MYB98 Positively Regulates a Battery of Synergid-Expressed Genes Encoding Filiform Apparatus–Localized Proteins [W]

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The synergid cells within the female gametophyte are essential for reproduction in angiosperms. *MYB98* encodes an R2R3-MYB protein required for pollen tube guidance and filiform apparatus formation by the synergid cells. To test the predicted function of MYB98 as a transcriptional regulator, we determined its subcellular localization and examined its DNA binding properties. We show that MYB98 binds to a specific DNA sequence (TAAC) and that a MYB98–green fluorescent protein fusion protein localizes to the nucleus, consistent with a role in transcriptional regulation. To identify genes regulated by MYB98, we tested previously identified synergid-expressed genes for reduced expression in *myb98* female gametophytes and identified 16 such genes. We dissected the promoter of one of the downstream genes, *DD11*, and show that it contains a MYB98 binding site required for synergid expression, suggesting that *DD11* is regulated directly by MYB98. To gain insight into the functions of the downstream genes, we chose five genes and determined the subcellular localization of the encoded proteins. We show that these five proteins are secreted into the filiform apparatus, suggesting that they play a role in either the formation or the function of this unique structure. Together, these data suggest that MYB98 functions as a transcriptional regulator in the synergid cells and activates the expression of genes required for pollen tube guidance and filiform apparatus formation.

INTRODUCTION

The angiosperm female gametophyte contains two synergid cells that lie adjacent to the egg cell and are essential for reproduction. During the angiosperm fertilization process, a pollen tube grows into one of the synergid cells, ceases growth, ruptures, and releases its two sperm cells into this cell. The synergid cell that interacts with the pollen tube typically undergoes cell death before or upon pollen tube penetration. The two sperm cells then migrate to and fuse with the egg cell and central cell, and ultimately, the fertilized female gametophyte gives rise to the embryo and endosperm of the seed (reviewed in Lord and Russell, 2002; Weterings and Russell, 2004).

During the final stages of pollen tube growth, a pollen tube grows along the placental surface, along the surface of the ovule's funiculus, into the ovule's micropyle, and finally into the female gametophyte. To determine whether the female gametophyte plays a role in pollen tube guidance, several groups have analyzed pollen tube growth in *Arabidopsis thaliana* mutants defective in embryo sac development. These studies showed that the female gametophyte is required for pollen tube guidance at two stages: guidance from the placenta to the funiculus (funicular guidance phase) and guidance from the funiculus to the micropyle (micropylar guidance phase) (reviewed in Higashiyama, 2002; Johnson and Preuss, 2002; Higashiyama et al., 2003).

To determine which cells within the female gametophyte produce the pollen tube attractant(s), Higashiyama and colleagues (2001) analyzed pollen tube attraction by *Torenia* ovules in which specific cells of the female gametophyte were removed by laser ablation. Pollen tubes were attracted by ovules lacking the central cell and egg cell but not by ovules lacking the synergid cells, indicating that the synergid cells are the source of a pollen tube attractant. Recently, the maize (*Zea mays*) synergid cell–expressed gene Zm *EA1* was shown to be required for pollen tube guidance (Marton et al., 2005), but its precise role in this process still needs to be determined (McCormick and Yang, 2005).

At the micropylar pole, the synergid cell wall is extensively thickened and elaborated, forming a structure referred to as the filiform apparatus (see Supplemental Figure 1 online). In *Arabidopsis* and many other species, the filiform apparatus has numerous finger-like projections into the synergid cytoplasm. Several functions for the filiform apparatus have been proposed, including pollen tube reception, import of metabolites, and export of the pollen tube attractant(s). However, a specific function of the filiform apparatus in pollen tube reception or transport has not been demonstrated (reviewed in Willemse and van Went, 1984; Huang and Russell, 1992; Higashiyama, 2002).

Genetic screens have identified several mutants that appear to be defective in synergid cell function. In *sirene* (Rotman et al., 2003) and *feronia* (Huck et al., 2003) mutants, wild-type pollen tubes enter mutant female gametophytes but fail to cease growth and release their contents. Although the corresponding genes have not been identified, they likely encode molecules involved in synergid cell–pollen tube interaction. *gametophytic factor 2* (*gfa2*) female gametophytes fail to undergo synergid cell death following pollination. *GFA2* encodes a J domain–containing protein

required for mitochondrial function, suggesting that functional mitochondria are required for synergid cell death (Christensen et al., 2002). Additional mutants affected in synergid function appear to be present in a recently identified collection of female gametophyte mutants (Pagnussat et al., 2005).

Genes functioning in the synergid cells have also been identified through expression-based screens. The *Arabidopsis* synergid-expressed genes include *MYB98* (Kasahara et al., 2005) and the 18 genes listed in Table 1. Additional synergid-expressed genes have been identified in maize (Cordts et al., 2001; Le et al., 2005; Marton et al., 2005; Yang et al., 2006).

MYB98 encodes a MYB family protein and is predicted to function as a transcriptional regulator. Within the female gametophyte, *MYB98* is expressed predominantly in the synergid cells. *myb98* female gametophytes are defective in pollen tube guidance: wild-type pollen tubes grow from the placenta to the funiculus but then fail to grow into the micropyle, indicating that the *myb98* mutation affects the micropylar guidance phase of pollen tube guidance. In addition, the filiform apparatus of *myb98* mutants lacks the finger-like projections observed in wild-type synergid cells. However, with the exception of the filiform apparatus defect, *myb98* synergid cells appear normal, suggesting that MYB98 regulates the expression of a specific set of genes that function in pollen tube guidance and filiform apparatus formation (Kasahara et al., 2005). Here, we provide evidence for this model. We show that MYB98 is localized to the nuclei of the synergid cells, binds DNA at a specific sequence, and is required for the expression of a battery of synergid-expressed genes. We also show that at least one of the downstream genes is likely a direct target of MYB98. Finally, we show that many of the downstream genes encode proteins that are secreted into the filiform apparatus. Together, these data suggest very strongly

that MYB98 activates a branch of the synergid gene regulatory network that is required for pollen tube guidance and filiform apparatus formation.

RESULTS

MYB98 Binds DNA and Is Localized to the Nucleus

MYB98 is predicted to encode a MYB family transcription factor (Stracke et al., 2001). If this prediction is correct, MYB98 should bind DNA and be localized to the nucleus. To determine the subcellular localization of MYB98, we analyzed transgenic plants containing a construct (*ProMYB98:GFP-MYB98*) comprising the *MYB98* promoter, a green fluorescent protein (GFP) coding sequence, and the entire *MYB98* coding sequence. This construct was sufficient to rescue the *myb98-1* mutant phenotype, suggesting that localization of this fusion protein mimics that of endogenous MYB98. Rescue was established by generating plants homozygous for both the *myb98-1* mutation and the *ProMYB98:GFP-MYB98* construct (see Methods); these plants had full seed set.

As shown in Figures 1A and 1B, in a mature, unfertilized, wild-type female gametophyte (stage FG7) (Christensen et al., 1997), GFP-MYB98 was localized to the nuclei of the two synergid cells.

