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REVIEW PAPER

From *Arabidopsis* to rice: pathways in pollen development

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Abstract

The control of male fertility is of vital importance for crop breeding, hybrid generation, and the control of pollen release. Recent development in the analysis of *Arabidopsis* male sterile mutants has meant that there is a greater understanding of the gene regulatory networks controlling maternal development of the anther and the resultant sporophytes. With the advent of the genome sequence and tools to allow the analysis of gene function, this knowledge base is now extending into the monocot crop rice. This has shown high levels of similarity between the networks of pollen development in *Arabidopsis* and rice, which will serve as valuable tools to understand and manipulate this developmental pathway further in plants.

Key words: Anther development, *Arabidopsis*, male sterility, PCD, pollen, programmed cell death, rice, tapetum.

Introduction

The development of functional pollen that is released at the appropriate stage to maximize pollination, is critical for plant reproduction and the creation of genetic diversity. Transcriptomic experiments in *Arabidopsis* using staged pollen have identified 13 977 genes that are expressed in the male gametophyte during development (Honys and Twell, 2004) and approximately 15 000 in the anther and male gametophyte (J Song and ZA Wilson, unpublished data). This clearly illustrates the diversity and extent of gene expression during anther and pollen development.

Controlling the process of pollen development and release is critical for selective breeding, the release of genetically modified (GM) pollen, and the commercial development of hybrid lines. Hybrids frequently exhibit heterosis or hybrid vigour, which means that the plants are stronger, develop more rapidly, and are higher yielding. However, the generation of hybrids is both time-consuming and expensive. A frequently used system for hybrid generation in self-fertilizing crop species, such as maize, rice, and cotton is Cytoplasmic Male Sterile (CMS) lines. CMS has been spontaneously identified in many plant species due to disturbed nuclear–mitochondrial interactions, both as a consequence of cross-hybridization strategies, or arising naturally in wild populations (Hanson and Bentolila, 2004; Pelletier and Budar, 2007). A number of CMS lines have now been characterized and the defects have frequently been linked to aberrant open reading frames (ORFs) in mitochondrial (MT) genomes (Hanson and Bentolila, 2004; Pelletier and Budar, 2007). Many of these mitochondrial defects can be recovered by nuclear-encoded genes that restore the plants to full fertility (Wang *et al.*, 2006), thus the control of fertility is possible by selecting the appropriate breeding lines. Although CMS lines have been used for the control of fertility, such traits have not been identified in all species and a lack of general understanding relating to the defects and the process of pollen development make their widespread application problematical. For example, in maize, Texas male sterile (T) cytoplasm the presence of the unusual mitochondrial gene *URF13* resulted in increased susceptibility to the pathogen *Bipolaris maydis* race T resulting in Southern Corn Leaf Blight devastation (Huang *et al.*, 1990; Wise *et al.*, 1999).

A number of transgenic approaches have also been developed for hybrid seed production, however, only one, for the generation of hybrid rapeseed (SeedLink®) has been used commercially. This technology is based upon the approach of anther specific expression of the toxic enzyme

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barnase (ribonuclease from *Bacillus amyloliquifaciens*) to confer sterility (Mariana *et al.*, 1990, 1992). Other approaches have relied upon barnase expression in two-component systems. For example, by the expression of split barnase as two inactive peptides and subsequent reconstitution (Burgess *et al.*, 2002), or more recently by expression of non-functional *barnase* and *acetolactate synthase* (*ALS*) and subsequent functional interaction through intein-based *trans*-splicing of the complementary protein fragments to generate male sterility and herbicide resistance (Gils *et al.*, 2008). Alternative systems have relied upon the suppression and restoration of the transcription of key genes associated with pollen development, for example, *MYB103* (Li *et al.*, 2007), however, such approaches are limited by the availability of suitable targets and an incomplete understanding of these pathways.

Over recent years, significant progress has been made towards understanding the process of pollen development, and much of this has come from the use of male sterile mutants in the model system *Arabidopsis*. However, the advent of the genome availability (Goff *et al.*, 2002; Yu *et al.*, 2002) and the development of tools for the analysis of gene function (Rensink and Buell, 2004) mean that such activities can now be carried out in rice. Rice performs a role both as a monocot model, but also as a crop of global economic and agronomic significance. It is estimated that approximately 645 million metric tons of rice was grown in 2007 (FAOstat, 2008; http://faostat.fao.org/) and this forms the staple diet of much of the world's population, providing 30% of the calorie intake of Asia (World Rice Statistics, International Rice Research Institute; Cheng *et al.*, 2007). Over the past 30 years the importance of hybrid rice has clearly been demonstrated with yield increases of approximately 20% over that of inbred rice (Zhong *et al.*, 2004; Cheng *et al.*, 2007). However, it is estimated that rice production will need to increase by ~30% by 2030 to feed the increasing population, so, for example, in the case of China, this would require an increase from 177 million metric tons in 2003 to 240 million metric tons by 2030 (Zhong *et al.*, 2004).

Stamen initiation and anther development

The process of stamen development involves a complex co-ordinated interaction between sporophytic and gametophytic tissues. The use of mutants with defects in specific cell types has shown that both the maternal anther tissues and gametophytic tissues are vital for the development of functional pollen. Commitment to gamete formation occurs late in plant development and is controlled by a series of developmental cues which ensure that flowering occurs at optimal stages for fertilization and subsequent seed set (Baurle and Dean, 2006; Giakountis and Coupland, 2008).

In eudicots, the floral structure occurs in successive whorls arising from the floral meristem and, in *Arabidopsis*, the flower consists of four whorls comprizing four sepals, four petals, six stamens, and a central carpel (Fig. 1A). Floral development has been divided into 12 stages (Smyth *et al.*, 1990) and stamen development is initiated alongside petal formation during stage 5. The stamen structure comprises two morphologically distinct parts, a stalk or 'filament', which serves to provide vascular links and facilitates extension of the stamen, and the anther, within which the pollen develops. The anther is composed of a number of different cell layers, which initiate by periclinal divisions in hypodermal cells in the anther primordium, to form archesporial cells in the corners of each anther primordium. Mitotic divisions of the archesporial cells then occur to form the different cell layers in the anther; initially two cell layers are formed, the inner primary sporogenous cells (PS) and the outer primary parietal cells (PP). These two cell types have different fates; the primary sporogenous cells undergo a small number of divisions to form pollen mother cells (PMCs), which go through meiotic divisions to form the meiocytes, whereas the primary parietal cells (PP) go through a series of further divisions to form the 'sterile' sporophytic cell layers of the anther wall. The PPs firstly divide to form endothecial cells and secondary parietal cells (SPC), the SPCs then divide to form the middle cell layer and the tapetum (Scott *et al.*, 2004) (Fig. 2A). The final structure results in the gametophytes being surrounded by

