

REVIEW PAPER

Identification of genes expressed in the angiosperm female gametophyte

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Abstract

Until recently, identification of gene regulatory networks controlling the development of the angiosperm female gametophyte has presented a significant challenge to the plant biology community. The angiosperm female gametophyte is fairly inaccessible because it is a highly reduced structure relative to the sporophyte and is embedded within multiple layers of the sporophytic tissue of the ovule. Moreover, although mutations affecting the female gametophyte can be readily isolated, their analysis can be difficult because most affect genes involved in basic cellular processes that are also required in the diploid sporophyte. In recent years, expression-based approaches in multiple species have begun to uncover gene sets expressed in specific female gametophyte cells as a means of identifying regulatory networks controlling cell differentiation in the female gametophyte. Here, recent efforts to identify and analyse gene expression programmes in the *Arabidopsis* female gametophyte are reviewed.

Key words: Angiosperms, *Arabidopsis*, cell differentiation, cell identity, central cell, female gametophyte, gene expression, gene networks, synergid cell, transcription factor.

The female gametophyte is essential for angiosperm reproduction

The plant life cycle alternates between a haploid gametophyte generation and a diploid sporophyte generation. In plants, specialized diploid cells (mother cells) within sporophytes undergo meiosis and give rise to haploid spores. Spores undergo cell proliferation and differentiation to develop into multicellular haploid gametophytes. A major function of the gametophyte generation is to produce haploid gametes. Fusion of egg and sperm gives rise to the sporophyte, thereby completing the life cycle (Raven *et al.*, 2005).

In lower plants, gametophytes typically are the dominant and free-living generation. In contrast, angiosperms have dramatically reduced gametophytes that are composed of very few cells and are embedded within the sexual organs of the flower. Angiosperms have two gametophytes: the female gametophyte (also referred to as the embryo sac or megagametophyte) and the male gametophyte (also referred to as the pollen grain or microgametophyte). The male

gametophyte develops within the stamen's anther and is composed of two sperm cells encased within a vegetative cell (McCormick, 2004; Singh *et al.*, 2008; Borg and Twell, 2010). The female gametophyte develops within the ovule, which is found within the carpels of the flower. The angiosperm female gametophyte most commonly consists of one egg cell, one central cell, two synergid cells, and three antipodal cells (Drews and Yadegari, 2002; Colombo *et al.*, 2008a; Sundaresan and Alandete-Saez, 2010; Yang *et al.*, 2010).

The female and male gametophytes are essential for the angiosperm reproductive process. Sexual reproduction is initiated when pollen is transferred from anther to stigma. Soon thereafter, the male gametophyte forms a pollen tube that grows through the carpel's internal tissues and eventually releases its two sperm cells into one of the two synergid cells. Soon after arrival, the pollen tube ceases growth and the synergid cell penetrated by the pollen tube

undergoes cell death. Next, one sperm cell migrates to the egg cell and the other to the central cell. When the plasma membranes of the male gametes fuse with those of the egg and central cells, the sperm nuclei are transmitted into these cells for karyogamy. Following fertilization, the egg and central cell give rise to the seed's embryo and endosperm, respectively (Lord and Russell, 2002; Weterings and Russell, 2004; Berger *et al.*, 2008; Sprunck, 2010).

Female gametophyte cells control many steps of the fertilization process. During pollen tube growth, the synergid cells produce a guidance cue that directs pollen tube growth to the ovule and female gametophyte (Higashiyama and Hamamura, 2008; Okuda *et al.*, 2009). Female gametophyte cells (mainly the synergid cells) contain factors that control arrest of pollen tube growth and release of pollen tube contents (Huck *et al.*, 2003; Rotman *et al.*, 2003, 2008; Escobar-Restrepo *et al.*, 2007; Capron *et al.*, 2008; Boisson-Dernier *et al.*, 2009; Miyazaki *et al.*, 2009; Tsukamoto *et al.*, 2010). Upon fertilization, the ovule is induced to develop into a seed. Central cell-expressed gene products control the activation of endosperm development via epigenetic mechanisms (Kohler and Grossniklaus, 2005; Huh *et al.*, 2008; Berger and Chaudhury, 2009; Jullien and Berger, 2009). Also, factors present in the egg cell may regulate the activation of embryo development, although these factors have yet to be identified (Curtis and Grossniklaus, 2008). Finally, genetic studies indicate that the female gametophyte plays a role in maternal control of embryo and endosperm development following fertilization (Evans and Kermicle, 2001; Grini *et al.*, 2002).

