMS-associated loci in the mitochondrial genomes of several plant species include an ATPase subunit gene and its neighboring gene (Hanson and Bentolila 2004; Linke and Börner 2005). In the case of PET1-CMS in sunflower, Texas-type CMS in maize, Owen CMS in sugar beet, and PCF CMS in petunia, *atp8*, *atp6/atp4*, *atp6* or *atp9* in mitochondrial genes are associated with each CMS (Dewey *et al.* 1986; Young and Hanson 1987; Sabar *et al.* 2003; Yamamoto *et al.* 2005). In addition, nuclear *Rf* genes that suppress or compensate for the CMS-associated mitochondrial genotype have been characterized, and these include aldehyde dehydrogenase in Texas type CMS (Liu *et al.* 2001; Liu and Schnable 2002) and a pentatricopeptide motif gene in PCF-CMS of petunia (Bentolila *et al.* 2002) and Boro-CMS of rice (Kazama and Toriyama 2003; Komori *et al.* 2004; Wang *et al.* 2006). Aldehyde dehydrogenase (RF2 protein) accumulates in the mitochondrial matrix of maize and in *Escherichia coli*, recombinant RF2 has been shown to catalyze oxidation of both acetaldehyde and glycolaldehyde (Liu *et al.* 2001). Thus, it appears that disruption of a metabolic process in mitochondria results in a defect of anther development in maize. Another significant pentatricopeptide repeat protein appears to perform RNA editing in the chloroplast (Kotera *et al.* 2005) and this protein is a member of a large gene family known from *Arabidopsis* and rice, which are predicted to target to mitochondria and/or chloroplasts. In the rice Boro-CMS system, a pentatricopeptide repeat protein corded onto the RF locus regulates transcriptional editing of the ATPase subunit *atp6* on the mitochondrial genome (Kazama and Toriyama 2003). These results suggest that mitochondria may alter the cellular metabolic processes of certain CMS systems, in particular affecting tapetum cells during microsporogenesis.

Effects of abiotic stresses on reproductive processes

Plants are directly and strongly affected by abiotic stresses that relate to water, temperature, light and nutrients. Since plants cannot move autonomously, their ability to adapt to environmental change may be better than that of animals. However, plant reproductive development is more sensitive to abiotic stresses than vegetative growth, and these sensitivities are often reflected in crop yields. Thus, the effects of abiotic stresses on plant reproductive development have been examined for many different plant species (**Table 2**). Given the extent of this literature, we have focused on high temperature injury. With increasing greenhouse gasses, this type of damage represents a serious problem for the future.

In *Arabidopsis*, heat shock disrupts pollen development in a stage-specific manner, with floral stage 9 (three anther wall layers are evident and PMCs undergo meiosis) primordia failing to produce any pollen grains (Kim *et al.* 2001). In tomato, moderate increases in temperature disrupt sugar metabolism and proline translocation during male reproductive development (Sato *et al.* 2006). In cowpea, male sterility occurs with high temperatures during floral development, due to premature degradation of the tapetum and lack of endothecial development (Ahmed *et al.* 1992). In wheat, two types of abnormal microsporogenesis are caused by high-temperature stress (raising the air temperature by 10°C for 3 days) and these abnormalities occur at the onset of meiosis (Saini *et al.* 1984). The first results from premature tapetal degeneration during meiosis. Although the PMCs complete meiosis, the microspores fail to orient along the periphery of the anther lumen and do not undergo pollen grain mitosis 1 (PGM1). The abnormal and immature microspores have an exine but no cytoplasm, and plants ultimately exhibit complete loss of spikelet fertility. In the second type of abnormal microsporogenesis, all microspores