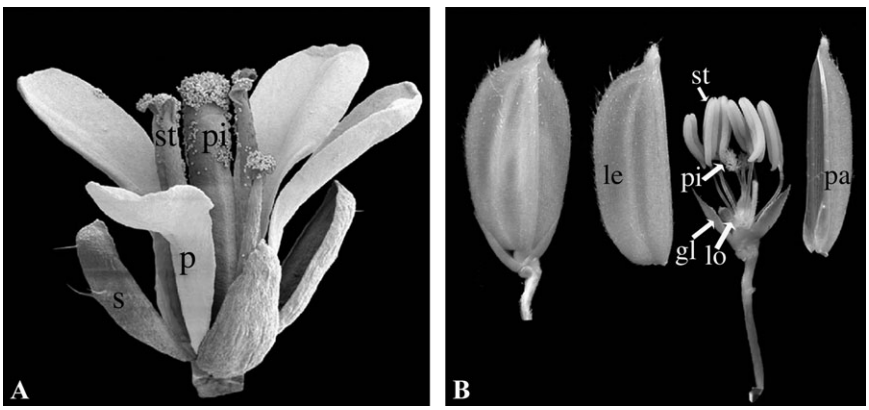

Fig. 1. Rice and *Arabidopsis*. (A) Arabidopsis flower, comprising four whorls of four sepals (s), four petals (p), six stamen (st), and a central pistil (pi). (B) Rice flower, comprizing a central pistil (pi), six stamens (st), an empty glume (gl), and two lodicules (lo) (petals) subtended by an inner bract, the palea (pa), and the outer bract, the lemma (le).

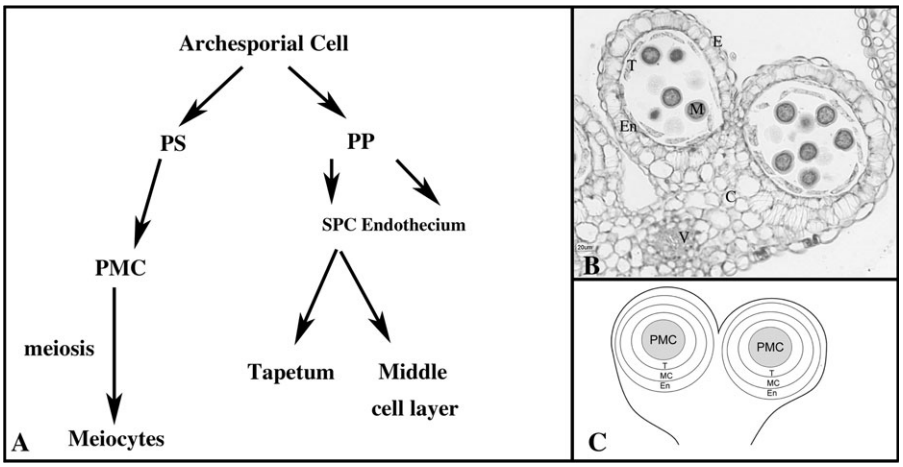

Fig. 2. Anther structure. (A) Archesporial cell divisions to form final anther cell layers. (B) *Arabidopsis* anther (bar=20 μm). (C) Diagram of the cell layers of the anther. PS, primary sporogenous cells; PP, primary parietal cells layer; PMC, pollen mother cells; SPC, secondary parietal cells; T, tapetum; MC, middle cell layer; En, endothecium, E, epidermis; C, connective; V, vasculature.

a series of cell layers, which are the tapetum, middle cell layer, endothecium, and outer epidermis (Fig. 2). *Arabidopsis* male sterile mutants, that have aberrations in some of these cell types, have shown that the various cell layers are critical for functional pollen development and release.

Archesporial specification and establishment of anther cell layers

One of the key steps associated with archesporial specification and the start of anther development is the induction of the transcription factor *NOZZLE/SPOROCYTELESS (NZZ/SPL)* (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999) by the *AGAMOUS* MADS box transcription factor (Ito *et al.*, 2004). NZZ/SPL has homology to MADS-like transcription factors and is thought to act in the L2 cell layer of the anther during archesporial division. *NZZ/SPL* is required for the initiation of sporogenesis in both stamen and carpel development and, in the *nzz/spl* mutant, the Pollen Mother Cells (PMCs) and the surrounding cell layers fail to form (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). *AG* expression occurs from early in floral initiation until late in flower development. Early expression acts in the specification of stamen and carpels (Ito *et al.*, 2004; Yanofsky *et al.*, 1990), however, the later expression is involved in anther morphogenesis and dehiscence, and filament formation and elongation (Ito *et al.*, 2007*b*). AG regulates stamen development at least in part by controlling the induction of *DEFECTIVE IN ANTHER DEHISCENCE1* (Ito *et al.*, 2007*b*), which encodes for a chloroplastic phospholipase A1 that catalyses the initial step in jasmonic acid (JA) biosynthesis. However, this regulation does not occur in young anthers and it is speculated that accessibility of the AG protein to *DAD1* may be developmentally regulated, possibly by chromatin modification of *DAD1* genomic DNA (Ito *et al.*, 2007*b*). The function of *AGAMOUS* appears to have been conserved in rice but has been partitioned into two MADS box genes, *OsMADS3* and *OsMADS58,* which are products of a recent gene duplication event (Yamaguchi *et al.*, 2006).

Archesporial cell number in the anther is strictly controlled by a similar mechanism to that seen in the shoot apical meristem. It is regulated, at least in part, by a Leu-rich repeat receptor kinase EXTRA SPOROGENOUS CELLS/EXCESS MICROSPOROCYTES1 (EXS/EMS1). In the *exs/ems1* mutant, additional L2 cells in the anther undergo archesporial specification, so that extra meiocytes are formed and the tapetal and middle cell layers are absent (Canales *et al.*, 2002; Zhao *et al.*, 2002). A similar phenotype is also seen in the *tapetal determinant1 (tpd1)* mutant (Yang *et al.*, 2003). *TPD1* encodes for a small secreted protein which is expressed predominantly in the microsporocytes and is thought to act in co-ordination with *EXS/EMS1* (Yang *et al.*, 2003). Over-expression of *TPD1* induces additional cell divisions, which are dependent upon *EXS/EMS1*; tapetal degeneration is also delayed in *tpd1* suggesting that TPD1 may function in both tapetal specification and the maintenance of tapetal cell fate (Yang *et al.*, 2005). Recently, the interaction between EMS1/EXS and TPD has been confirmed *in vitro* and *in vivo*, suggesting that TPD1 serves as a ligand for the EMS1 receptor kinase (Yang *et al.*, 2005; Jia *et al.*, 2008). Interestingly, sporogenous cells are able to form and meiosis can be initiated in the *exs/ems1* and *tpd* mutants in the absence of the tapetum and middle cell layer, however, cytokinesis fails to occur and subsequent degeneration of the meiocytes is seen. This indicates that the tapetal layer plays an essential role that is required for completion of the later meiotic events.

