AGL61 Interacts with AGL80 and Is Required for Central Cell Development in Arabidopsis[1][W][OA]

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The central cell of the female gametophyte plays a role in pollen tube guidance and in regulating the initiation of endosperm development. Following fertilization, the central cell gives rise to the seed's endosperm, which nourishes the developing embryo within the seed. The molecular mechanisms controlling specification and differentiation of the central cell are poorly understood. We identified *AGL61* in a screen for transcription factor genes expressed in the female gametophyte. *AGL61* encodes a Type I MADS domain protein, which likely functions as a transcription factor. Consistent with this, an AGL61-green fluorescent protein fusion protein is localized to the nucleus. In the context of the ovule and seed, *AGL61* is expressed exclusively in the central cell and early endosperm. *agl61* female gametophytes are affected in the central cell specifically. The morphological defects include an overall reduction in size of the central cell and a reduced or absent central cell vacuole. When fertilized with wild-type pollen, *agl61* central cells fail to give rise to endosperm. In addition, synergid- and antipodal-expressed genes are ectopically expressed in *agl61* central cells. The expression pattern and mutant phenotype of *AGL61* are similar to those of *AGL80*, suggesting that AGL61 may function as a heterodimer with AGL80 within the central cell; consistent with this, AGL61 and AGL80 interact in yeast two-hybrid assays. Together, these data suggest that AGL61 functions as a transcription factor and controls the expression of downstream genes during central cell development.

The central cell of the female gametophyte is critical for several steps of the angiosperm fertilization process. During the late stages of pollen tube growth, a pollen tube grows along the carpel's placental surface, onto the ovule's funiculus, and finally into the ovule's micropyle to reach the female gametophyte. Soon after entering the female gametophyte, the pollen tube releases its two sperm cells to effect double fertilization of the egg cell and central cell, which give rise to the seed's embryo and endosperm, respectively. Endosperm is an important component of the seed because it provides nutrients and other factors to the embryo during seed development and/or to the developing seedling following germination (for review, see Drews and Yadegari, 2002; Yadegari and Drews, 2004).

The central cell is required for pollen tube guidance. Mutations in the Arabidopsis (*Arabidopsis thaliana*) *CENTRAL CELL GUIDANCE* (*CCG*) gene affect the female gametophyte. *ccg* mutants undergo normal female gametophyte development but are defective in pollen tube guidance. *CCG* is expressed specifically in the central cell and encodes a protein with similarity to TFIIB. Although the role of *CCG* in pollen tube guidance is unclear, its expression pattern and mutant phenotype suggest that the central cell plays a critical role in pollen tube guidance (Chen et al., 2007).

The central cell also plays a role in controlling the initiation of endosperm development. The central cell expresses a set of genes that represses endosperm development in the absence of fertilization. These genes are collectively referred to as the *FERTILIZATION INDEPENDENT SEED* (*FIS*)-class genes and include *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*; Ohad et al., 1999), *FIS2* (Luo et al., 1999), *MEDEA* (*MEA*; Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999), *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*; Kohler et al., 2003a; Guitton et al., 2004), and *SWINGER* (*SWN*; Wang et al., 2006). *fis* mutant female gametophytes undergo endosperm development in the absence of fertilization. The FIS proteins are related to the Polycomb-group (PcG) proteins involved in heritable silencing of homeotic gene expression in Drosophila and mammals. These observations have led to a model in which the FIS proteins form a complex that represses genes involved in endosperm development within the central cell (for review, see Curtis and Grossniklaus, 2008).

The central cell forms during megagametogenesis. Most species including Arabidopsis and cereals undergo the *Polygonum* pattern of megagametogenesis.

During *Polygonum*-type megagametogenesis, a one-nucleate megaspore undergoes two rounds of mitosis, producing a four-nucleate cell. During a third round of mitosis, phragmoplasts and cell plates form between nuclei, initiating the cellularization process. Ultimately, the nuclei become completely surrounded by cell walls, resulting in formation of a seven-celled female gametophyte consisting of one central cell, one egg cell, two synergid cells, and three antipodal cells. The central cell inherits two nuclei, the polar nuclei. In Arabidopsis and many other species, the polar nuclei fuse to form the diploid central cell nucleus (secondary nucleus; for review, see Willemse and van Went, 1984; Huang and Russell, 1992; Yadegari and Drews, 2004).

