REVIEW PAPER

The final split: the regulation of anther dehiscence

Zoe A. Wilson1,*, Jie Song2, Benjamin Taylor1 and Caiyun Yang1

1 School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK
2 John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK

* To whom correspondence should be addressed. E-mail: zoe.wilson@nottingham.ac.uk

Received 14 October 2010; Revised 6 January 2011; Accepted 12 January 2011

Abstract

Controlling male fertility is an important goal for plant reproduction and selective breeding. Hybrid vigour results in superior growth rates and increased yields of hybrids compared with inbred lines; however, hybrid generation is costly and time consuming. A better understanding of anther development and pollen release will provide effective mechanisms for the control of male fertility and for hybrid generation. Male sterility is associated not only with the lack of viable pollen, but also with the failure of pollen release. In such instances a failure of anther dehiscence has the advantage that viable pollen is produced, which can be used for subsequent rescue of fertility. Anther dehiscence is a multistage process involving localized cellular differentiation and degeneration, combined with changes to the structure and water status of the anther to facilitate complete opening and pollen release. After microspore release the anther endothecium undergoes expansion and deposition of ligno-cellulosic secondary thickening. The septum separating the two locules is then enzymatically lysed and undergoes a programmed cell death-like breakdown. The stomium subsequently splits as a consequence of the stresses associated with pollen swelling and anther dehydration. The physical constraints imposed by the thickening in the endothecium limit expansion, placing additional stress on the anther, so as it dehydrates it opens and the pollen is released. Jasmonic acid has been shown to be a critical signal for dehiscence, although other hormones, particularly auxin, are also involved. The key regulators and physical constraints of anther dehiscence are discussed.

Key words: Anther development, Arabidopsis, dehiscence, endothecium, male sterility, pollen.

Introduction

The development and release of functional pollen is critical for plant reproduction and selective breeding. Hybrid vigour, heterosis, is often determined by non-mutually exclusive mechanisms that result in the superiority of a hybrid over its parents, with respect to rapid growth rate and increased yields (Lippman and Zamir, 2007). However, the emasculation process required for the generation of hybrids can be extremely time consuming and labour intensive, thus male-sterile lines are often favoured in commercial hybrid seed production (Wilson and Zhang, 2009). There is therefore a need for a better understanding of the processes involved in anther development that may provide effective mechanisms for the efficient generation of hybrids and for the general control of fertility. Providing a mechanism for rescuing fertility is also an essential requirement for hybrid production. Therefore, approaches for controlling male fertility that rely upon pollen release, rather than pollen generation, may provide good opportunities for future hybrid development.

The final stages of anther development and pollen release involve a switch from cellular differentiation to degeneration. Specialized cell types within the anther determine the site of anther opening, and synchronized development of the pollen with the anther wall tissues coordinates the timing of dehiscence. Previously there has been much focus on the development of functional pollen (Scott et al., 2004; Ma, 2005; Feng and Dickinson, 2007); however, there has been less discussion about the relatively uncharacterized events associated with anther dehiscence and pollen release. This review will therefore concentrate on these later events.
Anther and pollen development

Understanding of the basic process of anther and pollen development has increased over recent years, with much information coming from detailed analyses of male-sterile mutants in *Arabidopsis* (Sanders et al., 1999; Scott et al., 2004; Ma, 2005; Wilson and Zhang, 2009; Feng and Dickinson, 2010).

Pollen is formed within specialized organs, the stamens, in the flower. These comprise a wider upper region that forms the anther that contains the pollen, and a stalked region, the filament, containing the vasculature, which extends to ensure that pollen is released away from the region, the flower (Smyth et al., 1990; Goldberg et al., 1993). The major events in *Arabidopsis* anther development have been placed into 14 stages by Sanders et al. (1999). The initiation of floral organ primordia from pluripotent cells within the floral meristem involves AGAMOUS (*AG*), alongside other homeotic genes, to form organ initials according to the ABCDE model (Robles and Pelaz, 2005). Many of the key genes regulating the subsequent development of the different cell layers in anther and pollen formation have been identified (Fig. 1).

Initially divisions in the L1, L2, and L3 layers of the floral meristem bring about the formation of the stamen primordium. Divisions in the L1 layer increase the surface area of the anther and form the epidermis, whilst L3 cells divide to form connective and vasculature tissues. Percinical divisions of the L2 cells result in the formation of four clusters of archesporial cells in the anthers (Fig. 1). These then divide to form the sporogenous layer (Sp) and the primary parietal layer (PP), which goes through further divisions to form the maternal layers of the anther (Fig. 2A). This results in a final anther structure comprised of four maternal cell layers: the outer epidermis, endothecium, middle cell layer, and tapetum surrounding the inner sporogenous cells (Fig. 2B) (Sanders et al., 1999; Scott et al., 2004; Feng and Dickinson, 2007).

Fig. 1. Anther and pollen development pathway. WUSCHEL (WUS) activates AGAMOUS (AG) at the centre of the floral apex, the number of pluripotent cells increases, then AG represses WUS to promote differentiation. Stamen initiation is controlled by the homeotic genes APETALA3 (AP3), PISTILLATA (PI), and AG, with the primordia forming as a tetrad of archesporial cells. AG induces microsporogenesis via activation of NOZZLE/SPOROCYTELESS (NZZ/SPL) (Ito et al., 2004). The transcription factors JAGGED (JAG) and NUBBIN (NAB) are also involved in the process of defining stamen structure (Dinneny et al., 2006). Two CLAVATA 1-related leucine-rich repeat receptor-like protein kinases, BARELY ANY MERISTEM1 (BAM1) and BAM2, act in a regulatory loop with NLZ/SPL to promote somatic cell types and to restrict NZZ/SPL expression to the inner region of the locule (Hord et al., 2006; Feng and Dickinson, 2010). Archepsprial cell number and tapetal cell fate is controlled by a leucine-rich repeat receptor kinase EXTRA SPOROGENOUS CELLS/EXCESS MICROSPOROCYES1 (EXS/EMS1) (Canales et al., 2002; Zhao et al., 2002) and its ligand TAPETAL DETERMINANT1 (TPD1) (Jia et al., 2008). The SERK1 and SERK2 complex is thought to form a receptor complex with EMS1 in the tapetal plasma membrane (Albrecht et al., 2005; Colcombet et al., 2005), which then binds TPD1 (Yang et al., 2003, 2005) (indicated by the red dotted line). Tapetal development is initiated by DYSFUNCTIONAL TAPETUM1 (DYT1) (Zhang et al., 2006) and DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION1 (TDF1) (Zhu et al., 2008), with tapetal maturation, pollen wall formation, and tapetal programmed cell death (PCD) involving ABORTED MICROSPORES (AMS), MALE STERILITY1 (MS1) (Wilson et al., 2001), and ABCWBC27 (Sorensen et al., 2003; C. Yang et al., 2007; Xu et al., 2010) with the MALE GAMETOGENESIS IMPAIRED ANTHERS (MIA) gene, encoding a type V P-type ATPase, MALE STERILITY2 (MS2), and LEUCINE AMINOPEPTIDASE (LAP3). The final stage of dehiscence involves jasmonic acid (JA)-induced gene expression and transcription factors associated with endothecium secondary thickening, MYB26 (Steiner-Lange et al., 2003; Yang et al., 2007) and the NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST2 double mutant (Mitsuda et al., 2007). Regulation is indicated by arrows; green arrows indicate proven direct regulation; inhibition is shown by a T-bar.
The process of anther dehiscence