The mechanisms that control the differentiation of the female gametophyte and its individual cell types remain to be determined. However, recent work using molecular and genetic approaches has identified auxin as a critical signal for the proper development of the asymmetric structure of the female gametophyte in *Arabidopsis* (Pagnussat *et al.*, 2009). Independent processes have also been shown to control cell identity particularly in the micropylar cells of the female gametophyte (Gross-Hardt *et al.*, 2007; Pagnussat *et al.*, 2007; Moll *et al.*, 2008). Recent efforts in deciphering the regulatory networks that control female gametophyte cell differentiation and development are summarized below.

Identification of genes expressed in the *Arabidopsis* female gametophyte

As discussed above, each of the female gametophyte's four cell types contains unique structural, developmental, and physiological features. Our goal is to understand the gene regulatory circuitry operating in these cells that causes them to acquire their unique features and functions during cell differentiation. As a first step toward dissecting these gene-regulatory networks, genes expressed in the *Arabidopsis* female gametophyte were identified using differential expression screens.

The general strategy used to identify female gametophyte-expressed genes was to identify mRNAs present in normal ovules but not in mutant ovules lacking female gametophytes. *male sterility1 (ms1)* (Thorlby *et al.*, 1997; Wilson *et al.*, 2001; Ito *et al.*, 2007) was used as the source of normal ovules, and *determinant infertile1 (dif1)* (Bai *et al.*, 1999; Bhatt *et al.*, 1999; Cai *et al.*, 2003) as the source of mutant ovules lacking female gametophytes. *ms1* is male sterile but undergoes normal ovule and female gametophyte development (Steffen *et al.*, 2008). *dif1* is a recessive sporophytic mutation and all ovules lack female gametophytes in homozygous *dif1* mutants. The absence of a female gametophyte in *dif1* ovules results from a meiosis defect: *DIF1* encodes a homologue of yeast *REC8/RAD21* cohesin genes and the *dif1* mutation affects chromosomal segregation during meiosis (Bai *et al.*, 1999; Bhatt *et al.*, 1999; Cai *et al.*, 2003).

Female gametophyte-expressed genes were screened for with Affymetrix *Arabidopsis* ATH1 Genome Arrays using probes synthesized from the *ms1* and *dif1* ovule RNAs. These assays identified 86 genes whose expression was potentially enhanced in wild-type ovules. To validate this expression pattern, real-time reverse transcription-PCR (RT-PCR) assays were carried out with RNA from independently harvested ovules. Using the criteria of ≥ 8 -fold change, 71 of the 86 genes were validated as having reduced expression in *dif1* ovules (Steffen *et al.*, 2007).

To identify the female gametophyte cells that express these genes, 45 of the 71 genes were selected and transgenic *Arabidopsis* lines containing *promoter:GFP (Green Florescent Protein)* gene fusions were analysed (Steffen *et al.*, 2007). Of the 45 genes analysed, 43 showed expression in the female gametophyte. The female gametophyte expression patterns of these genes fell into five general categories: expression in the egg cell only (one gene), expression predominantly or exclusively in the synergid cells (17 genes), expression predominantly or exclusively in the central cell (11 genes), expression in the antipodal cells only (11 genes), and expression in multiple female gametophyte cell types (three genes).