complete PGM1 but only some of the microspores complete PGM2 and develop into normal pollen grains (Saini *et al.* 1984). The remaining microspores fail to complete PGM2 and do not accumulate starch. Therefore, the anthers contain a mixture of fertile and sterile grains. Interestingly, wheat male sterility is also induced by water deficit during PMC meiosis and both loss of reproductive cell orientation and abnormal vacuolization of the tapetum, are observed. Since this appears similar to high temperature stress injury in barley plants, we use the double-rowed barley (*Hordeum vulgare* L. cv. 'Haruna-nijyo') as a model for studies of floral development and high-temperature injury during anther development. Reproductive growth of individual plants and development of each spikelet in a panicle can be synchronized under controlled conditions in a growth cabinet (Sakata *et al.* 2000; Abiko *et al.* 2005; Oshino *et al.* 2007). 'Haruna-nijyo' is also the standard barley strain for genome and cDNA projects that are currently in progress. In later sections of this review, we introduce and discuss high temperature injury in barley plants.

HIGH TEMPERATURE INJURY

Experimental conditions for high temperature injury to barley reproductive development

Plants are grown in a growth cabinet at 20°C during the day and 15°C at night, with a 16 h photoperiod. Under these conditions, the panicle of the main stem develops to *ca*. 1 mm in length at 15 days after sowing. At this stage, differentiation of the inflorescence is nearly identical to the four-leaf stage (when the tip of the fourth leaf has emerged). At the five-leaf stage, the panicles are *ca*. 2–3 mm in length and each spikelet contains three stamen primordia and one pistil primordium (**Fig. 3**). Epidermal cells and archesporial cells are observed in the stamen primordia. Over the next 5 days, beginning at the five-leaf stage, the panicles progress to about 10 mm in length, with PMCs and tapetum cells developing in the anther (Sakata *et al.* 2000; Abiko *et al.* 2005; Oshino *et al.* 2007). A single 17 mm panicle contains PMCs at a developmental stage between prophase of meiotic division I (leptotene) and meiotic division II (tetrad). PMC development in the middle region of the panicle is slightly faster than at the proximal or distal regions (Sakata *et al.* 2000).

Following exposure of barley plants to high temperatures (30°C day/25°C night) for 5 days at the 4-leaf stage, pollen grains develop without a cytoplasm, an observation typical of Type 1 damage. Type 2 damage occurs after 5 days of high temperature treatment applied between the early stages of panicle differentiation and PMC meiosis (the 5-leaf stage), after which the anthers completely lack pollen grains at the heading stage. Type 3 damage occurs during the meiotic stage of PMCs (the 6- to 7-leaf stage) and high-temperature treatment causes formation of abnormal and immature microspores that do not accumulate starch (Sakata *et al.* 2000). Thus, these experimental systems represent a very useful means of studying male sterility induced by environmental stresses (**Fig. 3**). Under the same conditions, similar high temperature injury could be observed in other six-rowed barley cultivars (Higashitani *et al* unpublished).

Cytological analyses of high temperature injury in barley

Exposure of plants to high temperatures at the 5-leaf stage (2–3 mm panicles), results in the complete abortion of organ development, as well as of differentiation of tapetum cells and PMCs (Sakata *et al.* 2000; Abiko *et al.* 2005). Arrested proliferation in developing anther cells is more acute when high temperatures begin at the 5-leaf stage, than at the 4-leaf stage, suggesting that developing anther cells acclimatize during longer periods of high temperature stress. Under high-temperature conditions that begin at the 4-leaf stage and continue to the meiotic stage of PMCs (the six- to seven-leaf stages), the four cell layers (epidermal, endothecium, middle layer and tapetum) in the anther wall and the PMCs develop in 10 mm panicles. However, the anther wall cells exhibit increased vacuolization and over-development of chloroplasts. In PMCs, meiotic prophase chromosomes show premature synapses and nuclear membranes are partially disrupted. In the anther wall cells of 15 mm panicles, premature degradation of tapetum cells, abnormal swelling of mitochondria and irregular rough endoplasmic reticulum (RER) are observed (**Fig. 4**). In addition, a significant reduction in the nuclear density of microsporocytes is found in 15–20 mm panicles grown under high temperature conditions (Oshino *et al.* 2007).