Little is known about the regulatory processes controlling central cell development and few transcriptional regulators functioning in this cell have been identified. Those identified include the *FIS* genes discussed above, as well as *AGL80* (Portereiko et al., 2006) and *DEMETER* (*DME*; Choi et al., 2002). *DME* encodes a DNA glycosylase required for the activation of *FIS2*, *FWA*, and *MEA* expression in the central cell and endosperm (Choi et al., 2002; Jullien et al., 2006). *AGL80* encodes a Type I MADS-domain protein. *agl80* female gametophytes have defects in central cell morphology and fail to form endosperm when fertilized with wild-type sperm. *AGL80* is expressed in the central cell and is required for the expression of several central cell-expressed genes including *DME* and *DD46* (Portereiko et al., 2006).

To identify additional transcriptional regulators functioning in the central cell and female gametophyte, we performed a sensitive differential expression screen to identify such genes. Here, we report the identification of *AGL61*, which encodes a Type I MADS domain protein. We show that (1) *AGL61* is expressed exclusively in the central cell and endosperm during ovule and seed development, (2) *agl61* mutants have central cell defects similar to those of *agl80*, and (3) AGL61 interacts with AGL80 in yeast (*Saccharomyces cerevisiae*). Together, these results suggest that an AGL61-AGL80 heterodimer functions in the central cell to control the expression of downstream genes that are critical for central cell and endosperm development.

RESULTS

AGL61 Is Expressed in the Central Cell

We performed a screen to identify MADS box genes expressed in the female gametophyte. We harvested ovaries from *male sterility1* (*ms1*; Thorlby et al., 1997; Wilson et al., 2001; Ito and Shinozaki, 2002) and *determinant infertile1* (*dif1*; Bai et al., 1999; Bhatt et al., 1999; Cai et al., 2003), extracted RNA, and used real-time reverse transcription (RT)-PCR to assay the expression of genes within this gene family. *ms1* ovules are normal and *dif1* ovules lack female gametophytes (Steffen et al., 2007); thus, genes exhibiting reduced

expression in *dif1* ovaries relative to *ms1* ovaries are likely to be expressed in the female gametophyte.

These assays identified a gene, *AGL61*, exhibiting reduced expression in *dif1* ovaries relative to wild-type ovaries. The structure of *AGL61* is summarized in Figure 1 and the real-time RT-PCR data are provided in Figure 2A.

To determine which cells within the female gametophyte express *AGL61*, we generated and analyzed transgenic Arabidopsis plants containing a protein-fusion construct, *AGL61-GFP*, comprising the *AGL61* promoter and the entire *AGL61* coding region fused with a GFP coding sequence. Figure 3, A to C, show *AGL61-GFP* expression during female gametophyte development (female gametophyte stages are described in Christensen et al. [1997]). *AGL61-GFP* expression was first detected in the two polar nuclei just before fusion (late stage FG5; Fig. 3A). *AGL61-GFP* expression was not detected at earlier developmental stages. Expression in the central cell continued through stage FG6 (Fig. 3B) and into the mature stage (stage FG7; Fig. 3C). During all of these stages, the AGL61-GFP fusion protein was localized to the nucleus, consistent with a predicted function in transcriptional regulation.

To determine whether *AGL61* is also expressed in developing seeds, we analyzed *AGL61-GFP* expression at 12 to 48 h after pollination. During this period, *AGL61-GFP* expression was detected exclusively in the endosperm (Fig. 3D). During endosperm development, *AGL61-GFP* expression was strongest immediately after fertilization, diminished gradually at progressively older stages, and was not detected after
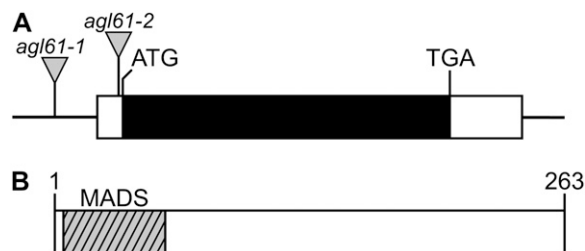