Pollen release requires careful timing and regulation so that there is synchronized development of the anther and the flower, to ensure that pollen release occurs at the optimal time to maximize either cross- or self-fertilization. Anther dehiscence is a multistage process that involves localized cellular differentiation and degeneration, combined with changes in the structure and water status of the anther to facilitate complete anther opening and pollen release.

Anther opening involves two types of specialized cells, the cells separating the two lobes of the anther (septum) that break down to form a single locale, and the stomium which is made from modified epidermal cells which splits to facilitate anther opening (Fig. 3). The differentiation of these cells occurs early during anther development stage 4, before pollen mother cell meiosis when the endothecium and connective walls are formed (Sanders et al., 1999, 2005; Ma, 2005). Initially the septum degenerates, resulting in a bilocular anther, which is followed by stomium cell breakage and pollen release (Sanders et al., 1999; Scott et al., 2004).

The endothecium and the localized secondary thickening within this cell layer also play a critical role in anther opening and pollen release. Endothecium development is coordinated with pollen maturation and the degeneration of the anther tapetum and middle layer. This process involves the expansion and subsequent deposition of ligno-cellulosic thickening in the endothelial cells, the breakdown of the septum in the anther wall, combined with anther dehydration and pollen swelling, resulting in stomium breakdown and anther opening (Keijzer, 1987; Bonner and Dickinson, 1989; Scott et al., 2004).

Stomium structure

The stomium is formed by the differentiation of epidermal cells within the anther, to form a single cell region, the location of which determines the position of anther opening (Fig. 3). Detailed microscopy in tomato revealed that the development of intersporangial septa and epidermal cell differentiation are early events occurring as the archesporial cells and tapetum differentiate, with subsequent development of the endothecium (Bonner and Dickinson, 1989). The endothecium then undergoes selective deposition of secondary thickening, whilst the stomium and septum region between the locules does not undergo thickening. This localized thickening is critical for subsequent anther opening. Prior to dehiscence the stomium undergoes cell death and splitting; this does not appear to require viable pollen to be present since splitting is still seen in male-sterile lines generated by tapetal cell ablation (Koltunow et al., 1990; Mariana et al., 1990).

The importance of a functional stomium for dehiscence has been demonstrated by cell ablation studies in tobacco.
surround the locule (Cheng 1989), whereas in maize the endothecium completely surrounds the stipe adjacent to the stomium (Bonner and Dickinson, 1979) only develops in the distal one-third of the anther in the male flowers to determine the form of stomium opening (Garcia et al. 2008). However, the same effect was observed if the surrounding tissues were protected by barstar expression, with only specific ablation of the stomium, indicating the importance and independent role of the stomium in anther opening, compared with the surrounding cells.

The majority of dehiscence research has been carried out on a limited number of species including Arabidopsis, Lilium, rice, maize, and members of the Solanaceae, including tobacco, tomatoes, and aubergine/eggplant. The basic process of anther dehiscence appears quite conserved across different plant species, with the dicot model plant Arabidopsis thaliana (Sanders et al., 1999) showing a similar dehiscence process to the Solanaceae (Bonner and Dickinson, 1989; Sanders et al., 2005) and to the monocots rice (Matsui et al., 1999) and maize (Keijzer et al., 1996). However, subtle differences exist between the species that influence anther structure and thus final opening position.

Analysis of anther dehiscence and stomium structure in 30 Solanum species identified variation in the shape and histological features of the stomium, with three main types of dehiscence mechanism observed: (i) poricidal, where dehiscence occurs through a small apical pore; (ii) poricidal-longitudinally dehiscing, where there is an apical pore but the opening continues down in a longitudinal split; and (iii) longitudinally dehiscing anthers where the stomium forms along the entire anther length (Garcia et al., 2008). However, in all cases the stomium consisted of small epidermal cells that serve as the only anther wall layer. The differentiation of these stomial cells and the distribution of thickening in the endothecium around the stomium determine the form of stomium opening (Garcia et al., 2008). Variation has also been observed in the structure of the endothecial layer; for example, in tomato the endothecium only develops in the distal one-third of the anther in the region adjacent to the stomium (Bonner and Dickinson, 1989), whereas in maize the endothecium completely surrounds the locule (Cheng et al., 1979).

In solanaceous species, specialized cell types are found in the ‘notch’ region under the stomium; these have been referred to by various names including the circular cell cluster, intersporangial septum, or hypodermal septum. Their development has been characterized in detail in tobacco (Sanders et al., 2005). They originate as subepidermal cells derived from the L2 layer that differentiate early during anther formation; these expand and accumulate small vesicles, whereas the flanking ‘pre-stomium’ cells do not divide and remain cytoplasmically dense. The circular cell clusters are typically 2–3 cells deep as a consequence of periclinal divisions; these then expand, the small vesicles fuse to form a large central vacuole, and calcium oxalate crystals accumulate. During stomium degeneration the calcium oxalate is released from the circular cell cluster onto the pollen and is subsequently transferred to the stigma upon pollination. The role of calcium oxalate has not been fully established, but it has been suggested that it provides calcium ions required for the pollen germination process (Iwano et al., 2004). Cell-specific ablation studies have, however, shown that the stomium functions independently from the circular cells to control dehiscence (Beals and Goldberg, 1997). The presence of the circular cells and calcium oxalate accumulation appear to be a feature of the Solanaceae, and do not occur in Arabidopsis or Lilium.