We used electrophoretic mobility shift assays (EMSAs) to determine whether MYB98 binds DNA. We previously showed that MYB98's Myb domain (R2 and R3 Myb repeats) is highly similar to that of mammalian c-Myb (Kasahara et al., 2005), suggesting that MYB98 binds DNA to a sequence similar to that of c-Myb. Several studies have shown that c-Myb binds to the consensus sequence YAACNG (Y = thymine or cytosine, N = any nucleotide) and that the AAC residues are critical for binding

**Table 1.** Synergid-Expressed Genes Discussed in This Article

Gene[a]	AGI[b]	Expression in the Female Gametophyte	Predicted Function[c]
DD2	At5g43510	SCs, (EC), (CC)	Defensin-like protein (CRP)
DD3	At3g56610	SCs, (EC), (CC)	Expressed protein (CRP)
DD4	At5g42955	SCs, (EC), (CC)	Expressed protein (CRP)
DD8	At5g52975	SCs, (EC), (CC)	Expressed protein (CRP)
DD11	At1g52970	SCs, (EC), (CC)	Expressed protein (CRP)
DD12	At2g21655	SCs, (EC), (CC)	Expressed protein (CRP)
DD17	At5g34885	SCs, (EC), (CC)	Expressed protein (CRP)
DD18	At1g45190	SCs, (EC), (CC)	Expressed protein (CRP)
DD27	At3g05460	SCs, CC	Sporozoite surface protein–related
DD31	At1g47470	SCs	Hypothetical protein (CRP)
DD32	At3g17080	SCs, (EC), (CC)	Self-incompatibility protein–related (CRP)
DD34	At4g07515	SCs, (EC), (CC)	Expressed protein (CRP)
DD35	At5g12380	SCs	Putative annexin
DD39/QRT3	At4g20050	SCs, (EC)	Polygalacturonase
DD42	At2g20660	SCs, (EC), (CC)	Rapid alkalinization factor family protein14
DD46	At1g22015	SCs, CC	Galactosyltransferase family protein
DD56	At4g30590	SCs, (EC), (CC)	Plastocyanin-like domain–containing protein
DD67	At5g11940	SCs, (EC), (CC)	Subtilase family protein

CC, central cell; CRP, Cys-rich protein; EC, egg cell; SC, synergid cell. Parentheses indicate weak expression.
[a] Genes were identified by Steffen et al. (2007).
[b] Arabidopsis Genome Initiative number.
[c] Based on The Arabidopsis Information Resource annotations (www.arabidopsis.org).
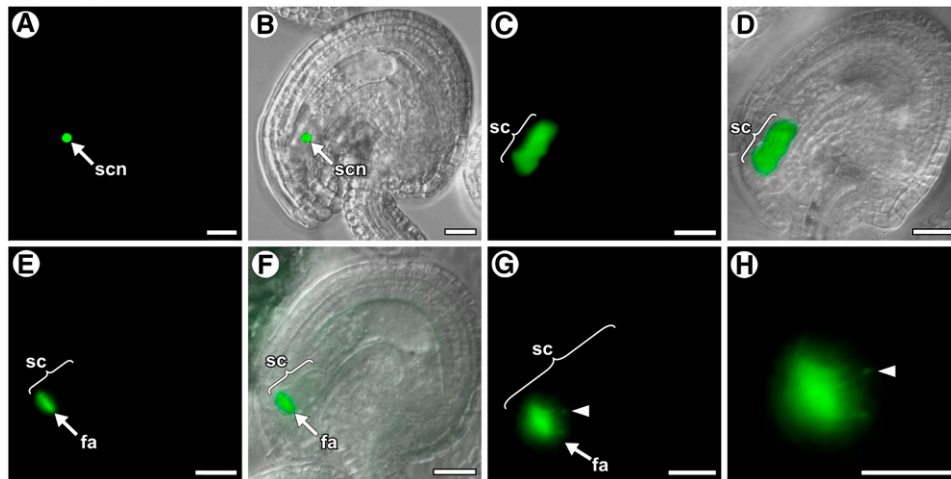
Figure 1. Localization of GFP Fusion Proteins in Wild-Type Ovules.

(A), **(C)**, **(E)**, **(G)**, and **(H)** show fluorescence images of the GFP signals. **(B)**, **(D)**, and **(F)** show fluorescence bright-field overlay images. fa, filiform apparatus; sc, synergid cell; scn, synergid cell nucleus. Bars = 20 μm in **(A)** to **(F)** and 10 μm in **(G)** and **(H)**.
(A) and **(B)** Localization of GFP-MYB98 in a mature female gametophyte (stage FG7). The GFP signal is localized to the nuclei of the synergid cells. Only one synergid nucleus is visible; the other is out of the focal plane.
(C) and **(D)** Localization of DD34-GFP in a mature female gametophyte (stage FG7). The GFP signal is present throughout the synergid cell.
(E) and **(F)** Localization of DD4-GFP in a mature female gametophyte (stage FG7). The GFP signal is localized to the filiform apparatus.
(G) and **(H)** Localization of DD11-GFP in a mature female gametophyte (stage FG7). These are high-magnification micrographs showing that the GFP signal is localized to the filiform apparatus. Arrowheads show a finger-like projection.

specificity (Biedenkapp et al., 1988; Weston, 1992; Tanikawa et al., 1993; Oda et al., 1998).

To determine whether MYB98 binds to the c-Myb consensus sequence, we generated the MYB98 Myb domain in *Escherichia coli* and analyzed interaction with a labeled oligonucleotide containing the sequence TAACGG using EMSAs. As shown in Figure 2A, addition of the MYB98 Myb domain caused a shift in the mobility of the labeled oligonucleotide, indicating that MYB98 binds to DNA. To determine whether this binding is specific for the sequence TAACGG, we added unlabeled oligonucleotides to the binding reaction. As shown in Figure 2A, the wild-type oligonucleotide competed fully, whereas an oligonucleotide containing mutations in the core AAC residues (M1) competed much less effectively. These data indicate that MYB98 binds to DNA in a sequence-specific manner.

To further define the MYB98 DNA binding sequence, we performed a series of competition assays in which binding of MYB98 to a labeled oligonucleotide containing the sequence TAACGG was competed with a 25-fold excess of unlabeled oligonucleotides containing mutations at various positions. As shown in Figure 2B, oligonucleotides containing mutations at positions 5 and 6 (M2, M3, and M4) competed as effectively as the wild-type oligonucleotide, an oligonucleotide containing a cytosine at position 1 (M7) competed but did so less effectively than the wild-type oligonucleotide, and oligonucleotides containing an adenine or a guanine at position 1 (M5 and M6) did not compete with the wild-type oligonucleotide. Together, these data suggest that MYB98 binds to the sequence YAAC with a preference for TAAC.

In summary, MYB98 is localized to the nucleus of the synergid cells and binds DNA at a specific sequence. These data suggest

very strongly that MYB98 functions as a transcriptional regulator in the synergid cells.

MYB98 Is Not Autoregulated

Autoregulation is a common feature of transcription factor gene regulation (Freeman, 2000; de Folter and Angenent, 2006). Upstream of the *MYB98* coding region are several putative MYB98 binding sites, suggesting that *MYB98* may regulate its own transcription. To test for this, we compared the expression of *ProMYB98:GFP* in *MYB98/MYB98* and *MYB98/myb98-1* pistils. In plants hemizygous for the *ProMYB98:GFP* construct, the percentage of female gametophytes expressing GFP was similar in *MYB98/MYB98* (48.4%; $n = 279$) and *MYB98/myb98-1* (44.9%; $n = 285$) pistils. Additionally, within *MYB98/myb98-1* pistils, the intensity of GFP fluorescence was similar among gametophytes. These data suggest that *MYB98* does not regulate its own expression.