Under control conditions, the mitotic index of anther wall cells reduces gradually in 5, 10 and 15 mm panicles; differentiation is almost completed in the latter. Sporogenous cells divide frequently in 5 and 10 mm panicles, but division decreases significantly in 15 mm panicles, as the cells proceed into meiotic prophase (Oshino *et al.* 2007). In contrast, high-temperature treatment causes a drastic reduc-

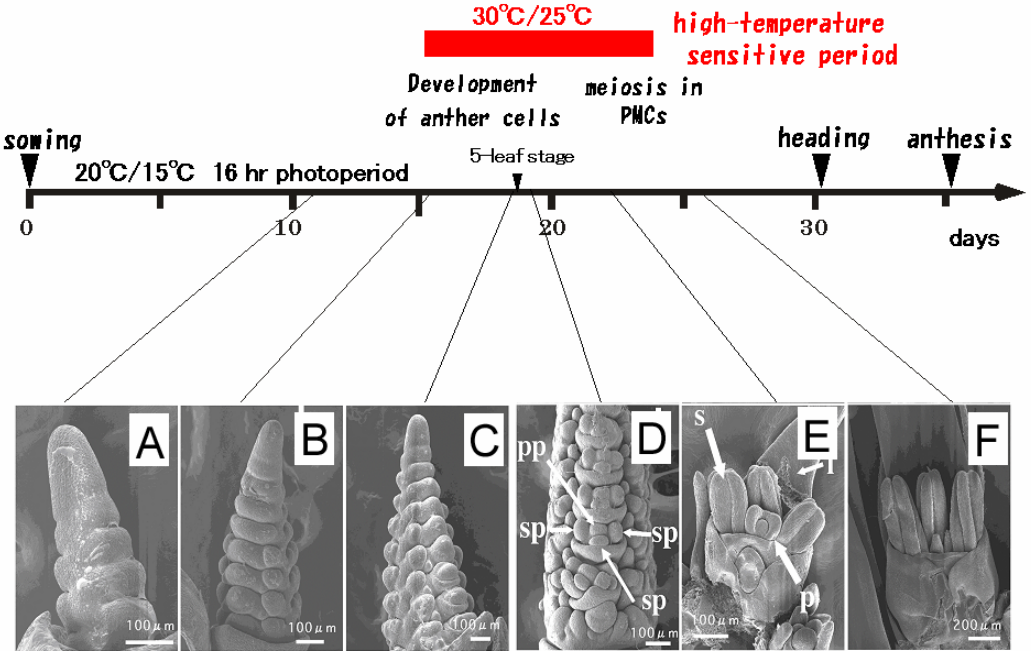

Fig. 3 Overview of reproductive development and high temperature sensitive periods in barley. Inflorescence apex is differentiated at Ca. 10 days after sowing (**A**: the 3-leaf stage) and is proliferated at the 4-leaf stage (**B**). Pistil primordium (pp) and stamen primordium (sp) are differentiated at the 5-leaf stage (**C**, **D**). Meiosis of pollen mother cells occurs when panicle lengths are 15 to 18 mm (**E**). Degradation of middle layer cells and tapetum cells is observed in 20 mm panicles (**F**). This figure is modified from the data described in Sakata *et al.* 2000.