Figure 1. Structures of the *AGL61* gene and AGL61 protein. A, *AGL61* gene structure. The black box represents the predicted coding sequence (633 nucleotides) and the white boxes represent the 5′ (49 nucleotides) and 3′ (139 nucleotides) untranslated regions. The *AGL61* open reading frame contains three in-frame start codons. The transcriptional start site is at position 10,588,112 within the genomic sequence (chromosome 2) and the three start codons are 46, 49, and 91 nucleotides downstream of the transcriptional start site. The second start codon (at position +49) is expected to initiate translation because it closely satisfies the consensus sequence criteria for a translation initiation codon (Kozak, 1991) and is positioned most closely to those of other MADS box genes (Parenicova et al., 2003). The insertion sites of the T-DNAs in the *agl61-1* and *agl61-2* mutants are marked by triangles. The T-DNA in *agl61-1* is inserted 81 nucleotides upstream of the transcriptional start site and is associated with a 17-bp deletion. The T-DNA in *agl61-2* is inserted immediately upstream of the predicted ATG and is associated with a 235-nucleotide insertion of unknown origin. B, AGL61 protein structure. AGL61 contains a MADS domain (gray hatched box; amino acids 7–68).
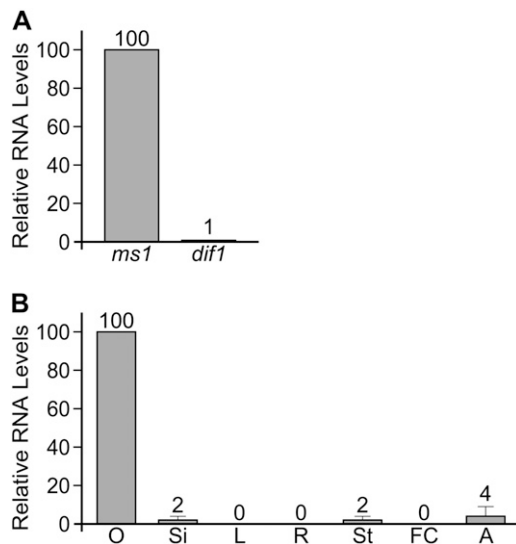
Figure 2. Real-Time RT-PCR analysis of *AGL61* expression. A, *AGL61* expression in *ms1/ms1* and *dif1-2/dif1-2* ovaries. B, *AGL61* expression in ovaries (O), siliques at 1 to 3 d after pollination (Si), leaves (L), roots (R), floral stems (St), floral clusters (FC), and anthers (A). In both A and B, each bar represents an average of three independent reactions, including both biological and technical replicates. In all cases, *AGL61* transcript levels were normalized to *ACTIN2* levels. Error bars indicate SD.

the eight-nucleate stage (stage IV) of endosperm development (endosperm stages are described in Boisnard-Lorig et al. [2001]). In reciprocal crosses with plants homozygous for the *AGL61-GFP* construct and wild type, expression was detected only when the reporter construct was present in the female parent.

We also analyzed expression of an *AGL61* promoter-fusion construct, *ProAGL61:GFP*. As with *AGL61-GFP*, *ProAGL61:GFP* was expressed exclusively in the central cell (Fig. 3E) and endosperm (Fig. 3F) during female gametophyte and seed development. In contrast to *AGL61-GFP*, *ProAGL61:GFP* expression persisted until the 16-nucleate stage (stage V) of endosperm development.

To determine whether *AGL61* is expressed elsewhere in the plant, we performed real-time RT-PCR with RNA from various organs. The results from these assays are shown in Figure 2B. Consistent with expression of *AGL61-GFP* and *ProAGL61:GFP* in the female gametophyte, strong *AGL61* expression was detected in ovaries. In addition, weak expression was detected in siliques, which correlates with limited *AGL61-GFP* and *ProAGL61:GFP* expression during seed development, and in stems and anthers. Expression was not detected by real-time RT-PCR in roots, leaves, and young flowers (Fig. 2B).

In summary, during ovule and seed development, *AGL61* is expressed exclusively in the central cell and endosperm, from late stage FG5 (just after central cell cellularization and before the polar nuclei fuse) of female gametophyte development to stage IV (eight-nucleate stage) or V (16-nucleate stage) of endosperm

development. Elsewhere in the plant, *AGL61* expression is extremely low or is not detected.