Similar approaches have been used by other groups to identify female gametophyte-expressed genes (Yu *et al.*, 2005; Johnston *et al.*, 2007; Jones-Rhoades *et al.*, 2007). More recently, these analyses have been complemented by the identification of individual-cell transcriptomes of the *Arabidopsis* female gametophyte using laser-capture microdissection coupled to microarray analysis (Wuest *et al.*, 2010). A summary of these expression patterns is shown in Table 1.

Identification of transcription factor genes expressed in the female gametophyte

The microarray screen used detected only one gene encoding a transcription factor (Steffen *et al.*, 2008). Although there has been no systematic study of transcription factor gene mRNA levels in plants, most mammalian and yeast

Table 1. The primary female gametophyte cell expression patterns

Pattern	Genes
A	<i>AT4G22050</i> (Yu <i>et al.</i> , 2005); <i>DD1</i> , <i>DD33*</i> (Yu <i>et al.</i> , 2005; Steffen <i>et al.</i> , 2007); <i>DD6</i> , <i>DD13</i> , <i>DD16</i> , <i>DD23</i> , <i>DD26</i> , <i>DD29</i> , <i>DD47</i> , <i>DD52</i> , <i>DD61</i> , <i>DD64</i> (Steffen <i>et al.</i> , 2007); <i>ARF15</i> , <i>NAC95</i> , <i>ZFP6</i> , <i>AT2G22750</i> , <i>AT5G01380</i> , <i>AT5G56200</i> (Wang <i>et al.</i> , 2010).
C	<i>MEA</i> (Vielle-Calzada <i>et al.</i> , 1999; Luo <i>et al.</i> , 2000; Wang <i>et al.</i> , 2006); <i>FIS2</i> (Luo <i>et al.</i> , 2000; Wang <i>et al.</i> , 2006); <i>FIE</i> (Luo <i>et al.</i> , 2000; Yadegari <i>et al.</i> , 2000); <i>PRL</i> (Springer <i>et al.</i> , 2000); <i>DME</i> (Choi <i>et al.</i> , 2002); <i>FWA</i> (Kinoshita <i>et al.</i> , 2004); <i>DD9*</i> (Yu <i>et al.</i> , 2005; Steffen <i>et al.</i> , 2007); <i>AGL80</i> (Porterioko <i>et al.</i> , 2006); <i>CCG</i> (Chen <i>et al.</i> , 2007); <i>AT4G14276</i> , <i>AT4G24974</i> , <i>AT5G27238</i> (Jones-Rhoades <i>et al.</i> , 2007); <i>DD19</i> , <i>DD22</i> , <i>DD25</i> , <i>DD28</i> , <i>DD36</i> , <i>DD41</i> , <i>DD65</i> , <i>DD66</i> , <i>DD73</i> (Steffen <i>et al.</i> , 2007); <i>DD7*</i> (Steffen <i>et al.</i> , 2007; Wuest <i>et al.</i> , 2010); <i>AGL61</i> (Berner <i>et al.</i> , 2008; Steffen <i>et al.</i> , 2008); <i>PABP</i> (Tiwari <i>et al.</i> , 2008); <i>ZOU/RGE1/BHLH95</i> , <i>AT5G27880</i> , <i>AT5G50490</i> (Wang <i>et al.</i> , 2010); <i>MYB64*</i> (Wang <i>et al.</i> , 2010; Wuest <i>et al.</i> , 2010); <i>AT2G33130</i> (Wuest <i>et al.</i> , 2010).