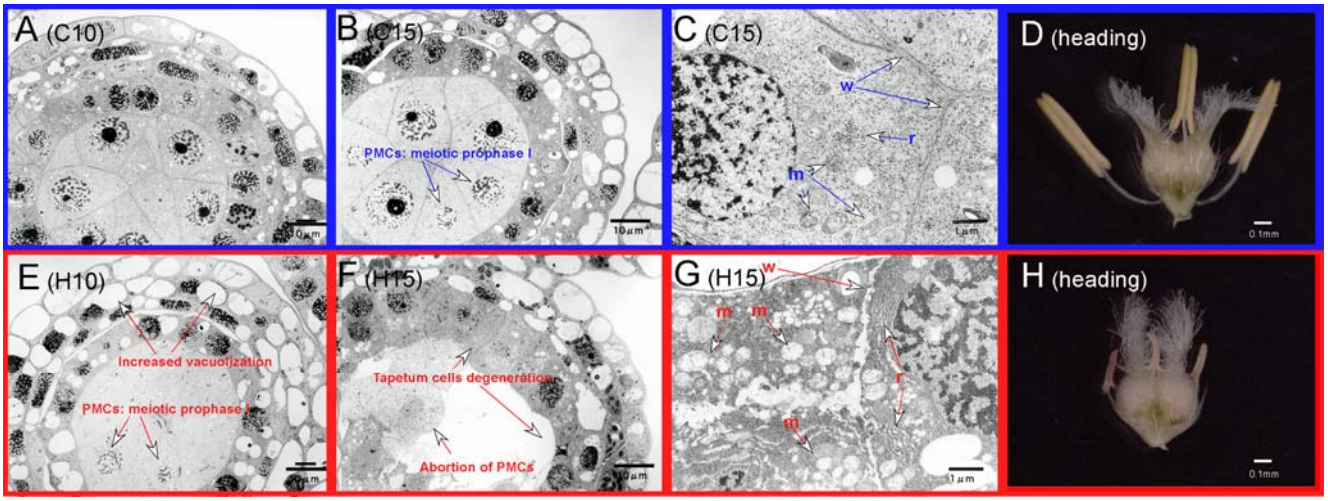

Fig. 4 Effect of high-temperature treatment on early development of barley anther cells. Anthers under normal temperatures (**A-D**) and exposed to high temperatures (**E-H**) are indicated. Ultra-thin sections were stained with uranyl acetate/lead citrate and were by TEM. Meiotic synapses of chromosomes of PMCs are observed in 15 mm panicles under normal temperatures (**B**). Increasing vacuolization and premature progression of meiosis of PMCs are revealed at high temperatures in 10 mm panicles (**E**). Premature degradation of tapetum cells with mitochondrial swelling (m), abnormal rough endoplasmic reticulum (r), and degraded wall (w) occurs at high temperatures in 15 mm panicles (**F, G**). Abnormal short anthers completely lacking pollen grains, and fertile and morphologically normal pistils, in plants exposed to high temperatures form the 4-leaf stage to the heading stage (**H**). This figure is modified from the data described in Oshino *et al.* 2007.

tion in the mitotic index of anther wall cells and primary sporogenous cells. Premature PMCs are rarely found to be dividing in 10 mm panicles and a similar observation can be made for anther wall cells in 15 mm panicles. In contrast, ovule cell division is observed under both control and high-temperature conditions (Oshino *et al.* 2007).

These results indicate clearly that high temperature injury beginning at either the 4- or 5-leaf stage, causes male tissue-specific arrest of cell proliferation. In addition, these results show that the cells most susceptible to high temperature stress are the secondary parietal and secondary sporogenous cells (**Fig. 2**). Moreover, cuticles in the outermost layer of the anther epidermis do not develop well under high temperature conditions (Sakata and Higashitani unpublished). This abnormality may involve Wda1, which is involved in wax synthesis during epidermal cuticle development, as well as for exine formation in pollen (reference in section 3-1: (Jung *et al.* 2006). Thus, it appears that the tapetum may perform important functions in formation of both pollen and the anther epidermis.

Programmed cell death during reproductive development and high temperature injury

Part of the plant PCD signaling pathway may be similar to that of animals (van Doorn and Woltering 2005; Kim *et al.* 2006; Vacca *et al.* 2006, 2007). For example, a *Bax inhibitor-1* mutant exhibited increased sensitivity to heat shock-induced cell death in *Arabidopsis*, indicating that the gene product (Bax inhibitor) functions identically in plants and animals, i.e., as a suppressor of cell death (Watanabe and Lam 2006). The cell death signal transduction pathway plays an important role in regulation of plant reproductive development (Wu and Cheun 2000). PCD degenerates ovule antipodal cells during female sexual organ development and synagid cells also degenerate after extension of the pollen tube into the female sexual organ. PCD is observed in some anther wall cells and anther dehiscence is initiated by programmed destruction of stomium. In rice and lily, tunnel analysis indicates that tapetum cells are generally degraded by PCD (Varnier *et al.* 2005; Li *et al.* 2006).