A number of leucine-rich repeat receptor-like protein kinases (LRR-RLKs) have also been shown to be important in anther cell specification. Two of these, *BAM1* (for BARELY ANY MERISTEM) and *BAM2* have been shown to act redundantly in cell fate specification in the anther, in determining the early stages defining the parietal cells that give rise to the endothecium, middle and tapetal cell layer. These cell layers are absent in the *bam1bam2*

double mutant, although central cells with properties associated with PMCs are seen but these subsequently degenerate, suggesting that *BAM1*/*BAM2* act redundantly to regulate sporogenous cell number negatively by promoting differentiation of the surrounding somatic cells (Hord *et al.*, 2006). It has been proposed that they may act in a positive-negative-regulatory loop with *SPL*/*NZZ* to balance the formation of sporogenous and somatic cell number in the anther (Sun *et al.*, 2007).

The functionally redundant LRR-RLKs Somatic Embryogenesis Receptor-Like Kinase 1 *(SERK1)* and *SERK2* also produce a similar phenotype to that of *ems1*/*exs* and *tpd1* mutants in that the tapetal layer is absent, suggesting that they may act in an analogous manner in specifying somatic cell identity (Albrecht *et al.*, 2005; Colcombet *et al.*, 2005).

Recently, the *ER*-family of LRR-RLKs (*ER, ERL1*, and *ERL2*) and MPK3 and MPK6 have both been shown to act redundantly in cell differentiation during early anther development (Hord *et al.*, 2008). In the absence of *ER*/*ERL1*/*ERL2* expression, aberrant cell patterning occurs with increased tapetal cells, and sometimes middle cell layer numbers, suggesting they may act in the division and regulation of signals needed for early tapetal development (Hord *et al.*, 2008). The *MPK3*/*MPK6* genes also affect cell division during these early stages of anther differentiation. These two families of genes produce mutant phenotypes similar to that seen in the *ems1*/*exs, serk1, serk2*, and *tpd1* mutants, however, the *mpk3*/*mpk6* and *er*/*erl1*/*erl2* mutants are able to produce a tapetal layer. *EMS1* and *TPD1* expression is not affected in the *mpk3*/+ *mpk6*/– mutant suggesting that *ER*/*ERL1*/*ERL2* and *MPK3*/*MPK6* may act independently to the previously characterized pathway of cell differentiation in the anther (Hord *et al.*, 2008).

The Receptor-Like Protein Kinase2 (RPK2) has also been shown to have a role in anther development, with male sterility observed in the *rpk2* mutant (Mizuno *et al.*, 2007). In the mutant, the inner secondary parietal cell layer fails to differentiate into a middle layer, meiosis occurs normally, however, the tapetum appears hypertrophic and secondary thickening is lacking within the endothecium. The limited number of pollen grains that form aggregate extensively and the locules are subsequently crushed without stomium lysis and dehiscence occurring (Mizuno *et al.*, 2007). Extensive changes in gene expression associated with metabolic processes and wall formation are observed in the *rlk2* mutant, suggesting that *RLK2* plays a prominent role in regulating key metabolic pathways associated with tapetal development (Mizuno *et al.*, 2007).

A lectin receptor-like kinase (At3g53810) has recently been shown to be critical in pollen development (Wan *et al.*, 2008). The resultant mutant phenotype causes 'small glued together collapsed' pollen grains (*sgc*). *SGC* is expressed in the anther during stages 6–7, but is not detected in the microspores or pollen. The LRK class of receptor-like kinases are distinct since they contain a legume lectin-like domain, which has been associated with carbohydrate binding activities. It is proposed that *SGC* may bind oligosaccharides generated during pollen development, for example, during callose degradation or pectin breakdown of PMC primary wall, and therefore serve in the signal transduction pathway during pollen development (Wan *et al.*, 2008).

ROXY1 and *ROXY2* are two plant specific CC type glutaredoxins, which act redundantly for anther development. They appear to act downstream of *NZZ*/*SPL* but upstream of *DYSFUNCTIONAL TAPETUM 1* (*DYT1*) and are required for interaction with glutathione for essential biochemical reactions in the tapetum, demonstrating an importance for redox regulation in male gametogenesis (Xing and Zachgo, 2008).

Tapetal development

A number of Arabidopsis genes have been identified that act after tapetal specification, which are required for normal tapetal function and viable pollen production, including *DYT1*, *ABORTED MICROSPORE* (*AMS*), and *MALE STERILITY 1* (*MS1*).

DYT1 appears to be one of the earliest players in tapetal development after initiation of the anther cell layers has occurred. *DYT1* encodes a putative basic helix-loop-helix (bHLH) transcription factor that is predicted to act downstream of *SPL*/*NZZ* and *EMS1*/*EXS* and to be required for normal expression of *AMS,* and *MS1* (Zhang *et al.*, 2006) (Fig. 3). *DYT1* is strongly expressed in the tapetum during anther stages 5 and 6, and also at a lower level in the meiocytes. In the *dyt1* mutant, the tapetum becomes highly vacuolated, PMC meiosis is initiated, but the callose wall is thin and cytokinesis rarely occurs (Zhang *et al.*, 2006). *DYT1* is critical for tapetal gene regulation, but is not sufficient for tapetal development since it does not rescue the phenotype if over-expressed in the *spl*/*nzz* background (Zhang *et al.*, 2006). No expression of *AMS* or *MS1* is seen in the *dyt1* mutant (Zhang *et al.*, 2006), however, the expression of two other genes, *MYB33* and *MYB65*, which have been associated with this stage of tapetal development, remained unaffected.