E	<i>WOX8*</i> (Haecker <i>et al.</i> , 2004; Wang <i>et al.</i> , 2010); <i>AT5G60270</i> (Johnston <i>et al.</i> , 2007); <i>DD45</i> (Steffen <i>et al.</i> , 2007); <i>ABI4</i> , <i>AT2G33710</i> (Wang <i>et al.</i> , 2010); <i>AT3G51690</i> , <i>AT5G16850</i> (Wuest <i>et al.</i> , 2010).
S	<i>MYB98*</i> (Kasahara <i>et al.</i> , 2005; Steffen <i>et al.</i> , 2007); <i>DD56*</i> (Yu <i>et al.</i> , 2005; Johnston <i>et al.</i> , 2007; Steffen <i>et al.</i> , 2007); <i>FER</i> (Escobar-Restrepo <i>et al.</i> , 2007); <i>AT1G28220</i> (Johnston <i>et al.</i> , 2007); <i>AT2G14378</i> , <i>AT2G21727</i> , <i>AT4G08025</i> , <i>AT5G35405</i> , <i>AT5G36340</i> , <i>AT5G42895</i> , <i>AT5G54062</i> (Jones-Rhoades <i>et al.</i> , 2007); <i>DD17*</i> (Jones-Rhoades <i>et al.</i> , 2007; Steffen <i>et al.</i> , 2007); <i>DD2</i> , <i>DD3</i> , <i>DD4</i> , <i>DD8</i> , <i>DD11</i> , <i>DD12</i> , <i>DD18</i> , <i>DD31</i> , <i>DD34</i> , <i>DD35</i> , <i>DD39</i> , <i>DD42</i> , <i>DD67</i> (Steffen <i>et al.</i> , 2007); <i>DD32*</i> (Steffen <i>et al.</i> , 2007; Wuest <i>et al.</i> , 2010); <i>LRE</i> (Capron <i>et al.</i> , 2008); <i>NAC23</i> , <i>AT3G01030</i> (Wang <i>et al.</i> , 2010); <i>AT1G65810</i> (Wuest <i>et al.</i> , 2010).
A, C	<i>AT5G01860</i> (Wang <i>et al.</i> , 2010)
C, E	<i>CK11</i> (Pischke <i>et al.</i> , 2002; Hejatkto <i>et al.</i> , 2003); <i>WOX2</i> (Haecker <i>et al.</i> , 2004); <i>LIS</i> (Gross-Hardt <i>et al.</i> , 2007); <i>AT3G61740</i> , <i>AT5G50915</i> (Johnston <i>et al.</i> , 2007).
C, S	<i>DD27</i> , <i>DD46</i> (Steffen <i>et al.</i> , 2007).
E, S	<i>AT5G27610</i> , <i>AT5G48650</i> , <i>AT5G56510</i> (Wuest <i>et al.</i> , 2010).
A, E, S	<i>AGP18</i> (Acosta-Garcia and Vielle-Calzada, 2004).
C, E, S	<i>SWN</i> (Wang <i>et al.</i> , 2006); <i>ATO</i> , <i>CLO/GFA1</i> (Moll <i>et al.</i> , 2008); <i>RL6</i> (Wang <i>et al.</i> , 2010).
FG	<i>PAB5</i> (Belostotsky and Meagher, 1996); <i>PAB2</i> (Palanivelu <i>et al.</i> , 2000); <i>MSI1</i> (Kohler <i>et al.</i> , 2003); <i>RBR1</i> (Ebel <i>et al.</i> , 2004); <i>CHR11</i> (Huanca-Mamani <i>et al.</i> , 2005); <i>LPAT2</i> (Kim <i>et al.</i> , 2005); <i>GPT1</i> (Niewiadomski <i>et al.</i> , 2005); <i>SWA1</i> (Shi <i>et al.</i> , 2005); <i>AT5G40260</i> (Yu <i>et al.</i> , 2005; Johnston <i>et al.</i> , 2007); <i>AT1G80370</i> , <i>AT4G01970</i> (Johnston <i>et al.</i> , 2007); <i>AGL23</i> (Colombo <i>et al.</i> , 2008b).

The primary female gametophyte-cell expression patterns are summarized here. A, antipodal cells; C, central cell; E, egg cell; FG, all cell types; and S, synergid cells.