Interestingly, when tapetum PCD is inhibited or delayed by extragenic induction of a PCD inhibiter such as the Bax inhibitor, pollen development aborts and male sterility occurs (Kawanabe *et al.* 2006). Abnormal enlargement of tapetum cells is observed with chilling injury in rice (Nishiyama 1970; Satake and Hayase 1974; Nishiyama 1976) and with Owen CMS in sugar-beet (Majewska-Sawka *et al.* 1993; Matsuhira *et al.* 2007). It is possible that such cell enlargement is due to inhibition of PCD.

In contrast, premature degradation of tapetum cells can be caused by miss-timed PCD. The association between male sterility and premature PCD of the tapetum cells has been observed in a thermosensitive sterile male rice line (Ku *et al.* 2003) and in a PET1-CMS mitochondrial mutation in sunflower (Balk and Leaver 2001).

Recently, certain genes have been implicated in PCD degeneration of tapetum and middle layer cells. Using rice, Li *et al*. (2006) isolated a sterile male *tapetum degeneration retardation* (*tdr*) mutant in a gene that encodes a putative bHLH transcription factor. Another rice bHLH transcriptional factor *Udt1* (*UNDEVELOPED TAPETUM1*) (Jung *et al.* 2005) is considered to be the upstream transcription factor for *TDR* (Li *et al.* 2006). In addition, chromatin immunoprecipitation assays have identified two downstream target genes of TDR, *OsCP1* and *Osc6*, which encode a Cys protease and a protease inhibitor, respectively (Li *et al.* 2006). Cys proteases and their inhibitors are known to be associated with PCD in various stress responses and during leaf senescence (Minami and Fukuda 1995; Solomon *et al.* 1999; Xu and Chye 1999).

In addition to anther wall cells and probably under more severe conditions than described here for high temperature stress, heat shock stress has been shown to induce PCD and cell cycle arrest in *Arabidopsis* vegetative cells and cultured tobacco cells (Panchuk *et al.* 2002; Kim *et al.* 2003; Coffeen and Wolpert 2004; Vacca *et al.* 2004; Kim *et al.* 2006; Vacca *et al.* 2006; Watanabe and Lam 2006; Vacca *et al.* 2007). It has also been reported that hydrogen peroxide is induced during the heat shock process (Jang *et al.* 2005; Volkov *et al.* 2006). These results suggest that in the case of high temperature injury to anthers, premature PCD causes premature degradation of tapetum cells and thus, proper timing of tapetum degeneration is essential for normal pollen development.

Global changes in gene expression are related to high temperature injury and male sterility

Genetic studies in *Arabidopsis* and rice have identified many genes related to plant reproductive development and some have been described above. Currently, some plant genome projects have been completed, while others remain in progress. However, several post genome analyses are now avail-

able, including one that used microarray technology to perform a parallel assessment of thousands of genes within a single experiment. Such technology is very useful for accelerating gene discovery and developing working hypotheses with respect to the regulation of environmental stress responses and developmental processes. In the pioneer model plants *Arabidopsis* and rice, microarrays have been used to perform genome-wide analyses of gene expression during floral development (Endo *et al*. 2002; Amagami *et al*. 2003; Endo *et al*. 2004; Wang *et al.* 2005; Wellmer *et al.* 2006). In addition, stage-specific expression profiles of reproductive plant organs have been reported (Mandaokar *et al.* 2003; Wang *et al.* 2005; Wellmer *et al.* 2006; Deyhle *et al.* 2007).

In contrast, there are few reports describing the alterations that environmental stresses cause on gene expression in plant reproductive organs (Dupuis and Dumas 1990; Mascarenhas and Crone 1996; Imin *et al.* 2004; Oshino *et al.* 2007). However, global alteration in vegetative tissues with respect to heat shock and/or high temperature stress, have been investigated more extensively. These studies have found induction of different physiological responses such as fluidity and transmission rate in cell membranes, as well as altered enzymatic activity and secondary physiological changes such as oxidative stress (Hopf *et al.* 1992; Dat *et al.* 1998a; Gray *et al.* 1998; Larkindale and Knight 2002; Sangwan *et al.* 2002; Baniwal *et al.* 2004; Charng *et al.* 2007; Swindell *et al.* 2007).