MYB33 and *MYB65* appear to act redundantly to facilitate anther development, although there does not appear to be an absolute requirement for them for male fertility (Millar and Gubler, 2005). In the *myb33myb65* double mutant the tapetum becomes hypertrophic during the PMC stage, resulting in premeiotic abortion of pollen, however, in high light or low temperature, fertility increases (Millar and Gubler, 2005). The *MYB33* and *MYB65* genes are closely related to the *HvGAMYB* gene, which is involved in gibberellin signalling in the aleurone layer (Gubler *et al.*, 1995). It has been proposed that *MYB33* and *MYB65* may act to limit pollen development under poor growth conditions and that they may play a role in tapetal starch mobilization in a manner akin to the role of the *HvGAMYB* gene in the seed aleurone layer (Millar and Gubler, 2005). In many cases bHLH proteins have been shown to form complexes with MYB and WD-40 proteins, therefore it has been proposed that DYT1 may act during

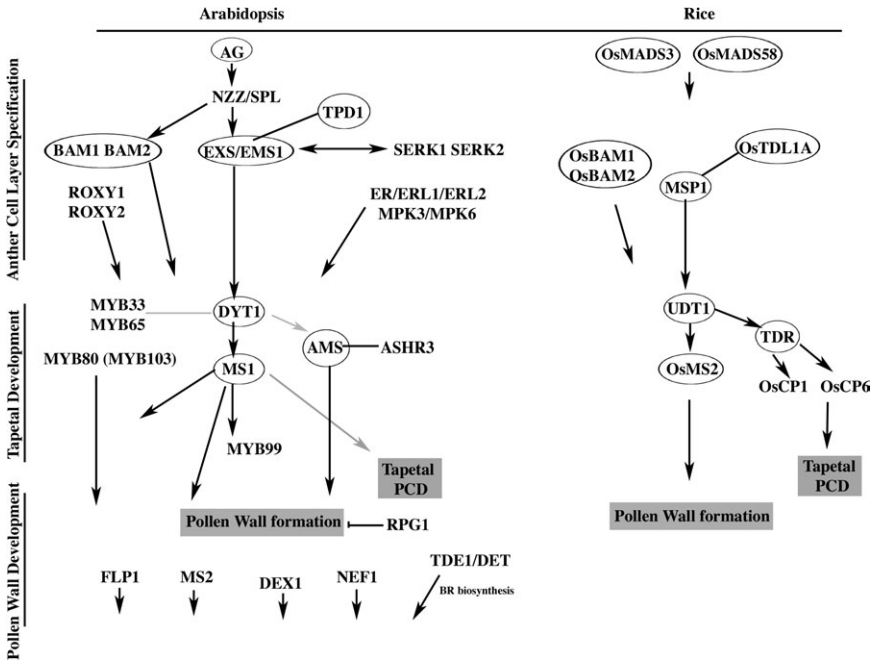

Fig. 3. Comparisons between the network of pollen development in *Arabidopsis* and rice. (AG, AGAMOUS; AMS, ABORTED MICROSPORE; BAM1, BARELY ANY MERISTEM 1; BAM2, BARELY ANY MERISTEM 2; DET, DE-ETIOLATED; DEX1, DEFECTIVE IN EXINE PATTERNING 1; DYT1, DYSFUNCTIONAL TAPETUM 1; EXS/EMS1, EXTRA SPOROGENOUS CELLS/EXCESS MICROSPOROCYTES 1; FLP1, FACELESS POLLEN-1; MS1, MALE STERILITY 1; MS2, MALE STERILITY 2; MSP1, MULTIPLE SPOROCYTE 1; NEF1, NO EXINE FORMATION 1; NZZ/SPL, NOZZLE/SPOROCYTELESS; RPG1, RUPTURED POLLEN GRAIN 1; SERK1, SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1; SERK2, SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 2; TDE1, TRANSIENT DEFECTIVE EXINE 1; TDR, TAPETUM DEGENERATION RETARDATION; TPD1, TAPETAL DETERMINANT 1; UDT1, UNDEVELOPED TAPETUM 1).

tapetal development as a homodimer with MYB33 and/or MYB65 (Zhang *et al.*, 2006). Rice *gamyb* mutants have also been shown to be blocked at a similar stage to *myb33myb65* just before meiosis and to undergo tapetal hypertrophy (Kaneko *et al.*, 2004). Thus the expression and function of GAMYBs appear to be highly conserved in cereals and *Arabidopsis* (Millar and Gubler, 2005).

AMS is also a bHLH protein, which is expressed in the tapetum, but shows prolonged expression, compared to many other tapetal specific genes, starting pre-meiotically and increasing post-meiotically (Sorensen *et al.*, 2003). In the *ams* mutant, meiosis occurs normally, however, there is a subsequent failure of pollen development due to tapetal and microspore degeneration. It has been speculated that *AMS* and *MS1* may be regulatory targets of DYT1 (Zhang *et al.*, 2006). Recently it has been shown that the *Arabidopsis* SET-domain protein ASHR3 can interact with AMS and, when over-expressed, results in reduced male fertility, suggesting that ASHR3 may serve to target AMS to chromatin and thereby regulate stamen development (Thorstensen *et al.*, 2008).

MS1 encodes for a Plant Homeodomain (PHD) transcription factor which shows tightly regulated expression in the tapetum as the callose breaks down, up to the free microspore stage (Yang *et al.*, 2007*a*). The *ms1* mutant fails to produce viable pollen (Wilson *et al.*, 2001; Ito and Shinozaki, 2002; Ariizumi *et al.*, 2005) and the tapetum shows alterations in secretion and in programmed cell death (PCD) (Vizcay-Barrena and Wilson, 2006). In the *ms1* mutant, there are large numbers of genes that show down-regulation, indicating the key role that *MS1* plays in regulating late tapetal gene expression and pollen wall deposition (Alves-Ferreira *et al.*, 2007; Ito *et al.*, 2007*a*; Yang *et al.*, 2007*a*). A number of transcription factors have been identified as possible downstream targets (Ito *et al.*, 2007*a*; Yang *et al.*, 2007*a*). Using dexamethasone-inducible *MS1* constructs, Ito *et al* (2007*a*) showed that *MYB99* was expressed 4 h after *MS1* induction and that this induction still occurred in the presence of the protein synthesis inhibitor cycloheximide (CHX), suggesting that *MYB99* is directly targeted by MS1. Insertional *myb99* mutants showed a reduction of fertility, with a reduced thickness of the tapetum, however, since the effect on pollen development was not severe it has been suggested that *MYB99* may act redundantly with other *MYB* factors which are expressed at this stage (Alves-Ferreira *et al.*, 2007).