Identification of 16 Genes Downstream of *MYB98*

The results presented above suggest that MYB98 regulates the expression of genes within the synergid cell. To test this further, we determined whether any of the synergid-expressed genes listed in Table 1 are downregulated in *myb98* female gametophytes. We used real-time RT-PCR to assay the expression of these 18 genes in *MYB98/MYB98* and *myb98-1/myb98-1* pistils. As shown in Table 2, all except *DD35* showed reduced expression in *myb98-1/myb98-1* pistils relative to *MYB98/MYB98* pistils, suggesting that the other 17 genes require *MYB98* for expression.
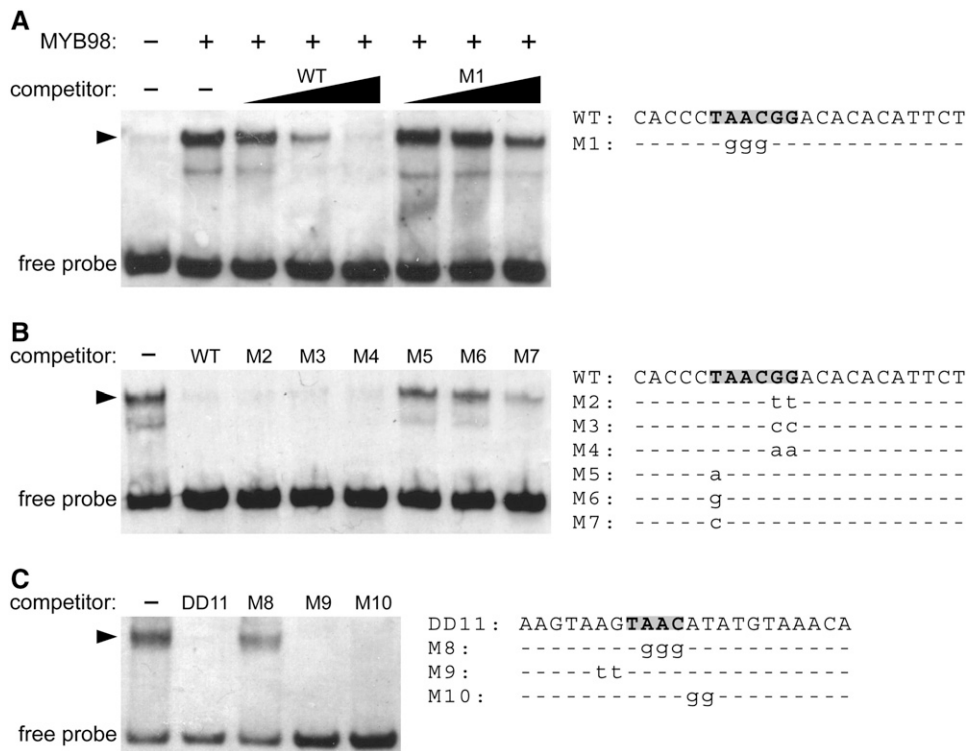
Figure 2. MYB98 Binds to the Sequence YAAC in Vitro.

All panels are from EMSAs using the MYB98 Myb domain and oligonucleotides containing wild-type and mutant versions of the MYB98 binding site. Arrowheads indicate the mobility shifts that occur upon the addition of the MYB98 protein. The minor bands below the major bands are present in a constant ratio to the major bands and may represent the binding of partially degraded MYB98 protein to the oligonucleotides. The sequences of the oligonucleotides used in these reactions are shown at right.

(A) MYB98 binds to the c-Myb consensus binding site. Lane 1 contains the labeled wild-type oligonucleotide alone. Lanes 2 to 8 contain the labeled wild-type oligonucleotide plus MYB98 protein. Lanes 3 to 5 contain unlabeled wild-type oligonucleotide in 1×, 10×, and 100× molar excess, respectively. Lanes 6 to 8 contain unlabeled mutated (M1) oligonucleotide in 1×, 10×, and 100× molar excess, respectively. The unlabeled wild-type oligonucleotide competes partially at 1× to 10× and fully at 100× molar excess. The unlabeled M1 oligonucleotide competes weakly at 100× molar excess.

(B) MYB98 binds to the sequence YAAC. All lanes contain labeled wild-type oligonucleotide plus MYB98 protein. Lanes 2 to 8 contain unlabeled oligonucleotides at 25× molar excess. Oligonucleotides M2, M3, and M4, which all contain mutations at positions 5 and 6, compete fully with the wild-type oligonucleotide. Oligonucleotides M5 and M6, in which the nucleotide at position 1 has been changed to an adenine or a guanine, fail to compete with the wild-type oligonucleotide. Oligonucleotide M7, in which the nucleotide at position 1 has been changed to a cytosine, competes at a reduced level.

(C) MYB98 binds to TAAC[−144]. All lanes contain labeled wild-type oligonucleotide (oligonucleotide DD11) plus the MYB98 protein. Lanes 2 to 5 contain unlabeled oligonucleotides at 25× molar excess. The wild-type oligonucleotide (DD11) competes fully. Oligonucleotide M8, in which the nucleotides in the core AAC residues have been mutated, fails to compete. Oligonucleotides M9 and M10, in which nucleotides outside the core AAC residues have been mutated, compete fully.

To validate these expression patterns, we analyzed the expression of the corresponding promoter:GFP constructs for these 17 genes in *MYB98* and *myb98-1* female gametophytes. Using crosses, we generated plants hemizygous for the promoter:GFP construct and homozygous for the *myb98-1* mutation. We then scored the number of female gametophytes expressing GFP in the synergid cells in these plants as well as in the parental line. As shown in Figure 3, the number of female gametophytes expressing *ProDD27:GFP* in the synergid cells was approximately equal in *MYB98/MYB98* and *myb98-1/myb98-1* pistils. By contrast, the other 16 genes showed reduced expression of the corresponding promoter:GFP construct in *myb98-1/myb98-1* pistils

(Figure 3). Together, these data indicate that *MYB98* is required for the expression of these 16 genes during synergid cell development.

Genes Downstream of *MYB98* Encode Proteins That Localize to the Filiform Apparatus

The predicted functions of the 16 genes downstream of *MYB98* are listed in Table 1. More than half (9 of 16 genes) encode proteins of unknown function. All except one (*DD34*) encode proteins with predicted N-terminal signal peptides. Most (11 of 16 genes) of the genes downstream of *MYB98* encode small, Cys-rich proteins (Table 1), 6 of which belong to two families: *DD11*, *DD18*, and

Table 2. Real-Time RT-PCR Analysis of 18 Synergid-Expressed Genes

Gene Name	C_T MYB98/MYB98 Pistils	C_T myb98-1/myb98-1 Pistils	Fold Change
DD2	19.2	28.8	776
DD3	21.6	25.3	13
DD4	18.7	32.4	13,308
DD8	23.8	27.7	15
DD11	24.2	32.1	239
DD12	20.9	26.3	42
DD17	25.7	39.0	10,086
DD18	22.2	>40	228,207
DD27	23.4	26.1	6.5
DD31	25.4	34.1	416
DD32	29.5	>40	1,448
DD34	30.3	38.1	223
DD35	24.9	25.8	1.9[a]
DD39/QRT3	24.7	27.9	9.2
DD42	25.9	29.7	14
DD46	25.9	29.8	15
DD56	25.1	33.1	256
DD67	26.5	33.3	111

C_T, threshold cycle.
[a] Not significantly different from 1.0.