Degeneration of cells in the anther

Enzymatic breakdown of the septum

At the cellular level, anther dehiscence is similar to siliqua dehiscence and, like microspore separation, is thought to involve cell wall-degrading enzymes which break down the pectin between cells (Roberts et al., 2002). Several hydrolytic enzymes and proteins linked to cell wall loosening are thought to be involved, including polygalacturonases (PGs), β-1,4-glucanases, and expansins (Bonghi et al., 1993; Taylor et al., 1993, 1994; Lashbrook et al., 1994; del Campillo and Bennett, 1996; Cho and Cosgrove, 2000).

These enzymes are part of large gene families that have not been fully characterized but have been shown to act redundantly in specific cell types; for example, in the Arabidopsis and rice genomes there are at least 69 and 59 predicted PGs, respectively (Kim et al., 2006; Gonzalez-Carranza et al., 2007). It has been suggested that one group of related PGs tend to be expressed in flowers and flower buds (Torki et al., 2000; Kim et al., 2006). A number of these have been characterized in Arabidopsis and linked to changes in pollen wall development; QUARTET1 (QRT1) and QRT2 (Rhee and Somerville, 1998) and QRT3 (Rhee et al., 2003) are required for degradation of the pollen mother cell wall as microspores are released from their tetrads. Three endo-PGs have been identified as involved in anther dehiscence, siliqua dehiscence, and floral abscission; ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1) and ADPG2 are both required for siliqua dehiscence, whilst all three (ADPG1, ADPG2, and QRT2) are required for anther dehiscence (Ogawa et al., 2009). Floral abscission is regulated by jasmonic acid (JA), ethylene, and abscisic acid (ABA); all three of these PGs have been shown to be regulated by JA, ADPG2 by ethylene (Gonzalez-Carranza et al., 2007) and QRT2 by ethylene and ABA (Ogawa et al., 2009). This suggests that anther dehiscence-related PG activity is also likely to be regulated by JA, ethylene, and ABA. Recently a PG gene, PS-2, which is expressed concurrently with anther dehiscence, has also been described in tomato, which when mutated results in a failure of anther dehiscence (Gorguet et al., 2009).
Programmed cell death (PCD) of the septum and stomium

The importance of tapetal PCD for successful pollen formation has been highlighted by a number of male-sterile mutants that fail to go through normal tapetal breakdown (Kawanabe et al., 2006; Li et al., 2006; Vizcay-Barrena and Wilson, 2006; Parish and Li, 2010). However, the anther septum and stomium go through a process of degeneration and cell death to facilitate pollen release, and this is also thought to be via a PCD-related process (Kuriyama and Fukuda, 2002; Sanders et al., 2005).

There have been a number of reports of dehiscence mutants resulting from changes to endothecium and stomium degeneration (Sanders et al., 1999). For example, anthers in the non-dehiscence1 mutant undergo an abnormal cell death programme, which results in endothecium degeneration and indirectly causes failure of stomium region breakage, although the pollen appears normal in this mutant (Sanders et al., 1999). Work in Lilium suggests that PCD appears to commence in the tapetal tissues and then extends to the outer anther tissues, including the middle cell layer, stomium region, and later, after dehiscence, to the endothecium and connective tissue (Varnier et al., 2005).

Ultrastructural analysis of the interlocular septum, the connective tissue, middle layer, and epidermis surrounding the stomium of Solanum lycopersicum revealed features consistent with PCD, including nuclear condensation, shrinkage of the plasma membrane, and aberrant mitochondrial morphology (Senatore et al., 2009). They also observed the accumulation of ricinosomes, precursors of protease vesicles, in these cells during the dehiscence process. Ricinosomes have been shown to harbour KDEL-tailed cysteine proteases, which are involved in the final process. Ricinosomes have been shown in the pistils and appear to be involved in pollen tube attraction; however, no phenotype has been observed in the T-DNA insertional knockout (Higashiyama, 2010). They have been shown to bind copper and to display a high redox potential; therefore it is likely that the Arabidopsis plantacyanin gene has the ability to produce reactive oxygen species (ROS). Overexpression of the plantacyanin gene brings about premature PCD of the endothecium during anther stage 14, at which point the wild-type endothecium is still active (based on staging by Sanders et al., 1999), and indehiscent anthers (Dong et al., 2005). Increased expression of the plantacyanin gene is also seen in the receptor-like protein kinase2 (rpk2) mutant, which displays a lack of the middle cell layer, tapetal hypertrophy of the anthers, and defects in endothecial thickening (Mizuno et al., 2007). These defects may therefore be a consequence of increased ROS activity in specific anther cell types, which alter the normal PCD processes within the anther.

Types of endothecial secondary thickening

In general, the process of dehiscence is similar in many species; however, there are subtleties associated with differences in anther structure (Garcia, 2002b) and types of anther endothecium thickening (Garcia, 2002a). Four main types of endothecium thickening have been observed, and these tend to occur in a species-specific manner: (i) annular rib types, which are single radial rings which are unconnected and run in parallel to each other; (ii) helical rib types, which form as a helix along the periclinal cell axis, which is also described as a U-shaped thickening; (iii) reticulate ribs, where irregular thickening forms on every face with multiple sites of branching and anastomising sites to form a network; and (iv) palmate ribs, where ribs and a solid plate form in the inner periclinal wall of every cell.