Large numbers of genes have been identified as linked to anther and pollen development and comparative analysis of expression data has been used to construct putative expression pathways (Alves-Ferreira *et al.*, 2007; Wijeratne *et al.*, 2007). Clustering analysis of expression data from a number of mutants, including *apetela3, spl/nzz*, and *ms1,* was used to group the genes into stages of expression (Alves-Ferreira *et al.*, 2007). Analysis of the *ms1* expression

changes identified groups of genes which were down-regulated and also up-regulated (Alves-Ferreira *et al.*, 2007), this was also seen in separate Affymetrix transcriptomic analyses of *ms1* expression (Yang *et al.*, 2007*a*) suggesting that MS1 may act as a repressor as well as an activator of transcription. A number of direct MS1 downstream targets have been proposed (Alves-Ferreira *et al.*, 2007; Ito *et al.*, 2007*a*; Yang *et al.*, 2007*a*) (Fig. 3).

Pollen wall formation

The pollen wall is formed of a number of layers, the outer exine, which can be further divided into two parts, the outer sculptured layer or sexine, and the inner nexine. The exine is formed principally of sporopollenin, an aliphatic polymer comprised of a series of polymers derived from long-chain fatty acids, phenylpropanoids, and oxygenated aromatic rings, which is synthesized predominantly by the tapetum (Scott, 1994; Piffanelli *et al.*, 1998). This outer layer can have highly ornate patterning, comprising columns or 'baculae' which support roof-like structures called the 'tectum'. The inner wall layer, the intine, is a pectocellulosic layer, which is synthesized primarily by the microspore.

Prior to the start of meiosis, the microsporocytes synthesize a transient callose (β-1,3-glucan) wall that continues to develop during meiosis to give the classic 'tetrad' appearance. This callose wall is thought to serve as a 'molecular filter' separating the individual microspores from the surrounding sporophytic tissues and to provide a physical protective barrier for the developing microspores, however, there are also suggestions that relatively large molecules can pass through it (Scott *et al.*, 2004). Whilst encased in this callose layer development of the primexine commences. The primexine which is generated by microspores is a microfibrillar polysaccharide matrix that serves as a template for subsequent sporopollenin deposition and exine formation. However, the callose layer is thought to provide a surface against which the tectum can form, rather than as a positional guide, since in *Brassica napus* correct columellae positioning still occurs in the absence of the callose wall (Scott *et al.*, 2004), although the presence of callose appears critical for exine formation.

There are 12 genes encoding callose synthase in the *Arabidopsis* genome. In the callose synthase5 (*cals5*) mutant, callose deposition during microsporogenesis is almost absent, suggesting that this gene is likely to be a principal player in microspore callose deposition (Dong *et al.*, 2005). Fertility is significantly reduced in the *cals5* mutant and the bacula and tectum (making up the sexine) fail to develop, the tryphine which normally fills the interstices of the sexine is also deposited abnormally in globular masses on the outer surface of the pollen (Dong *et al.*, 2005). However, in less extreme *cals5* mutants, abnormal exine formation and patterning is still seen, but there is no associated reduction in pollen viability, suggesting that the callose layer is needed for exine formation but not necessarily for pollen viability (Nishikawa *et al.*, 2005).

After tetrad formation callase synthesis occurs from the tapetum, the callose wall breaks down, and the microspores are released into the locules. The timing of callose breakdown also appears to be critical, since pollen viability is significantly affected by premature dissolution of the callose wall (Worrall *et al.*, 1992). Callose breakdown fails to occur in the *tapetal development and function1* (*tdf1*) mutant and no expression of *A6*, a putative β-1,3-glucanase (Hird *et al.*, 1993), is detected (Zhu *et al.*, 2008), providing further evidence that *A6* may be a callase, or functions as part of the callase enzyme complex. Map-based cloning has shown that *TDF1* corresponds to *AtMYB35* (Zhu *et al.*, 2008). Expression analysis indicates that *TDF1* is expressed in both tapetal cells and microspores, and may act downstream of *DYT1,* but upstream of *AMS* and *MYB103* (Zhu *et al.*, 2008).

MYB103 (*MYB80*) has also been shown to be important for tapetal development with the *myb103* mutant tapetum degenerating earlier without releasing oil bodies, vesicles, and plastids (Higginson *et al.*, 2003; Li *et al.*, 2007), resulting in a reduction in enzymes responsible for callose degradation and, subsequently, preventing exine formation (Li *et al.*, 2007). In the *ms188* allele of *myb103*, reduced breakdown of callose and A6 expression, and abnormal exine formation was also seen (Zhang *et al.*, 2007). *MS1* expression was strongly reduced in the *ms188* mutant (Zhang *et al.*, 2007), whilst there was no significant change of *MYB103* expression in the *ms1* mutant (Yang *et al.*, 2007*a*), suggesting that *MYB103* acts upstream of *MS1* and *MS2*.

Primexine deposition is also thought to be partially regulated by brassinosteroid (BR) biosynthesis. In the *transient defective exine 1 (tde1)* mutant, the early stages of primexine deposition and bacular formation are effected, although subsequently normal exine formation occurs (Ariizumi *et al.*, 2008). The *TDE1* gene was found to be identical to the *DE-ETIOLATED2* gene, which is involved in BR biosynthesis and treatment of the *tde* mutant with BR was able to rescue the exine deposition phenotype (Ariizumi *et al.*, 2008).

Precursors for sporopollenin biosynthesis are formed predominantly in the tapetum; these are secreted into the anther locule and are deposited on the primexine template to form the ornate, protective outer wall. Exine formation and production of viable pollen does not occur in the *ms2* mutant (Aarts *et al.*, 1997). The *MS2* gene is a putative fatty acid reductase which catalyses the conversion of fatty acyl groups into fatty alcohol groups and is thought to be involved in sporopollenin biosynthesis (Aarts *et al.*, 1997). *MS2* expression was reduced in the *ms188/myb103* mutant, however, this regulation was presumed as indirect since direct binding between MYB103 and a 1.1 kb region of the *MS2* promoter was not detected (Zhang *et al.*, 2007).

In between the gaps of the baculae, pollen coat, composed of pollenkit and tryphine is then deposited (Owen and Makaroff, 1995). This functions partly to help the pollen adhere to the stigma surface, but it can also be involved in self-incompatibility reactions. At this stage, the

tapetum functions as a highly active factory secreting the components for the wall formation. As wall formation reaches the final stages of development, the tapetum goes through a programmed cell death (PCD) pathway to release the full complement of compounds for wall formation into the anther locule.