Mutations in *AGL61* Affect the Female Gametophyte

To determine whether mutations in *AGL61* affect the female gametophyte, we analyzed lines containing T-DNA insertions in this gene. We analyzed two T-DNA alleles, *agl61-1* (SALK_009008) and *agl61-2* (GABI-Kat 642H10), which were obtained from the Arabidopsis SIGnAL (Alonso et al., 2003) and GABI-Kat (Rosso et al., 2003) collections, respectively. The T-DNA insertion sites in these mutants are shown in Figure 1A.

To determine whether the *agl61* mutations affect the female gametophyte, we crossed heterozygous mutant plants as females with wild-type males and scored the number of *AGL61/AGL61* and *agl61/AGL61* progeny. Table I shows that both mutations exhibited reduced transmission through the female gametophyte, indicating that they affect the female gametophyte (Supplemental Fig. S1).

To determine whether the *agl61* mutations also affect the male gametophyte, we crossed heterozygous mutant plants as male parents with wild-type females and scored the number of *AGL61/AGL61* and *agl61/AGL61* progeny. With both alleles, homozygous wild-type and heterozygous progeny were present in approximately equal proportions (Table I), indicating that these mutations do not affect the male gametophyte (Supplemental Fig. S1).

Table I shows that the *agl61* mutations transmit through the female gametophyte at low frequency. Based on the observed gametophytic transmission frequencies (Table I), homozygous mutants should be present at a frequency of 1.1% to 2.5%. However, homozygous mutants were not identified in >800 plants screened for each allele. These results along with the *AGL61* expression pattern suggest that the *agl61* mutations affect seed development.

Molecular Complementation of the *agl61-1* Mutation

To confirm that the female gametophyte defect is due to disruption of *AGL61*, we introduced a wild-type copy of this gene into the *agl61-1* mutant. We identified plants heterozygous for the *agl61-1* allele and hemizygous for the rescue construct; these plants contained 25% aborted seeds, as compared to 50% aborted seeds for *agl61-1* plants lacking the rescue construct. In the subsequent generation, we identified plants heterozygous for the *agl61-1* allele and homozygous for the rescue construct; these plants had full seed set. Together, these data indicate that disruption of the *AGL61* gene is responsible for the female gametophyte defect in *agl61-1* mutants.