The structure of the endothecial cell layer has also been shown to be critical for dehiscence. The insertion mutant of the maize HD-ZIP IV gene OCL4 (OUTER CELL LAYER4) shows a partial male sterility that is subject to
environmental changes; this reduced fertility is thought to result from the presence of an extra subepidermal cell layer with endothecium characteristics in the anther wall (Vernoud et al., 2009). During early development the outer secondary parietal layer divides periclinally in addition to the normal anticlinal division, resulting in a double endothecium-like layer. These periclinal divisions are restricted to the distal part of the anther (Vernoud et al., 2009). The epidermis-specific OCL4 expression in immature anthers is restricted to the region of the anther locule where the extra differentiation. In the subepidermal cell layers associated with endothecium, the epidermis-specific OCL4 expression is also observed in the distal part of the anther (Vernoud et al., 2009). The epidermis-specific OCL4 expression in immature anthers is restricted to the region of the anther locule where the extra differentiation. The epidermis can therefore regulate the events in the subepidermal cell layers associated with endothecium differentiation.

**Regulation of endothecium secondary thickening**

In the *Arabidopsis* anther, the endothecium is first established during anther stage 5. It undergoes expansion during anther stages 6-10 and develops secondary cell wall thickening during anther stage 11, at which point bar-like ligno-cellulosic fibrous bands are deposited (Sanders et al., 1999; Scott et al., 2004); however, the regulatory network controlling the biosynthesis and selective deposition of this thickening has not yet been fully elucidated.

Endothecium secondary thickening is essential for providing the mechanical force for anther dehiscence (Keijzer, 1987; Bonner and Dickinson, 1989). This has been demonstrated experimentally by analysis of *Arabidopsis* male-sterile mutants myb26 (Dawson et al., 1999; Steiner-Lange et al., 2003) and the *nac secondary wall thickening promoting factor1 (nst)* nst2 double mutant (Mitsuda et al., 2007). Disruption of endothelial thickening in these mutants results in a failure of anther dehiscence and male sterility. Phloroglucinol and ethidium acridine orange staining indicate that this thickening is composed of lignin as well as cellulose (Dawson et al., 1999; Yang et al., 2007). The composition of this thickening also seems important since disruption of monolignol biosynthesis, in the triple mutant (*ccc*), which carries mutations in cinnamoyl CoA reductase1 (CCR1), cinnamyl alcohol dehydrogenase c (CAD), and CAD d, results in a reduction of stem lignin by 50% and male sterility due to abnormal endothecial secondary thickening (Thevenin et al., 2010).

Many of the reported *Arabidopsis* dehiscence mutants have defects linked to JA biosynthesis; however, JA does not appear to regulate *MYB26* expression (Mandaokar et al., 2006) and methyl jasmonate treatment does not rescue the *myb26* mutation (Dawson et al., 1999), indicating that this pathway is distinct from the JA pathway. In the *myb26* mutant, anther development appears normal up to anther stage 11 (Sanders et al., 1999); however, during the later stages, the characteristic band-like ligno-cellulosic wall thickenings seen in the wild-type anther endothecium wall do not form. Degradation of the septum and formation of stomium take place normally in the mutant; however, the endothecium cells fail to expand, they collapse and the subsequent shrinkage of the anther walls does not occur, which results in failure of anther opening and pollen release (Dawson et al., 1999). Overexpression of *MYB26* results in ectopic secondary thickening in various above-ground tissues (Yang et al., 2007). *MYB26* therefore appears to be a critical factor regulating endothecium expansion and secondary thickening in a cell-specific manner in the anther.

Two NAC domain transcription factors, NST1 and NST2, have also been shown to be involved in the regulation of endothecium wall thickening (Mitsuda et al., 2005). NST2 is expressed predominantly in anther tissues, whilst NST1 is also expressed in vegetative tissues. The double mutant *nst1nst2* has an anther-indehiscent phenotype due to lack of secondary thickening in the endothecium, which is similar to the myb26 mutant (Mitsuda et al., 2005). Overexpression of *MYB26* induces ectopic secondary thickening and expression of *NST1NST2*, suggesting that they act downstream of *MYB26* (Yang et al., 2007).

The control of secondary wall formation in interfascicular fibres and secondary xylem also involves NST1 and SECONDARY WALL ASSOCIATED NAC DOMAIN PROTEIN1 (SN1, also called NST3) (Mitsuda et al., 2007; Zhong et al., 2007). NST1 and SN1 function redundantly in the regulation of secondary thickening in fibres (Mitsuda et al., 2007; Agalou et al., 2008) and siliques (Mitsuda and Ohme-Takagi, 2008). Dominant repression of NST1 or SN1 and the double knockout caused a severe reduction in the secondary thickening of fibres, whilst overexpression of either gene induced ectopic secondary thickening in various above-ground tissues (Zhong et al., 2006; Mitsuda et al., 2007). This and the phenotypes of ectopic secondary thickening as a consequence of overexpression of *MYB26* (Yang et al., 2007) and NST1 or NST2 (Mitsuda et al., 2005) suggest that there is high conservation in the secondary thickening pathways of floral and vegetative tissues.

The *Arabidopsis* UPCURLED LEAF 1 (UCL1) gene may act upstream to control secondary thickening by regulating the expression of *MYB26*. Dehiscence fails to occur in the *ucl1* mutant; this mutation is thought to be due to the up-regulation of one of the 16 *Arabidopsis* Class IV HD-ZIP genes, HOMEODOMAIN GLABROUS 3 (HDG3) (Li et al., 2007). Overexpression of HDG3 resulted in a down-regulation of *MYB26*, NST1, and NST2 expression, suggesting that HDG3 plays a negative role in regulating anther dehiscence. No phenotype was observed in the single *hdg3* mutant, suggesting possible redundancy with other members of the HD-ZIP family (Li et al., 2007). However, expression of HDG3 is low in wild-type anthers; therefore, the effect on dehiscence could also be a consequence of ectopic expression.

The RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) gene has also been found to be involved in secondary cell wall thickening in the endothecium, although the expression of RPK2 was not detected in this tissue during anther development (Mizuno et al., 2007). The *rpk2* mutant is male sterile and exhibits a number of defects associated with
pollen development, including abnormal tapetal development, a lack of middle cell differentiation, and a failure of anther dehiscence due to the endothecial cell collapsing because of reduced secondary thickening (Mizuno et al., 2007). It is thought that RPK2 functions as a plasma membrane-bound receptor controlling anther development (Mizuno et al., 2007), which may be required for endothecial thickening, or else the presence of an enlarged tapetum, or a lack of middle layer means that the endothecium in the rpk2 mutant fails to form normally. Expression analysis in the rpk2 mutant identified down-regulation of many genes linked to pollen development and secondary thickening, including a number of PGs, genes involved in lignin biosynthesis (e.g. phenylalanine ammonia-lyase and peroxidase), and a number of stress-related genes, particularly those associated with ABA (Mizuno et al., 2007). It has also previously been shown that the abscisic acid-insensitive 8 (abi-8) mutant is male sterile, exhibits defects linked to secondary thickening and cellulose biosynthesis, and is associated with cross-talk between the JA and ethylene pathways (Brocard-Giffer et al., 2004). This implies a direct, or indirect, link between these final events of anther development and stress responses.