Tapetal programmed cell death

During the later stages of pollen development, immediately after microspore release from the tetrad and before mitosis I, breakdown of the tapetum is observed. Tapetal breakdown occurs in a highly regulated manner and has the hallmarks of programmed cell death, including cytoplasm shrinkage and separation from the cell wall, chromatin condensation, and Endoplasmic Reticulum (ER) swelling (Rogers *et al.*, 2005). During this degradation process, vesicles containing sporopollenin precursors fuse with the plasma membrane to release their contents into the anther locule for construction of the pollen wall. The tapetal cells then produce tryphine and pollenkit, which is released into the locule as the tapetum degrades, and coats the mature pollen grains. Controlled tapetal breakdown is critical since alterations in this process have been frequently linked to reduced fertility (Kaul, 1988). TUNEL (terminal deoxynucleotidyl transferase-mediated UDP nick end-labelling) has been used to detect DNA fragmentation and a number of male-sterile mutants have been shown to have alterations in tapetal DNA fragmentation, indicative of a failure of tapetal PCD. For example, the *ms1* mutant shows delayed tapetal breakdown and a switch from PCD degradation to necrotic-based breakdown (Vizcay-Barrena and Wilson, 2006). The rice mutant *tapetal degeneration retardation (Ostdr)* shows delayed tapetal breakdown and significantly delayed PCD, resulting in a failure of pollen wall deposition and subsequent microspore degeneration (Li *et al.*, 2006; Zhang *et al.*, 2008). The correct timing of tapetal PCD is important and it has been proposed that the initiation signal for tapetal PCD may commence as early as the tetrad stage (Kawanabe *et al.*, 2006).

Cysteine protease activity has been associated with PCD, however, the identity of the proteases that induce the PCD process have not been defined in plant systems (Williams and Dickman, 2008). In the case of rice it has been shown that a cysteine protease (OsCP1) and a protease inhibitor (OsC6) are likely direct targets of OsTDR (Li *et al.*, 2006) and these are therefore implicated in the process of tapetal PCD induction. A number of cysteine proteases have also been identified as downstream from *MS1* (Yang *et al.*, 2007*a*), however, whether they play a role in the lack of tapetal PCD in the *ms1* mutant is unknown at present.

Exine formation and patterning

A number of genes have been identified which are involved in either the deposition and patterning of exine formation, or, alternatively, in the biosynthesis of sporopollenin. The defective in exine patterning (*dex1)* mutant demonstrates the critical role that the plasma membrane plays in exine patterning, since the regular undulations, which are seen in the wild-type plasma membrane fail to form in *dex1*. In the wild type, these undulations are associated with sites of sporopollenin deposition. Despite sporopollenin biosynthesis occurring in the *dex1* mutant, anchoring of sporopollenin fails to take place, resulting in aberrant primexine deposition and subsequent pollen degeneration (Paxson-Sowders *et al.*, 2001). *DEX1* is expressed during the early tetrad stage of pollen development and encodes a putatively plasma membrane localized protein with 12 potential N-glycosylation sites, suggesting that the protein can undergo significant modification and interacts with the cell wall. DEX1 also shows a region of limited similarity to calcium binding ligands implying that it may act as a calcium-binding protein. It has therefore been proposed that DEX1 may be a component of the primexine matrix, or the ER, and that it is involved in the assembly of primexine precursors (Paxson-Sowders *et al.*, 2001). The role of DEX1 in altering Ca^{2+} ion concentrations in the primexine fits with the idea of wall formation occurring via a self-assembly process, since it would provide the opportunity to vary micelle shape and thus alter patterning (Blackmore *et al.*, 2007).

The *RUPTURED POLLEN GRAIN1 (RPG1)* encodes a tapetal and microspore plasma membrane protein, which is required for exine pattern development. *RPG1* is expressed in the tapetum and microspores from anther stage 4 during meiosis, through to tapetal degeneration (stage 12) and, after tapetal degeneration, expression is only seen in the pollen grains (Guan *et al.*, 2008). In the *rpg1* mutant the microspore plasma membrane formation and development of membrane undulations is abnormal. This results in aberrant deposition of sporopollenin from the tetrad stage, resulting in microspore rupture and degeneration, however, the plant subsequently recovers some partial fertility (Guan *et al.*, 2008). RPG1 belongs to a conserved family of MtN3/saliva family proteins. In animals, they have been shown to associate with ion channel proteins and are proposed as playing a targeting, or chaperone-like role in ion channel development. RPG1 has been shown to contain phosphorylation sites that may suggest a role in protein regulatory networks. It has therefore been suggested that RPG1 may act through interactions with membrane proteins to regulate membrane traffic, or integrity and, therefore, primexine patterning (Guan *et al.*, 2008).

The *faceless pollen-1* (*flp1)* mutant was identified as a conditional male sterile that can be rescued by growth under high humidity. The pollen wall was found to be almost smooth with no reticulate pattern. Sporopollenin transfer, or polymerization to the primexine is abnormal and there is excessive tryphine deposition, which is the major cause of male sterility (Ariizumi *et al.*, 2003). FLP1 has homology to lipid transfer proteins and has been shown to have a role in cuticular wax biosynthesis. Sporopollenin comprises predominantly long-chain fatty acids and

phenolic monomers coupled by ester bonds as seen in polyphenolics such as suberin and lignin, suggesting that there is a conservation of biochemical pathways for cutin, lignin, and sporopollenin (Scott, 1994).

Another gene which has been shown to be critical during the early stages of exine formation is *NO EXINE FORMATION1 (NEF1)* (Ariizumi *et al.*, 2004). Primexine deposition occurs normally in the *nef1* mutant, however, sporopollenin aggregates in the locule and normal deposition onto the microspore plasma membrane and exine formation does not occur, resulting in pollen degeneration (Ariizumi *et al.*, 2004). It is speculated that *NEF1* acts early during exine formation before *MS2, DEX1*, and *FLP1,* since no exine deposition is observed in the mutant. Overall growth of the *nef1* mutant is reduced, the plastids are smaller, have fewer granal stacks, and contain reduced numbers of electron-transparent vesicles (plastoglobuli) with reduced lipid accumulation. NEF1 has homology to transporter proteins and has been shown to encode for a membrane protein, which may be involved in maintaining membrane integrity in the plastid. NEF1 is involved in lipid biosynthesis, which results in the observed defects in primexine formation and sporopollenin biosynthesis (Ariizumi *et al.*, 2004).