DD31 are within the CRP3700 subgroup; and DD4, DD17, and DD34 are within the CRP3740 subgroup (Silverstein et al., 2007).

To gain insight into the functions of the genes downstream of MYB98, we obtained and analyzed T-DNA mutants from the Salk Institute Genomic Analysis Laboratory collection (Alonso et al., 2003). Of the 16 genes, T-DNA lines were available for 6 genes (DD4, DD18, DD31, DD39/QRT3, DD56, and DD67). In all cases, the T-DNAs segregated normally (e.g., 1:2:1 within the progeny of self-pollinated heterozygous plants) and the heterozygous and homozygous mutant plants exhibited full seed set. These data suggest that the female gametophyte is not affected by these six mutations. The absence of mutant phenotypes for these T-DNA

lines is likely due to functional redundancy, as all are members of large gene families.

As a second approach, we selected six genes (DD2, DD4, DD11, DD12, DD32, and DD34) and determined the subcellular localization of the encoded proteins. DD34 was chosen because it lacks a putative signal peptide (discussed above). The other five have putative signal peptides. We analyzed transgenic lines expressing C-terminal GFP fusion proteins in the synergid cells. Each transgene construct contained the upstream regulatory sequences and the complete coding region fused with a GFP coding sequence. We analyzed the localization patterns in mature, unfertilized female gametophytes (stage FG7). As shown in Figures 1C and 1D, DD34-GFP, which lacks an N-terminal signal peptide, is distributed throughout the cytoplasm. By contrast, as shown in Figures 1E to 1H and Supplemental Figures 2A to 2F online, with the other five fusion proteins, which all contain N-terminal signal peptides, strong GFP signals were associated with the filiform apparatus and GFP was not detected in the cytoplasm. These data suggest that DD34 is a cytoplasmic protein, whereas the DD2, DD4, DD11, DD12, and DD32 proteins are secreted into the filiform apparatus.

The results presented above suggest that within the synergid cells, secretion into the filiform apparatus is the default secretory pathway. To test this, we analyzed transgenic plants containing a protein fusion construct (ProMYB98:SP-GFP) comprising the MYB98 promoter, the coding sequence of a signal peptide (from DD11), and a GFP coding sequence. As shown in Supplemental Figures 2G and 2H online, this fusion protein was localized to the filiform apparatus. Thus, an N-terminal signal peptide appears to be sufficient to localize a protein to the filiform apparatus in the synergid cells.

The DD11 Promoter Contains a MYB98 Binding Site Necessary for Synergid Expression

To identify in vivo MYB98 DNA binding sites, as well as direct targets of MYB98, we chose one gene and dissected its
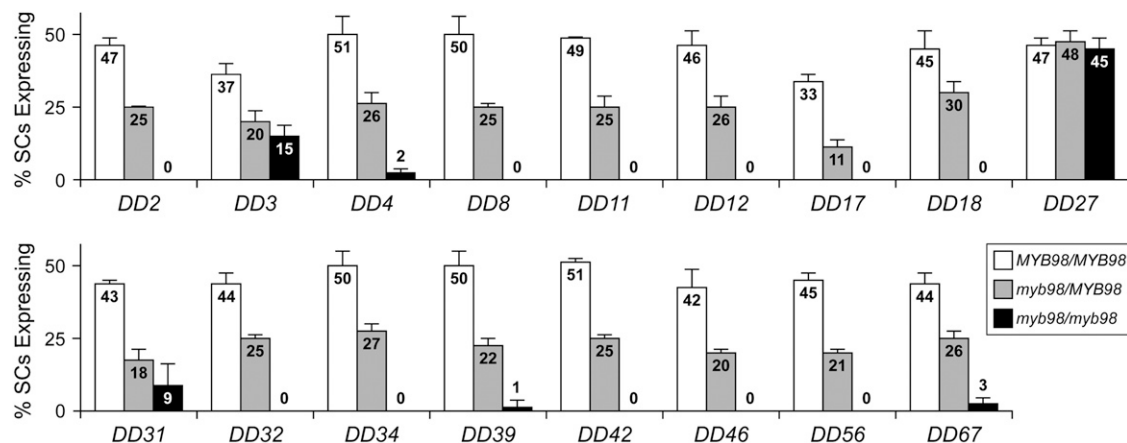
Figure 3. Expression of Synergid-Expressed Genes in MYB98 and myb98-1 Female Gametophytes.

Percentage of synergid cells (SCs) expressing the corresponding promoter:GFP construct in MYB98/MYB98 (white bars), myb98-1/MYB98 (gray bars), and myb98-1/myb98-1 (black bars) pistils. All plants scored were hemizygous for the promoter:GFP construct. Error bars indicate SD.

promoter. We chose *DD11* because the location of the adjacent upstream gene (Figure 4A) suggests that the regulatory sequences necessary for synergid expression are located within a small (409 bp) region. Consistent with this, we previously showed that a promoter fragment containing 398 bp upstream of the translational start site is sufficient to direct expression to the synergid cells (Steffen et al., 2007). In addition, as shown in Figure 4A, the *DD11* promoter contains six YAAC sites, which are potential MYB98 binding sites (Figure 2); therefore, this gene could be a direct target of MYB98.

To determine whether any of these YAAC sites, or any other sequences, are necessary for *DD11* expression, we generated a series of 5′ (Figure 4B) and 3′ (Figure 4C) deletions of the *DD11* promoter, fused these with a GFP coding region, introduced these promoter:GFP constructs into wild-type *Arabidopsis* plants, and analyzed ≥10 transgenic lines per construct. With the 5′ deletion series, a promoter fragment containing 174 bp upstream of the translational start site was sufficient to drive the expression of GFP in the synergid cells, but removal of an additional 37 bp (to position −137) abolished expression (Figure 4B). With the 3′ deletion series, a promoter fragment containing −352 to −139 bp relative to the translational start site was sufficient to drive the expression of GFP in the synergid cells, but removal of an additional 40 bp from the 3′ end (to position −179) abolished expression (Figure 4C).

The regions identified in the 5′ and 3′ deletions are compared in Figure 4D. These regions overlap by 36 bp, at positions −174 to −139, suggesting that this 36-bp region contains sequences necessary for synergid expression. As shown in Figure 4D, this region contains a single putative MYB98 binding site (TAAC) at position −144. We refer to this site as TAAC^{-144}.

To determine whether MYB98 can bind to TAAC^{-144}, we performed EMSAs with the MYB98 Myb domain and an oligonucleotide containing a region of the *DD11* promoter encompassing this site. As shown in Figure 2C, MYB98 bound to this oligonucleotide, and this binding was competed with the wild-type competitor and competitors with mutations outside the core AAC residues (oligonucleotides M9 and M10) but not with competitors bearing mutations within the core AAC residues (oligonucleotide M8). These data indicate that MYB98 binds to TAAC^{-144} in vitro.