Cytokinins have also been implicated in the regulation of secondary wall formation, since Arabidopsis histidine-containing phosphotransferase 4 (AHP4) has been shown to negatively regulate secondary thickening in the endothecium (Jung et al., 2008). The AHP proteins are mediators of the multistep phosphorelay pathway of cytokinin signalling. Overexpression of AHP4 results in a reduction of endothecial thickening, while the ahp4 mutant showed a slight reduction in thickening. AHP4 expression also correlated to levels of IRREGULAR XYLEM1 (IRX1), 6, and 8 gene expression, suggesting a link to the cellulose biosynthesis pathway (Jung et al., 2008).

Opening the anther

Anther opening involves differential forces on the anther walls to facilitate rupture of the septum and stomium and then subsequent opening of the anther (Fig. 4). Observations of the process of dehiscence in Gasteria verrucosa suggest that as pollen intine formation occurs, the epidermal and endothecial cells of the anther lose some of their starch and start tangential and radial expansion, which is then followed by endothecial secondary thickening (Keijzer, 1987). However the stomium and septum region between the locules does not undergo secondary thickening. The septum undergoes enzymatic lysis of the middle lamellae, with the mechanical swelling of the bordering epidermal cells (Keijzer, 1987) facilitating stomium opening. Tangential swelling of the epidermis and endothecium increases the circumference of the locule wall; however, because the endothecium walls have secondary thickening the inner locule wall dimensions are fixed. This outer enlargement combined with the inner fixed dimensions causes the locule wall to bend inwards, resulting in disruptions to the stomium cells (Fig. 4). The small epidermal cells facing the septum (stomium) are then mechanically broken by inward bending of the adjacent locule walls.

In rice it has been shown that the force created by anther desiccation does not appear sufficient for stomium and septum rupture; however, the swelling of the pollen grains generates the force that is required for septum lysis (Matsui et al., 1999) (Fig. 4). On the other hand, the later stages of locule opening appear dependent upon desiccation of the anther, with shrinkage of the tangential outer wall, which is limited by secondary thickening, providing the force for opening (Keijzer, 1987).

Dehydration of the anther wall

The final stages of anthesis involve the dehydration of the endothecium and epidermal cells, which cause the locule to bend outwards. It has been suggested that this occurs, at least in part, as a consequence of evaporation via stomata on the adaxial side of the anthers, since anther dehydration can be affected by relative humidity (Keijzer, 1987). Keijzer (1987) observed in G. verrucosa that evaporation was more rapid from older anthers, and suggested that this may be as a consequence of low relative humidity inside the bud. This could also be due to active removal of water, since depletion of starch from the anther filaments in G. verrucosa coincided with osmotic retraction of water from the anthers (Keijzer, 1987).

Observations of the water status in tomato anthers has revealed differential regions of anther dehydration, suggesting relocation of water within the anthers and petals, and that desiccation is unlikely to be a major factor in the dehydration of the anther walls (Bonner and Dickinson, 1990). At the time of anther wall dehydration, Bonner and Dickinson (1990) also observed the depletion of starch from the connective tissue surrounding the vasculature and proposed that the conversion of starch to sugar would serve to increase the osmotic potential of the anther tissues and could provide a mechanism for dehydration within specific cell types and regions of the anther. More recent work in Arabidopsis has shown localized accumulation of the H+-sucrose transporter, AtSUC1, around the connective tissues of anthers, which may serve to increase osmotic potential and induce dehydration of the surrounding regions in the anther (Stadler et al., 1999).

Analysis of dehiscence in Petunia suggests that this dehydration may partly be caused by retraction of water from the anthers to the nectaries. It has been proposed that the Petunia NECTARY1 (NEC1) and NEC2 genes may function in the upper part of the filament and stomium cells to alter the starch to sugar balance and regulate water potential in the stomium and nectaries, resulting in anther dehydration (Ge et al., 2000, 2001). It therefore seems likely that an active process of dehydration is occurring within the anther to provide the final force for anther opening (Fig. 4).

Active water movement in the anther may also be due to localized accumulation of cations; Matsui et al. (2000) observed the transfer of potassium ions from the anther locule onto barley pollen grains post-anthesis. They
proposed that the swelling of pollen, which is at least partly responsible for septum rupture, was due to the osmotic effect of the potassium ion accumulation in the pollen grains. This has also been supported by observations of high levels of potassium in the stomium area of barley anthers (Rehman and Yun, 2006), suggesting that the potassium ions may play a role in attracting water from the surrounding regions and causing the swelling of the endothecium and pollen prior to anther opening. Potassium ion accumulation has also been suggested to play a role in filament extension in lily (Heslop-Harrison et al., 1987). High levels of potassium have also been identified in mature pollen of other species, for example Lilium and Tradescantia paludosa (Bashe and Mascarenhas, 1984; Heslop-Harrison et al., 1987), and the presence of potassium channels and changes in potassium level have been linked to subsequent pollen tube germination (Bashe and Mascarenhas, 1984).

Other factors may also be involved in anther dehiscence. Flavonoids are known to play a wide variety of roles in plants, from protection from ultraviolet (UV) light, to the pigmentation of flowers to attract pollinators (Shirley, 2006) and in the regulation of auxin transport (Jacobs and Rubery, 1998). In Arabidopsis the absence of FLOWER FLAVONOID TRANSPORTER (FFT) affects flavonoid levels, with the fft-1 mutant exhibiting altered root growth, seed and pollen development, and anthers that fail to dehisce (Thompson et al., 2010). The FFT transcript is localized to epidermal guard cells and it has been proposed that it may control dehiscence by playing a role in anther dehydration (Thompson et al., 2010).