A recent mutant screen which analysed pollen wall structure by SEM identified 12 novel exine mutants, *kaonashi (kns) 1-12*, unlike other exine mutants which have been identified due to reduced male fertility, most of these produce viable pollen and are fully fertility (Suzuki *et al.*, 2008). These mutants have been grouped into (i) those which showed a collapsed exine structure, reminiscent of callose synthase mutants, (ii) thin exine, due to deficient primexine development, (iii) defective tectum formation, and (iv) densely arranged baculae. One of these, *kns2,* has subsequently been identified as defective in sucrose phosphate synthase, which is proposed as functioning in primexine or callose synthesis (Suzuki *et al.*, 2008). These mutants will provide an interesting resource to analyse the effects that subtle alterations in exine structure and pattern have on pollen development.

Hormonal regulation of pollen development and dehiscence

Auxin has been shown to play an important role during both early and late pollen development (Cheng *et al.*, 2006; Cecchetti *et al.*, 2008). The principal source of auxin appears to be local synthesis rather than transport into the anther, since the auxin biosynthesis genes *YUC flavin monooxygenases* (*YUC2* and *YUC6)* are expressed within the anther. Inhibiting anther auxin transport also does not appear to affect the expression of the auxin reporter DR5::GUS significantly, or alter anther development (Cecchetti *et al.*, 2008). In the *yuc2yuc6* auxin biosynthesis double mutant, no pollen grains form, stamen elongation does not occur, and the flowers are male sterile (Cheng *et al.*, 2006). However, the auxin receptor mutants (*tir1 afb* triple and quadruple mutants) show retarded preanthesis filament growth (stages 10–13), but premature anther development with early endothecium thickening, which occurs prior to tapetal degeneration, premature pollen mitotic divisions, stomium splitting, and dehiscence (Cecchetti *et al.*, 2008). Auxin therefore appears to be involved both in co-ordinating pollen development and maturation by regulating entry into the cell cycle and controlling dehiscence, and also by controlling stamen filament growth (Cecchetti *et al.*, 2008). These late stages of filament elongation and dehiscence also involve regulation by the hormone jasmonic acid.

Jasmonic acid is required for male fertility and many aspects of flower and pollen development, including the regulation of anther development, dehiscence, and flower opening. JA mutants (e.g. *coi1, opr3/dde1*; Sanders *et al.*, 2000; Stintzi and Browse, 2000; Devoto *et al.*, 2002) have frequently been isolated because of changes in pathogen response, however, an additional phenotype is that of reduced male fertility, due to short filaments, a lack of dehiscence, and reduced pollen viability (Mandaokar *et al.*, 2006). In the JA biosynthetic mutant *dad1*, pollen maturation combined with dehiscence and flower opening fails to occur due to a lack of JA in the anther (Ishiguro *et al.*, 2001). It has been proposed that JA acts by controlling water transport in the anther, possibly partly via induction of the *AtSUC1* gene (Ishiguro *et al.*, 2001).

Male fertility in JA biosynthesis mutants can be rescued by JA treatment, however, this is very stage-specific and is limited to mid-stage 12, which equates to pollen mitosis II stage, approximately 48 h prior to dehiscence (Mandaokar *et al.*, 2006). Analysis of stamen gene expression in the *opr*3 mutant identified 821 genes that were specifically induced and 480 repressed by jasmonate treatment, and of these 13 were transcription factors (Mandaokar *et al.*, 2006). MYB21 and MYB24, two related subgroup 19 MYB factors, were identified in this group. The *myb24* mutant appeared phenotypically normal whilst the *myb21* mutant had reduced male fertility, which was associated with reduced filament length and delayed dehiscence. However, the *myb21myb24* double mutant showed an increase in male sterility partly due to a reduction in stamen filament length which could not be rescued by jasmonate, indicating a critical role for MYB21 and MYB24 in the jasmonate response during stamen development (Mandaokar *et al.*, 2006). The *myb108* mutant, which was also identified from the JA transcriptomic experiments (Mandaokar *et al.*, 2006), showed only a slight reduction in filament length, however, when combined with the *myb24* mutation, it showed a significant reduction in stamen length and an associated reduction in fertility (Mandaokar and Browse, 2009).

MYB24 is expressed during early floral development in the flowers, particularly in microspores and ovules, but single *myb24* mutants do not show altered phenotypes (Yang *et al.*, 2007*c*). However, when combined with the *myb21* mutation, which shows a similar wild-type expression pattern to *MYB24*, a male sterile phenotype was observed, with the flowers failing to dehisce and also

a reduction in petal opening (Mandaokar *et al.*, 2006). Over-expression of *MYB24* results in general aberrant growth and, specifically, reduced male fertility because of a failure of anther septum and stomium breakdown, and a lack of fibrous bands in the anther endothecium and connective tissues (Yang *et al.*, 2007*c*). Yang *et al* (2007*c*) showed altered expression of genes associated with the phenylpropanoid biosynthesis pathway in *MYB24* over-expressing lines, suggesting that MYB24 may act in conjunction with MYB21 to regulate anther development and dehiscence. MYB32 and the closely related MYB4 have also been reported as influencing pollen development, with structural distortions and a lack of cytoplasm observed in pollen from the knockout lines (Preston *et al.*, 2004). In these lines, there is also altered expression of components of the phenylpropanoid pathway and it has been proposed that MYB32 and MYB4 may function in the determination of pollen wall composition and development by altering the phenylpropanoid pathway flux (Preston *et al.*, 2004).

A similar effect of alteration of secondary thickening was seen in the *ms35/myb26* mutant which fails to dehisce due to the absence of secondary thickening in the anther endothecium (Dawson *et al.*, 1999; Steiner-Lange *et al.*, 2003; Yang *et al.*, 2007*b*). However, this mutation can not be rescued by JA, indicating that a JA independent pathway to endothecium differentiation and the deposition of secondary thickening occurs in the anther (Yang *et al.*, 2007*b*). A similar phenotype of anther indehiscence due to a lack of endothecial secondary thickening, is also observed in the double NAC domain mutant, *nst1nst2* (Mitsuda *et al.*, 2005). Overexpression of MYB26 induces ectopic secondary thickening by inducing the expression of genes associated with secondary thickening and lignification, and is thought to act by regulating the expression of *NST1* and *NST2* (Yang *et al.*, 2007*b*).