To determine whether TAAC^{-144} is necessary for *DD11* expression, we generated a construct (mTAAC^{-144}) in which this site is mutated in the context of a promoter fragment containing 174 bp upstream of the translational start site and introduced this construct into wild-type plants. As shown in Figure 4E, the wild-type promoter fragment was sufficient to drive the expression of GFP in the synergid cells. By contrast, the same construct containing a mutation in TAAC^{-144} was not expressed in the synergid cells. Together, these data indicate that the *DD11* promoter contains a MYB98 binding site necessary for synergid expression.

DISCUSSION

The synergid cells are required for pollen tube guidance and fertilization and have several structural specializations, including a filiform apparatus. We are dissecting the gene regulatory network of the *Arabidopsis* synergid cell to understand how this important cell type acquires its unique features and func-

tions. Here, we show that MYB98 functions as a transcription factor, that 16 genes lie downstream of *MYB98* in the synergid gene regulatory network, and that at least 1 of these genes, *DD11*, is a direct target of MYB98. We further show that many of the genes downstream of *MYB98* encode proteins that are secreted into the filiform apparatus, suggesting that they play a role in the formation or function of this structure. Together, these data establish that *MYB98* activates a branch of the synergid gene regulatory network required for pollen tube guidance and filiform apparatus formation.

MYB98 Encodes a Synergid Cell Transcription Factor

MYB98 is a member of the R2R3-MYB gene family, which comprises 125 genes in *Arabidopsis* (Stracke et al., 2001) and 80 to 84 genes in rice (*Oryza sativa*) (Jia et al., 2004; Jiang et al., 2004). R2R3-MYB proteins are thought to function as transcription factors because they contain a structurally conserved DNA binding domain referred to as the Myb domain. Consistent with this, many R2R3-MYB proteins have been shown to function in transcriptional regulation (Stracke et al., 2001; Dias et al., 2003). Here, we show that MYB98 is localized to the nuclei of the synergid cells (Figures 1A and 1B) and is required for the expression of a battery of genes in the synergid cells (Figure 3). We further show that MYB98 binds to a specific DNA sequence (Figure 2) and that a MYB98 binding site is necessary for synergid expression (Figure 4E). Together, these results strongly suggest that MYB98 functions as a transcriptional regulator within the synergid cells.
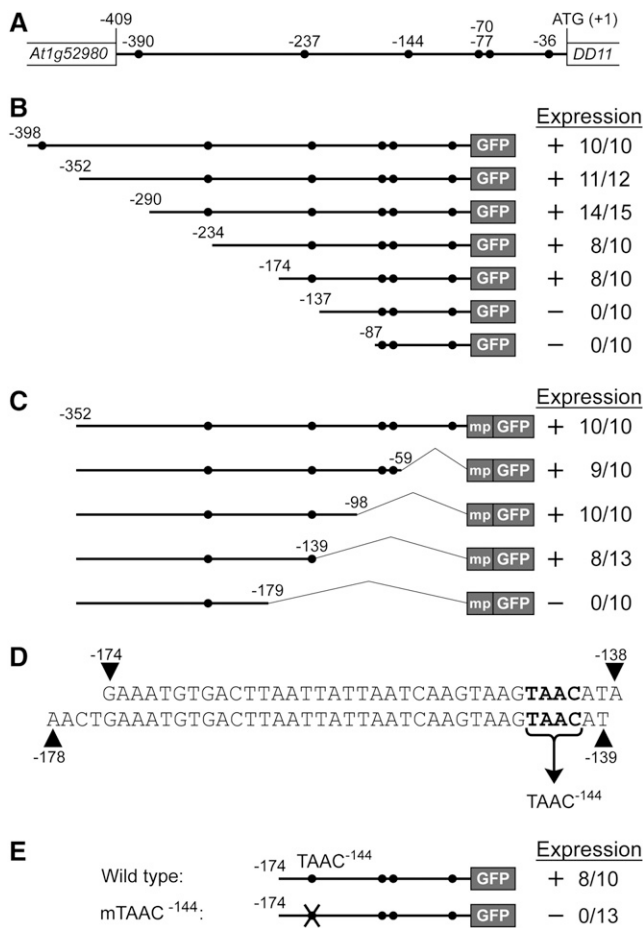
MYB98 Positively Regulates a Battery of Genes within the Synergid Cells

We previously identified a battery of synergid-expressed genes (Steffen et al., 2007), including *MYB98* (Kasahara et al., 2005). However, the relationships among these genes within the synergid gene regulatory network were not established. Here, we show that 16 of these genes are downregulated in *myb98* synergid cells (Table 2, Figure 3), establishing that they are positively regulated by *MYB98*. These data also show that *MYB98* regulates a large number of genes, at least 16 and presumably more.

Five of the genes downstream of *MYB98* (*DD3*, *DD4*, *DD31*, *DD39/QRT3*, and *DD67*) are expressed in the absence of *MYB98*, albeit at much reduced levels (Figure 3; see Supplemental Figure 3 online). The dramatically reduced expression of these genes in *myb98* female gametophytes indicates that MYB98 is a central regulator of these genes. The low but detectable expression of these genes in *myb98* female gametophytes indicates that other synergid factors can compensate, in part, for the absence of MYB98. Thus, these genes appear to be activated in the synergid cells by multiple factors.

MYB98 Binds to the Sequence TAAC and Directly Regulates *DD11*

The genes downstream of *MYB98* could be regulated either directly or indirectly by MYB98. As a first step toward understanding MYB98 regulation of the downstream genes, we identified MYB98 binding sites in vitro and in vivo. In vitro, MYB98

A

-409
-390
-237
-144
-70
-77
-36
ATG (+1)

At1g52980
DD11

B

Expression

-398 ── GFP + 10/10
-352 ── GFP + 11/12
-290 ── GFP + 14/15
-234 ── GFP + 8/10
-174 ── GFP + 8/10
-137 ── GFP − 0/10
-87 ── GFP − 0/10

C

Expression

-352 ── mp GFP + 10/10
-59 ── mp GFP + 9/10
-98 ── mp GFP + 10/10
-139 ── mp GFP + 8/13
-179 ── mp GFP − 0/10

D

-174 ▼ -138 ▼

GAAATGTGACTTAATTATTAATCAAGTAAG**TAAC**ATA
AACTGAAATGTGACTTAATTATTAATCAAGTAAG**TAAC**AT

-178 ▲ -139 ▲

TAAC⁻¹⁴⁴

TAAC^{-144}

E

Expression

Wild type: -174 TAAC^{-144} ── GFP + 8/10
mTAAC^{-144}: -174 ──✗── GFP − 0/13

Figure 4. Identification of Sequences Necessary for Synergid Expression within the *DD11* Promoter.

(A) Schematic of the genomic region encompassing the *DD11* promoter including the upstream gene (*At1g52980*). Closed circles represent putative MYB98 binding sites (YAAC). All contain the sequence TAAC, and they are designated TAAC^{-390}, TAAC^{-237}, TAAC^{-144}, TAAC^{-77}, TAAC^{-70}, and TAAC^{-36}.