For some genes (*), different patterns of expression may have been described in independent studies using one or more methods. For a more detailed description, please refer to the relevant publications.

transcription factor genes produce mRNAs that occur at low levels (Holland, 2002; Vaquerizas *et al.*, 2009). Thus, the low number of transcription factor genes identified in the above-mentioned microarray-based approach was probably due to insufficient sensitivity. This is especially relevant to the general approach used as the female gametophyte cells comprise only seven of ~1,000 cells in a mature ovule—in effect, diluting the female gametophyte RNAs ~150-fold. As an alternative, in a second screen, real-time RT-PCR was used to detect individual transcription factor gene mRNAs in ovules or ovaries followed by reporter gene assays for analysis of cell-specific expression (Kasahara *et al.*, 2005; Steffen *et al.*, 2008; Wang *et al.*, 2010).

Real-time RT-PCR assays were performed with nearly 1,400 *Arabidopsis* transcription factor genes using RNA from *ms1* and *dif1* ovules or ovaries (Wang *et al.*, 2010). These assays identified 69 genes with elevated mRNA levels in *ms1* as compared with *dif1* mutant ovaries. Using additional biological replicates and more stringent statistical analyses, 26 genes were confirmed to be down-regulated in *dif1* ovaries (Wang *et al.*, 2010).

To determine the female gametophyte cells within which these genes are expressed, promoter fusion analyses with single or double nuclear-localized GFP constructs were used (Wang *et al.*, 2010). This approach was taken because many transcription factor genes produced weak or ambiguous levels of promoter-reporter activity using standard,

untargeted, cytosolic GFP (cGFP) as a reporter (Wang *et al.*, 2010). Moreover, for genes expressed in multiple cell types within the female gametophyte, determination of the exact patterns using cGFP reporters proved difficult due to low resolution of the cGFP signal. This was particularly the case for genes with reporter activity in the egg cell, the central cell, and/or the synergid cells.

Using *promoter:GFP* gene fusions, the expression of 18 transcription factor genes was assayed (Wang *et al.*, 2010). Of these, 17 showed expression in the female gametophyte. Ten genes were exclusively or predominantly expressed in the egg cell (two genes), the central cell (two genes), or the antipodal cells (six genes), whereas the remaining seven genes were expressed in two (four genes) or more (three genes) cell types, usually with one cell type showing a higher level of expression. The expression of a subset of these genes was also analysed during megagametogenesis and early seed development. For example, 12 genes were transcriptionally active in the developing embryo and/or endosperm.

These real-time RT-PCR assays identified a number of transcription factor genes that had not been identified in other published studies, presumably because their mRNA levels are too low for effective detection using standard microarray-based approaches (Wang *et al.*, 2010). Therefore, a combined expression-based analysis of regulatory and non-regulatory gene sets that are expressed in specific cell types (Table 1) should enable identification of the

regulatory pathways controlling cell differentiation in the female gametophyte.

Analysis of the synergid cell and central cell gene networks

Functional studies have been carried out with two of the transcription factor genes identified in the screens discussed above. These genes are *MYB98* (Kasahara *et al.*, 2005; Punwani *et al.*, 2007, 2008) and *AGL61/DIANA* (Bemer *et al.*, 2008; Steffen *et al.*, 2008). As discussed below, analysis of these genes provided initial insight into the gene networks of the synergid and central cells.

MYB98 encodes an R2R3 MYB protein. In the context of the ovule, the *MYB98* gene is expressed predominantly in the synergid cells and *myb98* mutations affect the synergid cells specifically. *myb98* synergid cells fail to attract pollen tubes and have an abnormal filiform apparatus, which is a thickened and elaborated cell wall located at the micropylar poles of these cells. However, with the exception of the filiform apparatus defect, *myb98* synergid cells are otherwise normal. These observations suggest that *MYB98* regulates a subcircuit of the synergid gene regulatory network that functions to activate the expression of genes required for pollen tube guidance and filiform apparatus formation (Kasahara *et al.*, 2005; Punwani *et al.*, 2007).