In developing barley panicles, we have found up-regulation of several genes in response to altered temperature conditions (Oshino *et al.* 2007). These up-regulated genes can be categorized within certain groups, such as a stress-induced protein group, plant hormone-related protein group, photosystem- and chloroplast-related protein group and a mitochondria-related protein group. These analyses indicate that high temperature brings about many physiological changes in plant cells. For example, heat tolerance in seedlings is related to hormones via involvement with the ethylene- and ABA-signaling pathways (Larkindale *et al.* 2005; Kotak *et al.* 2007).

We also observe that following exposure to high temperatures, genes encoding anther-specific lipid transfer protein (LTP) and certain other unknown proteins, exhibit shifts in transcription to an earlier stage in panicle development (Oshino *et al.* 2007). Some of these genes may be related to the PCD process in anther cells. Interestingly, Crimi *et al*. (2006) use an *in vitro* mammalian mitochondrial system to show that maize LTP elicits a pro-apoptotic effect (release of cytochrome *c* from the mitochondrial membranes). They also suggest functional and structural similarities between plant LTPs and the mammalian BH-3 protein Bid (Crimi *et al.* 2006). In contrast, the transcriptional repression of anther-specific LTP is observed in Owen CMS of sugar-beet plants, which have abnormally enlarged tapetum cells that do not degenerate (Matsuhira *et al.* 2007). Anther-specific LTPs are highly expressed in mature tapetum cells just prior to degeneration and these results suggest that transcriptional control and function of anther-specific LTPs are important for PCD-mediated degeneration of the tapetum. Thus, a diagnostic of high temperature injury may be premature degeneration of tapetum cells.

Interestingly, our microarray analysis also reveals that high temperatures cause transcriptional repression of several genes involved in DNA replication and cell proliferation such as histones, DNA polymerase, replication licensing factors and ribosomal proteins (Oshino *et al.* 2007). This transcriptional repression occurs specifically in developing anther cells such as secondary sporogenous cells, tapetum and middle layer cells, but not in female reproductive development or growth of vegetative tissues. This tissue-specific repression is closely linked to a drastic reduction in the mitotic index of anther wall and primary sporogenous cells caused by increasing temperatures.

CONCLUDING REMARKS

In this review, we introduced the complex mechanisms underlying reproductive development and differentiation in plants with respect to male sterility. We also showed that this defect can be caused by several factors including proteins encoded by both nuclear and mitochondria genomes, as well as environmental stresses. Accumulating molecular and genetic information has allowed the identification of many genes related to development and differentiation, as well as indicating certain widely-conserved processes in flowering plants.

In particular, we discussed molecular mechanisms relating to high-temperature injury during barley anther development. We demonstrated how sterility is related to premature progression of the anther early development program and cell fates, as well as to comprehensive alterations in transcription patterns. It seems that involvement of PCD in anther-specific development may have caused the male reproductive process to be more sensitive to certain environmental stresses than the female reproductive development process or vegetative growth.

In *Arabidopsis* seedlings, *hot1*, *hot2*, *hot3*, and *hot4* mutants are isolated as defects of basal heat tolerance. *HOT1* and *HOT2* encode HSP101 and an endochitinase-like protein, respectively (Hong and Vierling 2000, 2001). *hot2* mutant seedlings are sensitive not only to heat shock but also to salt and water stresses. Free cytosolic calcium also plays a role in the thermotolerance of mustard plants (Dat *et al.* 1998b) and salicylic acid and HSPs are both required for basal and acquired thermotolerance (Larkindale *et al.* 2005; Kotak *et al.* 2007). Further forward and reverse genetic analyses on the reproductive and vegetative tissues of model plants such as *Arabidopsis* and rice, will be important for developing our understanding of tolerance to environmental stresses in male reproductive development. It is likely that these findings will eventually be applied to other crops.

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