(B) and **(C)** Expression of the 5′ **(B)** and 3′ **(C)** promoter deletion constructs. Schematics of the constructs are shown at left. The 5′ promoter deletion constructs **(B)** contain 5′ end points of between −398 and −87 bp relative to the translational start, a 3′ end point of +21 bp relative to the translational start, and are fused with a GFP coding region. The 3′ promoter deletion constructs **(C)** contain a 5′ end point of −352 bp relative to the translational start, 3′ end points of between −1 and −179 bp relative to the translational start, and are fused with a 35S minimal promoter (mp) and a GFP coding region. Expression levels of the corresponding constructs in transgenic plants are shown at right.

(D) Sequences of the regions identified as necessary for synergid expression in the 5′ (top) and 3′ (bottom) deletions of the *DD11* promoter. These regions contain a single TAAC site (TAAC^{-144}; boldface).

(E) Expression of the mTAAC^{-144} construct. A schematic of this construct is shown at left. mTAAC^{-144} contains a mutation in the core AAC residues (TAAC mutated to TGGG) of the MYB98 binding site and is otherwise identical to the −174 5′ promoter deletion construct **(B)**. Expression levels of the corresponding constructs in transgenic plants are shown at right.

In **(B)**, **(C)**, and **(E)**, expression levels are indicated as follows: + indicates

binds to the sequence YAAC (Y = thymine or cytosine), with a higher affinity for TAAC (Figure 2B). The sequence YAAC is contained within the binding sites of several other R2R3-MYB proteins, including WEREWOLF (Koshino-Kimura et al., 2005; Ryu et al., 2005), MYB2 (Abe et al., 1997), GAMyb (Gubler et al., 1995), MYBGA (Chen et al., 2006), C1 (Roth et al., 1991), P (Grotewold et al., 1994), and MYB.Ph3 (Solano et al., 1997).

To identify in vivo MYB98 binding sites, we dissected the promoter of the *DD11* gene. This analysis identified a 36-bp region that contains a MYB98 binding site at position −144 (TAAC^{-144}). Mutations in the core AAC residues of TAAC^{-144} abolish both MYB98 binding (Figure 2C) and synergid expression (Figure 4E). These data suggest very strongly that MYB98 directly regulates the *DD11* gene, although we cannot eliminate the possibility that MYB98 activates the expression of another MYB protein that binds to TAAC^{-144} in the *DD11* promoter.

The MYB98 binding site (TAAC) is unlikely to confer synergid expression on its own. Analysis using Patmatch (http://www.Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) indicates that this sequence is present one or more times in the promoter of essentially every gene within the *Arabidopsis* genome. Therefore, it is likely that additional *cis* elements are required to confer synergid-specific expression. Consistent with this, the *DD11* promoter contains six TAAC sites (Figure 4A), and only TAAC^{-144} was shown to be necessary for synergid expression. None of the other TAAC sites appear to be functional: TAAC^{-77}, TAAC^{-70}, and TAAC^{-36} do not compensate for the loss of TAAC^{-144} in construct mTAAC^{-144} (Figure 4E); TAAC^{-237} does not compensate for the loss of TAAC^{-144} in the −179 3′ promoter deletion construct (Figure 4C); and deletion of TAAC^{-390} does not affect expression (Figure 4B). These observations suggest that only TAAC^{-144} is functional. TAAC^{-144} may contain adjacent sequences required for optimal MYB98 binding or may be located at an optimal distance from other *cis* elements. Several other R2R3-MYB proteins, including WEREWOLF and GLABRA1 (Schiefelbein, 2003) and C1 (Grotewold, 2005), interact in a combinatorial fashion with cofactor proteins.

Currently, we do not know whether the other 15 genes are regulated directly or indirectly by MYB98. All of these genes contain multiple TAAC sites in their promoters. However, as discussed above, the same is true for essentially every gene in the *Arabidopsis* genome, and our analysis of the *DD11* promoter has shown that not all TAAC sites are functional; thus, the presence of the TAAC sites in the downstream genes is not indicative of direct regulation. Promoter analysis of the other downstream genes will be required to determine which, if any, are direct targets.

Functions of the Genes Downstream of *MYB98*

myb98 synergid cells have defects in pollen tube guidance and filiform apparatus formation (Kasahara et al., 2005), suggesting

that GFP was detected in the synergid cells in some transgenic plants, − indicates that GFP was not detected in the synergid cells in any transgenic plants, the numerator of the ratio indicates the number of transgenic lines expressing GFP, and the denominator of the ratio indicates the total number of transgenic lines analyzed.

that some or all of the downstream genes play a role in these processes. Consistent with this, five of the MYB98-regulated genes we identified encode proteins that localize to the filiform apparatus. Furthermore, 10 additional genes encode proteins containing putative N-terminal signal peptides, and an N-terminal signal peptide is sufficient to localize GFP to the filiform apparatus, suggesting that some of these untested proteins may also be localized to the filiform apparatus.

Many of the MYB98-regulated genes could feasibly encode a pollen tube attractant. Several of these genes are predicted to encode proteins involved in cell signaling. *DD32* encodes a protein similar to the S proteins from *Papaver*. These proteins are expressed in the stigma, interact with incompatible pollen, and induce a Ca^{2+}-dependent signaling pathway that results in cell death of the incompatible pollen tubes (Thomas et al., 2003; Thomas and Franklin-Tong, 2004). *DD2* is a member of the defensin-like family (Thomma et al., 2002). This family encodes proteins similar to defensins, which are small (typically 45 to 50 amino acids), Cys-rich, basic, secreted peptides with conserved secondary structure that function as antimicrobial peptides (Thomma et al., 2002) and signaling molecules (Li et al., 2001; Dresselhaus, 2006). *DD42* is predicted to encode a rapid alkalinization factor, and a related protein, RALF, is a secreted polypeptide that may function in signaling (Pearce et al., 2001; Haruta and Constabel, 2003). *DD67* encodes a subtilase, and members of this family are involved in the generation of extracellular peptide signals (Berger and Altmann, 2000). In addition, many of the genes of unknown function may encode signaling molecules. Most encode small, Cys-rich proteins with putative N-terminal signal peptides, suggesting that they may be secreted and may play a role in cell signaling (reviewed in Dresselhaus, 2006).

Several of the MYB98-regulated genes may play a role in filiform apparatus formation. Two of these genes are predicted to encode cell wall–modifying proteins. *DD39/QRT3* encodes a polygalacturonase involved in the degradation of pectin in plant cell walls (Rhee et al., 2003). *DD46* is predicted to encode a galactosyltransferase, and related proteins in *Arabidopsis* modify cell wall polysaccharides (Edwards et al., 1999; Li et al., 2004). Although the localization of these proteins has not been determined, they may be secreted into the filiform apparatus, because they also have putative N-terminal signal peptides (discussed above). These proteins may function to form or stabilize the specialized cell wall of the filiform apparatus.