To identify genes regulated by *MYB98*, the synergid-expressed genes identified in the microarray screen were tested for reduced expression in *myb98* female gametophytes. Specifically, the 17 genes expressed predominantly or exclusively in the synergid cells plus one gene expressed highly in both the synergid cells and the central cell were tested. Of the 18 genes tested, 16 exhibited reduced expression in *myb98* ovules. Most of these genes encode cysteine-rich proteins within four gene families (CRP810, CRP3700, CRP3730, and CRP3740). Further analysis by us and others (Jones-Rhoades *et al.*, 2007) showed that the *MYB98* subcircuit comprises at least 83 downstream genes including 48 genes within the CRP810, CRP3700, CRP3730, and CRP3740 families. All genes tested exhibited no mutant phenotype, most probably due to functional redundancy. However, five of six proteins tested localized to the filiform apparatus, suggesting that they play a role in formation or function of this structure (Punwani *et al.*, 2007, 2008).

The DNA-binding properties of *MYB98* were also examined *in vitro* and *in vivo*. Using electrophoretic mobility shift assays, it was shown that MYB98 binds to a specific DNA sequence (TAAC) *in vitro*. Through promoter dissection of several of the downstream genes, it was demonstrated that the *in vivo* MYB98 binding site is GTAACNT (N=any nucleotide). Many of the genes downstream of MYB98 contain the GTAACNT element, and site-directed mutagenesis of these elements abolishes synergid expression, very strongly suggesting that the corresponding genes are direct targets of MYB98 (Punwani *et al.*, 2007, 2008).

The second transcription factor gene analysed was *AGL61*, which encodes a type I MADS transcription factor.

During ovule and seed development, *AGL61* is expressed exclusively in the central cell and early endosperm. The *agl61* mutation affects the central cell specifically. *agl61* central cells exhibit an overall reduction in size and a reduced or absent vacuole. *agl61* central cells fail to give rise to endosperm when fertilized with wild-type pollen. These observations suggest that AGL61 controls the expression of central cell-expressed genes that are required for development of the central cell into endosperm following fertilization (Bemer *et al.*, 2008; Steffen *et al.*, 2008).

MADS-domain proteins often function as heterodimers with other MADS-domain proteins (de Folter and Angenent, 2006). The expression pattern and mutant phenotype of *AGL61* are similar to those of *AGL80*, which were previously analysed (Portereiko *et al.*, 2006). These observations suggest that AGL61 may function as a heterodimer with AGL80 within the central cell. AGL61–AGL80 interactions were determined in yeast two-hybrid assays and in bimolecular fluorescence complementation assays using *Arabidopsis* protoplasts. In both assays, a specific interaction was observed, suggesting very strongly that an AGL61–AGL80 heterodimer functions in the central cell (Bemer *et al.*, 2008; Steffen *et al.*, 2008).

To identify genes regulated by *AGL61* and *AGL80*, several of the central cell-expressed genes identified in the microarray screen were tested for reduced expression in *agl61* and *agl80* female gametophytes. Of four genes tested, three showed reduced expression in both *agl61* and *agl80* central cells. During the course of these studies, it was also found that several synergid- and antipodal-expressed genes are ectopically expressed in *agl61* and *agl80* central cells. Together, these data suggest very strongly that an AGL61–AGL80 heterodimer functions to both activate and repress genes during central cell development (Bemer *et al.*, 2008; Steffen *et al.*, 2008).

Summary

Each of the female gametophyte's four cell types contains unique features that are essential for angiosperm reproduction. As a first step toward understanding the gene networks that cause these cells to acquire their unique features and functions during cell differentiation, large numbers of both regulatory and non-regulatory gene sets expressed in the *Arabidopsis* female gametophyte were identified (summarized in Table 1). Functional analysis of two of these genes, *MYB98* and *AGL61*, has provided an initial view of the gene networks operating in the synergid and central cells. Analysis of the gene networks operating in the other female gametophyte cells is currently in progress.

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