Water translocation frequently occurs via plasmodesmata connections; however, the large aquaporin gene family has been shown to mediate the passive movement of water across membranes. Many of these have not yet been characterized;
The involvement of JA in anther dehiscence is also supported by the JA signal transduction mutant coronatine insensitive (coi1); its sterility is due to non-dehiscence that cannot be rescued by exogenous jasmonate (Feyts et al., 1994; Xie et al., 1998; Devoto et al., 2002). COI1 is an F-box protein, which recruits JAs and forms a complex with JAZ proteins (denoted for their ZIM and Jas motifs) in the presence of JAs. JAZ proteins repress transcription of JA-responsive genes; in the COI1–ligand–JAZ ternary complex they are polyubiquitinated and subsequently degraded by the 26S proteasome, and this allows JA signal transduction (Katsir et al., 2008). The coi1 mutant fails to respond to JA and coronatine because JAZ proteins are not degraded in the presence of these signals (Katsir et al., 2008). JA synthesized in the filaments is thought to act partly by regulating water transport in the stamens and petals, which subsequently plays a role in filament extension and dehiscence (Ishiguro et al., 2001). The DADI gene encodes a PLA1 that catalyses the production of free LA from cellular lipids as the first step in JA biosynthesis that is catalysed by several enzymes, including phospholipase A1 (PAL1), 13-lipoxygenase, allene oxide synthase, allene oxide cyclase, and 12-oxo-phytodienoic acid reductase (Fig. 5). Delayed dehiscence or non-dehiscence phenotypes have been observed in mutants defective in JA biosynthetic enzymes, including the fatty acid desaturation (fad) mutants (McConn and Browse, 1996), opr3 (mutation in 12-oxo-phytodienoic acid reductase) (Stintzi and Browse, 2000), delayed-dehiscence1 (dde1) and dde2 (Sanders et al., 2000; von Malek et al., 2002), defective in anther dehiscence 1 (dad1) (Ishiguro et al., 2001), and allene oxide synthase mutants (Park et al., 2002) (Fig. 5). In general, defects in all stages of the JA pathway (Fig. 5) appear to cause similar phenotypes of reduced filament elongation and a lack of dehiscence, although detailed morphological analysis of indehiscent anthers has only been conducted for the fad1 mutant (Ishiguro et al., 2001). In the Arabidopsis dad1 mutant the stomium fails to open and elongation of the filament is delayed; however, other features required for dehiscence, such as tapetal degeneration, septom breakdown, endothecium thickening, and formation of trinucleate pollen, occur normally (Ishiguro et al., 2001). In the dde1 mutant, which carries a defect in OPDA-reductase 3, dehiscence occurs but is delayed; however, this may be due to an incomplete block in the JA pathway and the gradual accumulation of jasmonates via alternative OPR gene family members (Sanders et al., 2000). Stomium opening and fertility can be rescued by exogenous application of jasmonate; however, specific treatments of dde1 suggest that the anthers are responsive to jasmonate treatment only during stages 9–11 (Sanders et al., 2000).

Roles of phytohormones in anther dehiscence

Jasmonic acid

Studies on a large number of dehiscent mutants have shown that jasmonates contribute to the control of anther dehiscence, filament elongation, and pollen viability (Scott et al., 2004). JA is derived principally from the 18-carbon fatty acid linolenic acid (LA), and its biosynthesis is catalysed by several enzymes, including phospholipase A1 (PAL1), 13-lipoxygenase, allene oxide synthase, allene oxide cyclase, and 12-oxo-phytodienoic acid reductase (Fig. 5). Delayed dehiscence or non-dehiscence phenotypes have been observed in mutants defective in JA biosynthetic enzymes, including the fatty acid desaturation (fad) mutants (McConn and Browse, 1996), opr3 (mutation in 12-oxo-phytodienoic acid reductase) (Stintzi and Browse, 2000), delayed-dehiscence1 (dde1) and dde2 (Sanders et al., 2000; von Malek et al., 2002), defective in anther dehiscence 1 (dad1) (Ishiguro et al., 2001), and allene oxide synthase mutants (Park et al., 2002) (Fig. 5). In general, defects in all stages of the JA pathway (Fig. 5) appear to cause similar phenotypes of reduced filament elongation and a lack of dehiscence, although detailed morphological analysis of indehiscent anthers has only been conducted for the fad1 mutant (Ishiguro et al., 2001). In the Arabidopsis dad1 mutant the stomium fails to open and elongation of the filament is delayed; however, other features required for dehiscence, such as tapetal degeneration, septom breakdown, endothecium thickening, and formation of trinucleate pollen, occur normally (Ishiguro et al., 2001). In the dde1 mutant, which carries a defect in OPDA-reductase 3, dehiscence occurs but is delayed; however, this may be due to an incomplete block in the JA pathway and the gradual accumulation of jasmonates via alternative OPR gene family members (Sanders et al., 2000). Stomium opening and fertility can be rescued by exogenous application of jasmonate; however, specific treatments of dde1 suggest that the anthers are responsive to jasmonate treatment only during stages 9–11 (Sanders et al., 2000).

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produced within the stamen filament prior to anther dehiscence. Ishiguro et al. (2001) suggest that DAD1 acts by regulating JA levels to control water transport into the vascular tissues from the endothecium, connective tissue, and anther locules. This in turn influences the development of the flowers and anthers to facilitate the correct timing of petal opening and anther dehiscence. They propose that stamen filament extension and petal elongation are initiated by production of JA via DAD1 induction in the upper part of the filament; this promotes water uptake from the locules, endothecium, and connective tissue into this region. During the later stages of development, cells in the upper and lower parts of the filament express DAD1, inducing JA and causing water removal from the anther cell walls into the filament, resulting in filament extension and subsequent petal elongation and flower opening (Ishiguro et al., 2001). This may be, at least partly, responsible for the dehydration and expansion of the anther cell layers required for anther opening. Ishiguro et al. (2001) propose that JA acts by inducing the expression of genes required for water transport in the anther, for example the plasma membrane H⁺-sucrose transporter, AtSUC1, which has been found in the parenchymatous tissues surrounding the connective tissues of anthers and has been proposed to facilitate water removal from the walls of the anther (Stadler et al., 1999). Keijzer (1987) reported that the stamen filament remains hydrated during anther wall dehydration, and this may be explained by the observed cell-specific modification of water potential in the anther prior to dehiscence (Stadler et al., 1999).