The filiform apparatus dramatically increases the surface area of the plasma membrane at the micropylar pole of the synergid cells (see Supplemental Figure 1 online) and is associated with an elaborated endoplasmic reticulum, Golgi stacks, and numerous vesicles. Based on these structural features, it has been proposed that the filiform apparatus facilitates the transport of substances into and out of the synergid cells (Willemse and van Went, 1984; Huang and Russell, 1992). In support of this proposal, micropylar exudates likely from the synergid cells have been observed in *Gasteria verrucosa* (Franssen-Verheijen and Willemse, 1993). Here, we provide additional evidence. We show that five proteins are secreted into the filiform apparatus (Figures 1E to 1H; see Supplemental Figure 2 online). In addition, it is likely that some of the other 10 signal peptide–containing proteins are

also secreted into the filiform apparatus (discussed above). These data indicate that the filiform apparatus functions in transport and that it is the site at which numerous proteins are secreted from the synergid cells.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana seeds were surface-sterilized with chlorine gas and germinated on plates containing 0.5× Murashige and Skoog salts (Sigma-Aldrich M-9274), 0.05% MES, 1.0% sucrose, and 0.8% Phytagar (Life Technologies). Ten-day-old seedlings were transferred to Scott's Redi-Earth or Sunshine Mix No. 2 supplemented with MiracleGro and grown under 24 h of illumination. The following T-DNA lines were used: *DD4* (SALK_073152), *DD18* (SALK_117305), *DD31* (SALK_114435), *DD39/QRT3* (SAIL_912_G05), *DD56* (SALK_023946), and *DD67* (SALK_067858).

Plant Transformation

T-DNA constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. *Arabidopsis* plants (accession Columbia-0) were transformed using a modified floral dip procedure (Clough and Bent, 1998). Transformed progeny were selected by germinating surface-sterilized T1 seeds on growth medium containing antibiotics (30 µg/mL kanamycin sulfate or 5 µg/mL BASTA) supplemented with 15 µg/mL cefotaxime. Resistant seedlings were transplanted to soil at 10 d after germination. Transgene identity was verified by PCR using a gene-specific primer and a T-DNA–specific primer.

Expression and Purification of MYB98

A 372-bp region spanning the R2R3 DNA binding domain of MYB98 was amplified from a *MYB98* cDNA clone (Kasahara et al., 2005) using primers 98CF (5′-TTACGCCAAGCTCATATGTCATCATGGAAAGAAAC-3′) (introduces a *Nde*I site) and 98CR (5′-GGAGCTCGAATTCGGATCCTAATACT-TAGATCTAC-3′) (introduces a *Bam*HI site). The resulting fragment was ligated into pET28a(+) (Novagen) at the *Nde*I and *Bam*HI sites to generate clone pET28a.98. The resulting construct was sequenced and introduced into *Escherichia coli* strain BL21 (DE3) pLysS (Stratagene).

The transformed bacteria were grown to a density of 1.5 at OD_{600} at 37°C in 50 mL of Luria-Bertani medium, isopropyl-1-thio-β-galactopyranoside was added to a final concentration of 100 nM, and the bacterial culture was incubated at room temperature for 1 h. The culture was then centrifuged to pellet the bacteria, the supernatant was removed, and the bacterial pellet was resuspended in 2 mL of HIS-Bind buffer (20 mM Tris-Cl, 0.5 M NaCl, and 5 mM imidazole, pH 8.0) plus 25 µL of *E. coli* protease inhibitors (Sigma-Aldrich).

To isolate the recombinant protein, the cells were disrupted using a probe sonicator and centrifuged at 20,000g for 15 min. One hundred microliters of nickel-nitrilotriacetic acid agarose slurry (GE/Amersham) was then added to the supernatant. This mixture was incubated with gentle rocking for 20 min at 4°C. The mixture was then centrifuged, the supernatant was removed, and the pellet was washed three times, once with 1 mL of HIS-Bind buffer followed by two washes with 1 mL of HIS-Wash buffer (20 mM Tris-Cl, 0.5 M NaCl, and 60 mM imidazole, pH 8.0). The pellet was then resuspended in 200 µL of HIS-Elute buffer (20 mM Tris-Cl, 0.5 M NaCl, and 1 M imidazole, pH 8.0) for 10 min at room temperature. This solution was then centrifuged, and the supernatant was dialyzed in storage buffer (20% glycerol, 0.5 mM DTT, 6 mM $MgCl_2$,

50 mM KCl, 0.1 mg/mL BSA, 10 mM Tris-Cl, and 1 mM EDTA, pH 8.0) using a Centriprep centrifugal filter unit with an Ultracel YM-3 membrane (Millipore).

EMSAs

Oligonucleotides for the gel mobility shift assays were obtained from Operon (www.operon.com) and made double-stranded by annealing sense and complementary oligonucleotides in TEN buffer (10 mM Tris-Cl, 1 mM EDTA, and 0.1 M NaCl, pH 8.0) by heating to 95°C for 5 min and slowly cooling to room temperature (~30 min). Double-stranded oligonucleotides were labeled with digoxigenin using the Roche digoxigenin gel shift kit, second generation (Roche), following the manufacturer's protocol. The binding reactions were performed in binding buffer (10% glycerol, 0.5 mM DTT, 6 mM $MgCl_2$, 50 mM KCl, 0.1 mg/mL BSA, 10 mM Tris-Cl, and 1 mM EDTA, pH 8.0) for 15 min at room temperature. For all reactions, 77 fM of digoxigenin-labeled oligonucleotides, the appropriate concentration of unlabeled oligonucleotides, and 20 ng of protein were mixed in a 10-μL binding reaction. The binding reactions were then incubated on ice for 5 min and were resolved on 1-mm-thick 8% native polyacrylamide gels in 0.5× TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA, pH 8.0) at 250 V for 20 min using the mini Protean 3 system (Bio-Rad). The oligonucleotides were transferred to Hybond-N+ nylon transfer membranes (GE/Amersham) by electro-blotting at 400 mA for 30 min in 0.5× TBE. The oligonucleotides were cross-linked to the nylon membranes using a UV Stratalinker 1800 (Stratagene) at the standard setting (120,000 μJ/cm²). Digoxigenin-labeled oligonucleotides were detected according to the manufacturer's protocol (Roche).

Analysis of GFP Expression Patterns

Plants containing GFP constructs were analyzed using a Zeiss Axioplan compound microscope. GFP was excited using a UV lamp and detected with the 38 HE EGFP filter set; images were captured using an Axiocam MRm REV2 camera with the AxioVision software package version 4.5 (Zeiss). Analysis was performed at 24 h after emasculation of stage 12c flowers (Christensen et al., 1997). For analysis of GFP fluorescence in the synergid cells, we followed the dissection procedure outlined by Steffen et al. (2007).

Protein Fusion Constructs

To generate the *MYB98* protein fusion construct, we amplified the promoter region (−686 bp from the start of translation) using primers p98EF1 and p98ER1. This PCR product was cloned into pEGAD (Cutler et al., 2000) using the *Sac*I and *Age*I sites (replacing the 35S promoter), resulting in construct *ProMYB98:GFP*. Next, the *MYB98* coding region was amplified from a cDNA clone (Kasahara et al., 2005) using the primers 98Fegad and 98Regad. This fragment was cloned into *ProMYB98:GFP* using *Eco*RI and *Bam*HI, resulting in construct *ProMYB98:GFP-MYB98*. Sequences of primers are listed in Supplemental Table 1 online.