Two auxin response transcription factors ARF6 and ARF8 have been shown to act partially redundantly in many stages of development, including stamen elongation and anther dehiscence. This action appears to be via repression of gene expression and specific targets are thought to be small auxin up-regulated genes (*SAUR62*, *SAUR63*, *SAUR64*, *SAUR65*, *SAUR67*, *IAA3*, and *IAA4*) (Nagpal *et al.*, 2005). It is thought that ARF6 and ARF8 act in part by inducing JA production, or by decreasing JA conjugation or breakdown, which, in turn, regulates anther dehiscence and flower opening (Nagpal *et al.*, 2005). *ARF6* and *ARF8* expression has been shown to be under the control of miRNA167 (Ru *et al.*, 2006; Wu *et al.*, 2006; Yang *et al.*, 2006).

From models to crops: male sterility in rice

As previously discussed, extensive information is now available on the process of pollen development in *Arabidopsis*. However, over recent years significant progress has also been made to elucidate pollen development in monocots, focusing principally on rice. The rice inflorescence architecture is quite different from those of other major cereal crops, instead of two or more florets in one spikelet as seen in other cereal crops, such as maize and wheat, a rice spikelet has only one floret surrounded by a pair of empty glumes. Each rice floret typically consists of a central pistil, six stamens, and two lodicules (petals) subtended by an inner bract, the palea, and the outer bract, the lemma (Rudall and Bateman, 2004; Itoh *et al.*, 2005) (Fig. 1B). Pollen formation in rice appears to follow a similar developmental pathway to that observed in *Arabidopsis* (Chen *et al.*, 2005; Itoh *et al.*, 2005) with the development of a secretory tapetum. A number of male sterile mutants have now been identified in rice pollen development and these are revealing a high degree of conservation in the early regulatory network of pollen formation (Fig. 3).

The early stages of rice pollen development show a high degree of similarity with *Arabidopsis*. *MULTIPLE SPOROCYTE1 (MSP1),* which encodes a LRR receptor-like kinase, appears to be the orthologue of the *Arabidopsis EMS1/EXS* gene. The phenotype of the *msp1* mutant strongly resembles that of *ems1/exs*, with increased numbers of male and female sporocytes, and disrupted anther wall layers which lack the tapetum cell layer (Nonomura *et al.*, 2003). MSP1 therefore appears to function in an analogous way to EMS1/EXS in initiating anther wall development and restricting the numbers of cells which enter into male and female sporogenesis. In *Arabidopsis* TPD1 has recently been identified as the ligand for EMS1/EXS (Jia *et al.*, 2008). In rice, two *TPD1*-like genes have been identified and one of these, *OsTDL1A,* has been shown to be co-expressed with *MSP1* and to bind to MSP1, suggesting that it acts as the corresponding rice ligand to limit sporocyte numbers (Zhao *et al.*, 2008). Rice orthologues of the BAM1 and BAM2 LRR-RLKs have been identified, suggesting that this signalling pathway is also conserved between *Arabidopsis* and rice (Hord *et al.*, 2006).

UNDEVELOPED TAPETUM (*OsUDT1*) encodes a bHLH protein, which acts after initiation of the tapetum in an analogous manner to *DYT1*. It has been shown to be expressed in the anther wall, meiocytes, and weakly in vegetative tissues, and to be critical for tapetum development (Jung *et al.*, 2005). OsUDT shows high sequence homology to AMS and also to DYT1, but is likely to act as the rice orthologue of DYT1.

The role that the *OsTDR* gene plays during rice tapetal development and pollen formation has been characterized in detail. *OsTDR* is expressed in the tapetum during late pollen development and encodes for a bHLH protein, which has been proposed as a potential trigger for PCD in the rice tapetum (Li *et al.*, 2006). The *Ostdr* mutant tapetum becomes highly vacuolated, PCD is greatly retarded and microspore degeneration occurs. Interestingly, two genes, *OsCP1* and *Osc6*, encoding a cysteine protease and a protease inhibitor, respectively, have been identified by Chromatin Immunoprecipitation-PCR (CHIP-PCR) and the electrophoretic mobility shift assay (EMSA) as direct targets for OsTDR (Li *et al.*, 2006). The *OsCP1* gene has

previously been shown to play a critical role around the stage of microspore release from tetrads (Lee *et al.*, 2004) and *Osc6* to be tapetally expressed (Tsuchiya *et al.*, 1992). OsTDR may therefore act to regulate PCD via induction of the cysteine protease (Li *et al.*, 2006). In addition, OsTDR regulates a number of genes involved in aliphatic metabolism for pollen wall development. In *Ostdr* the aliphatic composition of the anther locules was altered. In particular, less accumulation of fatty acids, primary alcohols, alkanes, and alkenes, and abnormal increases of secondary alcohols with carbon length from C29 to C35 were seen, suggesting OsTDR's unique role in regulating aliphatic components required for anther/pollen development (Zhang *et al.*, 2008). Similarity analysis indicates that OsTDR is part of the bHLH super-family of transcription factors. A number of these have been shown to be involved in pollen development in rice and have corresponding genes in *Arabidopsis.* Phylogenetic analysis suggests that OsTDR is closest to the *Arabidopsis* AMS protein, OsUDT is also closely related to these proteins, however, it seems to have greater similarity to the *Arabidopsis* DYT1 protein (Jung *et al.*, 2005) suggesting that the bHLH protein associated with tapetal development are functionally conserved between *Arabidopsis* and rice.

Future developments

Significant numbers of genes have been identified by forward genetic screens in both *Arabidopsis* and rice, which have allowed the identification of many key players in male reproductive development. Comparative analysis between species is providing additional tools to aid in the understanding of these complex pathways. Recent developments in high through-put sequencing and genome analysis will facilitate further step-changes in the level of characterization and understanding of these processes. Extensive transcriptome data is available in *Arabidopsis* and for a number of mutants in both *Arabidopsis* and rice, however, recent developments have meant that equivalent information is now available for rice anther development. Recent transcriptome analyses in rice have been conducted to identify genes that are associated with phytohormone and signalling pathways of pollen development (Hirano *et al.*, 2008). Analysis of the rice anther and pollen transcriptome has recently been conducted at six developmental stages by sequencing-by-synthesis (SBS) technology providing a means to dissect the roles of the tapetum in secretion and lipid exine synthesis and by *in silico* analysis to differentiate between the tapetal and pollen transcriptomes (Huang *et al.*, 2008). Laser capture microdissection of anther tissues has also been conducted to provide a valuable resource for tissue-specific analysis of gene expression in rice (Hobo *et al.*, 2008; Suwabe *et al.*, 2008). These data will provide opportunities for further comparative analysis, greater network development, and the future manipulation of fertility in model and crop plants.

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