The activation-tagged mutant of SHI-RELATED SEQUENCE7 (SRS7) also shows disrupted anther dehiscence; viable pollen is produced but tapetal breakdown and anther opening do not occur, indicating a link between tapetal degeneration and anther dehiscence (Kim et al., 2010). SRS7 is predominantly expressed in the filament at the same stage as DAD1 and has similar fertility defects to JA mutants, and Kim et al. (2010) propose that SRS7 may be involved in JA signalling.
JA-induced gene expression

JA synthesis and signalling have important functions in the temporal coordination of late stamen development. Studies show that in addition to acting early in the floral primordial initiation and initiation of microsporogenesis, AGAMOUS (AG) (Bowman et al., 1991) plays a regulatory role in controlling late-stage stamen development (Ito et al., 2007), including anther morphogenesis and dehiscence, as well as filament formation and elongation. During late-stage development it has been shown that AG acts at least partly by directly regulating the transcription of DADI and therefore JA biosynthesis (Ito et al., 2007).

Expression analysis in the opr3 mutant has identified 821 genes in the stamen that are regulated by JA application and, of these, 13 are transcription factors (Mandaokar and Browse, 2009). Pollen from the opr3 mutant is inviable if manually extracted from the anther, indicating that JA is playing a role in pollen development as well as release. Two of these transcription factors, MYB21 and MYB24, act in an overlapping manner to regulate anther dehiscence. The myb21myb24 double mutant has short filaments, and the anthers and petals fail to open, but when manually extracted the pollen is viable; these defects could not be rescued by JA treatment (Mandaokar and Browse, 2009). AtMYB21 has also been shown to be repressed by the light signalling factor CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), and COP1 is required for correct tissue-specific expression of MYB21 (Shin et al., 2002). MYB21 has also been shown to directly regulate PHENYLALANINE LYSASE (PAL) and ALTERNATIVE OXIDASE (AOX) expression (Shin et al., 2002). Expression of AtMYB24 is tightly regulated during anther development, and overexpression results in various floral defects including retarded anther development and non-dehiscence. Stomium and septum lysis do not occur in the AtMYB24 overexpression lines, and reduced amounts of endothecial secondary thickening are seen, with disruption in the expression of genes in the phenylalanine pathway (Yang et al., 2007). MYB21 and MYB24 appear to act downstream as transcription factors that mediate the JA response.

Microarray analysis of gene expression in anther development in rice showed that 314 genes responded to either gibberellin (GA) or JA treatment, and 24 GA- and 82 JA-responsive genes showed significant changes in expression between meiosis and the mature anther stages (Wang et al., 2005). This suggests significant cross-talk between the JA and GA pathways.

Gibberellins

GAs have a major role in male fertility, with GA-deficient mutants showing abnormal stamen development (Chhun et al., 2007; Hu et al., 2008; Rieu et al., 2008b). GA mutants show reduced filament elongation, which is thought to be due to reduced cell extension (Cheng et al., 2004). GA is needed for the coordination and synchrony of floral organ development, as shown by analysis of the five Arabidopsis C19-GA 2-oxidases mutants (Rieu et al., 2008a). GA is also required for pollen formation, with development halting in the ga1-3 mutant post-meiosis, but prior to pollen mitotic divisions (Cheng et al., 2004). The tapetum appears to be a major site for GA production (Itoh et al., 1999; Kaneko et al., 2003; Hu et al., 2008), with a peak of GA3ox and GA3ox4 expression detected prior to tapetal breakdown and the initiation of dehiscence (Hu et al., 2008). GA biosynthesis also appears to occur in the filament, with a number of GA3oxidases showing expression there during late anther development. However, one of the early single-copy genes involved in GA biosynthesis, A1CPS, appears only to be expressed in the anther (Silverstone et al., 1997), suggesting that the anther may exert regulation over GA biosynthesis in the filaments (Mutasa-Gottgens and Hedden, 2009).

GA and GAMYB are known to be involved in the regulation of anther development (Murray et al., 2003; Cheng et al., 2004; Kaneko et al., 2004). HvGAMYB is a transcription factor shown to be up-regulated by GA, and anthers of transgenic barley overexpressing HvGAMYB are male sterile due to a failure in dehiscence with the stomium remaining intact (Murray et al., 2003). This is thought to be at least partly due to a lack of pollen expansion in these lines, resulting in reduced pressure on the stomium and a resulting failure of stomium lysis (Murray et al., 2003). GA promotion of floral organ expansion and anther development is mediated, in part, by these homeotic transcription factors. GA induction of their expression, however, did not occur in the presence of the translation inhibitor cycloheximide, indicating that it is indirect (Yu et al., 2004). Regulation of these homeotic transcription factors may also be mediated by miRNA159 and MYB33, which have been shown to be involved in GA-regulated anther development. GA enhances miR159 levels by opposing DELLA function. Interestingly, overexpression of miRNA159 caused a reduction in MYB33 transcript in Arabidopsis flowers, and anthers failed to release pollen (Achard et al., 2004). The role of MYB33 is currently unclear, except that it is known to act redundantly with MYB65, and has a facultative role during the earlier stages of tapetal development (Millar and Gubler, 2005).

Auxin

There is increasing evidence suggesting that other phytohormones are also involved in anther dehiscence. Auxin plays a key role in general floral development, organ formation, and pollen development, and also has a major effect on coordinating the maturation of pollen and the dehiscence of the anther (Cheng et al., 2006; Cecchetti et al., 2008).

Targeted expression of the rolB (root loci B), an Agrobacterium oncogene that increases auxin sensitivity, in a cell-autonomous manner in tobacco suggested that local increases in auxin caused a delay in anther dehiscence (Cecchetti et al., 2008). The regulatory role of auxin on
anther development was later confirmed by analysis of *Arabidopsis* auxin receptor mutants. In the *tir1 afb1 2 3* auxin receptor multiple mutant, early pollen maturation and anther dehiscence was seen. Endothelial lignification occurred prematurely before tapetal degeneration, and septum and stomium degeneration were early and simultaneous, rather than sequential; pollen mitotic divisions were also premature, due to induction of the cell cycle by auxin, and stamen filament elongation was reduced (Cecchetti et al., 2008). Accumulation of auxin, as a consequence of tapetal degeneration and alterations in secondary wall thickening in the anther endothecium. The orientation of thickening changed from longitudinal to transverse, thus hindering septum breakdown, resulting in male sterility; this effect could also be induced by altering auxin transport in cotton anthers (Yasuor et al., 2006).