To determine whether the GFP-MYB98 protein fusion complements the *myb98-1* phenotype, we introduced the *ProMYB98:GFP-MYB98* construct into *myb98-1/myb98-1* plants by *Agrobacterium*-mediated transformation. In the T1 generation, we observed rescue of the pollen tube guidance defect in at least 50% of ovules in 17 independent transformants. In addition, in the T2 generation, we identified plants homozygous for both the *myb98-1* mutation and the *ProMYB98:GFP-MYB98* construct; these plants had full seed set.

To generate the protein fusion constructs for *DD2*, *DD4*, *DD11*, *DD12*, and *DD32*, we amplified the promoter regions and coding regions using

the following primers: DD2F and DD2R for *DD2*, DD4F and DD4R for *DD4*, DD11F and DD11R for *DD11*, DD12F and DD12R for *DD12*, and DD32F and DD32R for *DD32*. Sequences of primers are listed in Supplemental Table 1 online. In all cases, the PCR primers added unique restriction enzyme sites that were used to ligate the PCR products into the pBI101.gfp vector. These constructs were introduced into wild-type *Arabidopsis* plants by *Agrobacterium*-mediated transformation, and at least 10 transgenic lines were analyzed per construct.

To generate the protein fusion construct for the DD11 signal peptide (*ProMYB98:SP-GFP*), we amplified 700 bp of the MYB98 regulatory region using the following primers: 5941-98F and 5941-98R3. This PCR product was cloned into pFGC5941 (Kerschen et al., 2004) using the *Eco*RI and *Nco*I sites (replacing the 35S promoter), resulting in the construct fFGC98. We amplified the GFP coding sequence from pBI101.gfp using the following primers: GFPF1 and GFPR1. This PCR product was cloned into pGFC98 using the *Bam*HI and *Xba*I sites, resulting in the construct pFGC98-GFP. We synthesized complementary oligonucleotides encompassing the putative N-terminal signal peptide from *DD11* (DD11-SP1, 5′-CGCGCCATGGAGAAAGCAATTCTCATAACGTTTCTCATAGCCACC-ACATCGATGGTTTATCAAACTG-3′; DD11-SP2, 5′-GATCCAGTTTGAT-AAACCATCGATGTGGTGGCTATGAGAAACGTTATGAGAATTGCTTTC-TCCATGG-3′). These oligonucleotides were annealed creating compatible overhangs, which were used to clone the *DD11* signal peptide into pGFC98-GFP using the *Asc*I and *Bam*HI sites, resulting in the construct *ProMYB98:SP-GFP*. All constructs were sequence-verified. The putative DD11 signal peptide consists of the following amino acid sequence: 5′-MEKAILITFLIATTSMVYQT-3′ (PSORT, http://psort.nibb.ac.jp/). This construct was introduced into wild-type *Arabidopsis* plants by *Agrobacterium*-mediated transformation, and 17 transgenic lines were analyzed.

Analysis of Promoter:GFP Expression in *MYB98/MYB98*, *MYB98/myb98*, and *myb98/myb98* Pistils

We first identified single-locus lines for each reporter construct. For each promoter:GFP construct, T2 seeds from multiple T1 lines were plated on growth medium containing kanamycin sulfate (30 μg/mL), the resistant:sensitive ratio was scored, and lines with a resistant:sensitive ratio of 3:1 were transplanted to soil. Eight plants from each line were scored for the percentage of synergid cells expressing GFP to confirm that the reporter construct was present at only a single locus and to identify homozygous plants.

Plants homozygous for the promoter:GFP transgenes were used in a cross with *myb98-1/myb98-1* pollen. The resulting F1 plants were hemizygous at the promoter:GFP locus and heterozygous at the *MYB98* locus. F1 plants were used as pollen donors in a second cross with *myb98-1/myb98-1* females. The resulting F1 plants were genotyped by PCR to identify plants hemizygous for the promoter:GFP locus and homozygous for the *myb98-1* mutation using primers described by Kasahara et al. (2005).

When scoring the percentage of female gametophytes with GFP fluorescence in the synergid cells, at least three pistils were analyzed from at least three individuals per generation.

Promoter Deletion Constructs

Promoter fragments were generated by PCR using the primers listed in Supplemental Table 2 online. The primers introduced unique restriction enzyme sites. The PCR products were digested and ligated with pBI101.GFP (for 5′ deletions) or 35SmpGFP (for 3′ deletions) (Yadegari et al., 2000). All constructs were sequence-verified. The 5′ deletion constructs were fused with a GFP coding region. The 3′ deletion constructs were fused with the minimal promoter:GFP construct (mpGFP),

which contained a GFP coding region fused at its 5′ end with a minimal promoter derived from the cauliflower mosaic virus 35S promoter (Benfey and Chua, 1990).

Real-Time RT-PCR

Pistils were harvested from *ms1/ms1* and *myb98-1/myb98-1* plants and placed immediately into liquid nitrogen. Pistils were harvested from the oldest stage 12 flower (Smyth et al., 1990) on each plant as well as the next two older flowers.

RNA was extracted from the pistil tissue using the Qiagen RNeasy kit following the manufacturer's protocol (www.qiagen.com). DNA contamination was removed from samples using the Ambion TURBO DNA-*free* DNase kit following the manufacturer's protocol. After DNase treatment, RNA samples were repurified using the Qiagen RNeasy kit following the manufacturer's protocol. Aliquots of RNA (1 μg) were reverse-transcribed using the RETROscript kit (Ambion) following the manufacturer's protocol.

For real-time RT-PCR, PCR was performed using the Roche FastStart DNA Master SYBR Green I master mix (www.roche.com) in a volume of 10 μL on a Roche LightCycler system. The PCR mixture consisted of 0.5 μL of cDNA, 0.5 μM primers, and 1× master mix. For each real-time RT-PCR run, *ACTIN2* (At3g18780) was used as an internal control to normalize for any pipetting error of the cDNA template. The PCR program consisted of 95°C for 5 min followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 10 s. To determine the specificity of the PCR, the amplified products were subjected to melt curve analysis using the machine's standard method as well as run on a gel. The reported threshold cycle values are averages of two independent trials (biological replicates). For *DD18* and *DD32*, expression was not detected in *myb98-1* ovules. In these cases, a threshold cycle value of 40 was used to calculate fold change. All real-time RT-PCR primers were the same as reported by Steffen et al. (2007).

Identification of TAAC Sites within the *Arabidopsis* Genome

We used Patmatch 1.1 (http://www.Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) to search for the sequence TAAC within the Locus Upstream Sequences −500bp (DNA) database. This analysis identified 28,180 genes containing this sequence in their promoters.

Supplemental Data

The following materials are available in the online version of this article.

   Supplemental Figure 1. Depiction of the Synergid Cells and the Filiform Apparatus.

   Supplemental Figure 2. Localization of GFP Fusion Proteins in Wild-Type Ovules.

   Supplemental Figure 3. Intensity of the GFP Signal from *ProDD3:GFP* in *MYB98* and *myb98-1* Synergid Cells.

   Supplemental Table 1. Primers Used to Generate the Protein Fusion Constructs.

   Supplemental Table 2. Primers Used to Generate the 5′ and 3′ Deletions of the *DD11* Promoter.

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