Early during floral development in *Arabidopsis*, prior to differentiation of the pollen mother cells, expression of the auxin biosynthesis genes (YUCCA flavin monooxygenases) *YUC2* and *YUC6* was detected in the anthers (Cheng et al., 2006; Cecchetti et al., 2008). Free auxin levels, based upon expression of the DR5::GUS reporter, were seen in the tapetal tissues during stages 8 and 9 (Aloni et al., 2006) and were shown to be very high in the anther from stage 10 (tapetal degeneration and the first pollen mitotic division) to stage 12 (bilocular anther) (Aloni et al., 2006; Feng et al., 2006; Cecchetti et al., 2008). It is thought that this auxin is synthesized within the anther, since *YUC2* and 6 expression also occurs at this stage (Cheng et al., 2006; Feng et al., 2006; Cecchetti et al., 2008) and auxin transport inhibitors/mutants (*mdr1 pgpl*) have minimal effects on the late events of anther dehiscence and pollen maturation (Noh et al., 2001). Stamen filament elongation and pollen viability were reduced in the *mdr1 pgpl* auxin transport mutant (Noh et al., 2001), but was not significantly affected by the premature dehiscence observed in the *tir1 afb* multiple mutant (Cecchetti et al., 2008). These data suggest that anther dehiscence and pollen maturation are coordinated and regulated by endogenously anther-synthesized auxin, and that their development is independent of the pre-anthesis anther filament elongation, which requires auxin transport into the vasculature (Cecchetti et al., 2008). Auxin levels are highly regulated during anther development, with auxin serving to limit precocious pollen maturation and dehiscence, and to coordinate the timing of these events (Cecchetti et al., 2008).

Auxin is unlikely to act in isolation from the other hormonal pathways and is thought to regulate anther dehiscence through JA. Loss of AUXIN RESPONSE FACTOR 6 (ARF6) and ARF8 disrupts JA production and thus causes delayed or non-dehiscence, and reduced filament and petal elongation as seen in the *dad1* mutant (Tabata et al., 2010), which can be rescued by exogenous application of JA (Nagpal et al., 2005; Cecchetti et al., 2007). ARF6 and 8 have been shown to be required for activation of *DAD1* expression and therefore to regulate JA biosynthesis (Tabata et al., 2009).

However, it is also likely that another auxin-mediated pathway may affect anther dehiscence. Alterations in local auxin homeostasis, as a consequence of the up- or down-regulation of the GT trihelix DNA-binding transcription factor PETAL LOSS-D (*PTL-D*) gene resulted in a failure of septum and subsequent stomium degeneration and consequential failure of dehiscence (Li et al., 2008). However, the *ptl* mutant, which is fully sterile, cannot be rescued by JA treatment, suggesting that *PTL* is acting via an alternative auxin-mediated pathway to the JA pathway.

**Ethylene**

Retardation of dehiscence has also been observed by alteration of ethylene levels. Studies in tobacco have revealed that ethylene has an effect on the final events of dehiscence, degeneration of the stomium cells and dehydration (Rieu et al., 2003). Ethylene-insensitive tobacco flowers, resulting from mutation of the *ethylene receptor1* (*etr1*) mutant, or by treatment with the ethylene-perception inhibitor 1-methyl-cyclopropene (MCP), resulted in loss of dehiscence synchrony, with flower opening and delay in stomium cell degeneration and dehydration (Rieu et al., 2003). Two *Petunia* genes, *PhERS1* and *PhERS2*, encoding ethylene receptor homologues are proposed to regulate the timing/synchronization of stomium degeneration and anther dehiscence. Antisense suppression of *PhETR2* in *Petunia* led to stomium cell degeneration and anther dehiscence before anthesis (Wang and Kumar, 2007). The role of ethylene in regulating dehiscence in tobacco flowers has also been supported by analysis of ethylene-insensitive plants, or treatment with the ethylene perception inhibitor MCP, which resulted in delayed dehiscence, while ethylene treatment accelerated dehiscence (Rieu et al., 2003). It is therefore proposed that ethylene may act as a signal regulating anther dehiscence in tobacco and *Petunia*, in a manner similar to JA in *Arabidopsis* (Rieu et al., 2003).

**Morphological effects of stress**

Anther dehiscence has been shown to be a highly regulated process, which appears to be particularly sensitive to abiotic stress. Environmental conditions, particularly increased temperature, have a dramatic effect on pollen fertility and anther dehiscence (Sakata and Higashitani, 2008; Jagadish et al., 2010; Zinn et al., 2010). This reduction in fertility appears to be at least partly due to tissue-specific auxin reduction, since auxin treatment can at least partly compensate for high temperature stress (Sakata et al., 2010); however it is currently not known whether this is acting via alterations in endothecium thickening. Heat stress has been shown to result in protein expression changes, particularly associated with dehiscence (Jagadish et al., 2010). High temperature stress has also been linked to a reduction in the swelling of pollen, which provides the force for anther opening (Matsui et al., 2000).
Conclusions

Molecular understanding of anther dehiscence has increased over recent years. It has been shown to involve a number of key developmental processes including cellular differentiation, cell expansion, degradation, and PCD, alongside selective modifications of water status in specific cell types to facilitate pollen dispersal. The key events of this process are shown in Fig. 6. Many of these processes involve pathways similar to those found in other tissues, for example secondary thickening in the anther and vegetative tissues, and enzymatic lysis of abscission and dehiscence zones. There are many aspects of the dehiscence process which have still not been fully characterized, for example the regulation of stomium and septum formation, the precise delimitation of thickening within the anther, and the regulatory networks that determine differentiation and water movements in the anther. This means that currently it is also difficult to predict the molecular effects that physical and environmental stresses have upon the anther during opening. Providing greater understanding of such processes will provide valuable insight into controlling crop fertility in a world of changing environmental stresses.

Acknowledgements

We would like to thank the BBSRC for funding.

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