Mutations in *AGL61* Affect the Central Cell

To determine whether the *agl61* mutations affect megagametogenesis, we analyzed *agl61-1* and *agl61-2*
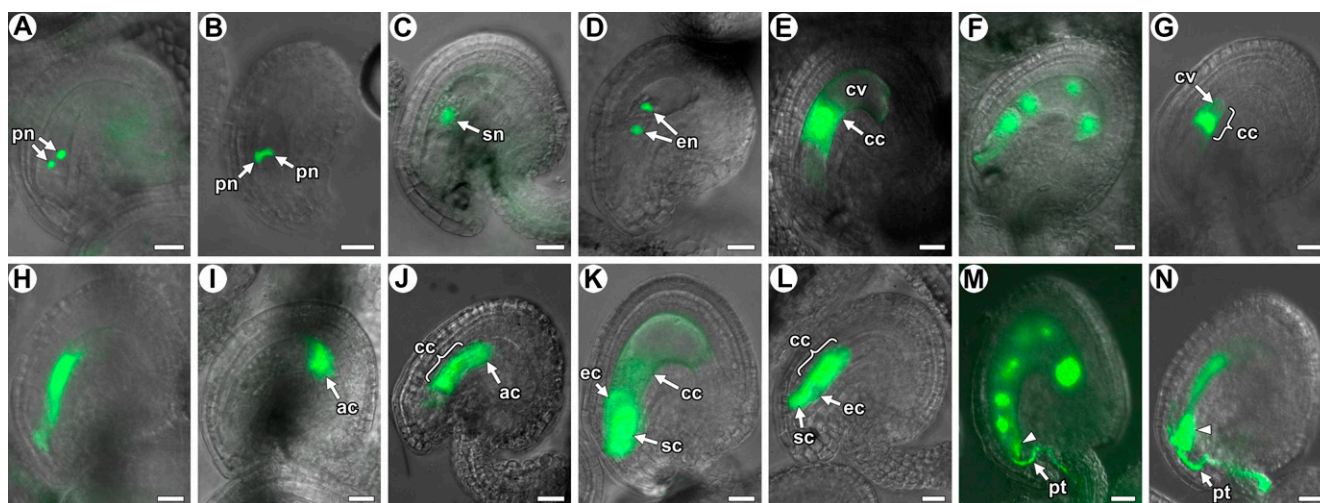
Figure 3. Analysis of protein- and promoter-fusion constructs in wild-type and *agl61* female gametophytes and endosperm. All panels show fluorescence bright-field overlay images. A to D, *AGL61-GFP* expression in wild-type female gametophytes and endosperm. A, *AGL61-GFP* expression at late stage FG5 (after cellularization but before fusion of the polar nuclei). B, *AGL61-GFP* expression at early stage FG6, during fusion of the polar nuclei. C, *AGL61-GFP* expression in a mature female gametophyte (stage FG7). D, *AGL61-GFP* expression in a seed at stage II (two-nucleate stage) of endosperm development. E and F, *ProAGL61:GFP* expression in wild-type female gametophytes and endosperm. E, *ProAGL61:GFP* expression in a mature female gametophyte (stage FG7). F, *ProAGL61:GFP* expression in a seed at stage III (four-nucleate stage) of endosperm development. G and H, *ProAGL61:GFP* expression in *agl61-1* female gametophytes and endosperm. G, *ProAGL61:GFP* expression in a mature (stage FG7) *agl61-1* female gametophyte. H, *ProAGL61:GFP* expression in a seed at 24 h after pollination. I and J, *ProDD1:GFP* expression in wild-type (I) and *agl61-1* (J) female gametophytes at stage FG7. In wild-type female gametophytes (I), *ProDD1:GFP* is expressed only in the antipodal cells. In *agl61-1* female gametophytes (J), *ProDD1:GFP* is expressed in both the antipodal cells and the central cell. K and L, *ProDD3:GFP* expression in wild-type (K) and *agl61-1* (L) female gametophytes at stage FG7. In wild-type female gametophytes (K), *ProDD3:GFP* is expressed strongly in the synergid cells and weakly in the egg cell and the central cell. In *agl61-1* female gametophytes (L), *ProDD3:GFP* is expressed strongly in the synergid cells, strongly in the central cell, and weakly in the egg cell. M and N, Fluorescence images of pollen tubes on wild-type (M) and *agl61-1* (N) seeds at 24 h after pollination. Endosperm and pollen tube fluorescence are due to expression of *ProAGL61:GFP* and *ProLAT52:GFP*, respectively. Arrowheads indicate the GFP bolus released from the pollen tube. Female gametophyte and endosperm stages are described in Christensen et al. (1997) and Boisnard-Lorig et al. (2001), respectively. ac, Antipodal cells; cc, central cell; cv, central cell vacuole; ec, egg cell; en, endosperm nuclei; pn, polar nuclei before fusion; pt, pollen tube; sc, synergid cell; sn, secondary nucleus of the central cell. Scale bars = 20 μm.

female gametophytes using confocal laser scanning microscopy (CLSM; Christensen et al., 1997). *agl61-1* and *agl61-2* had similar phenotypes. Here, we report a description of *agl61-1*.

We first analyzed female gametophytes at the terminal developmental stage (stage FG7). We emasculated *agl61-1/AGL61* flowers at stage 12c (Christensen et al., 1997), waited 24 h, fixed ovule tissue for confocal analysis, and analyzed >100 female gametophytes. Of the observed female gametophytes, approximately 50% (55/107) were normal and approximately 50% (52/107) were abnormal, suggesting that that the ab-

Table I. *Segregation of the agl61-1 and agl61-2 mutations*

Parental Genotypes		Progeny Genotypes		
Male	Female	*AGL61/AGL61*	*agl61/AGL61*	*agl61/agl61*
agl61-1/AGL61	*agl61-1/AGL61*	49% (93[a])	51% (95[a])	0% (0[a])
AGL61/AGL61	*agl61-1/AGL61*	98% (95[b])	2% (2[b])	–
agl61-1/AGL61	*AGL61/AGL61*	52% (43)	48% (40)	–
agl61-2/AGL61	*agl61-2/AGL61*	50% (98[a])	50% (97[a])	0% (0[a])
AGL61/AGL61	*agl61-2/AGL61*	95% (77)	5% (4)	–
agl61-2/AGL61	*AGL61/AGL61*	45% (45[b])	55% (55[b])	–

[a] χ^2 values are not significantly different at a threshold of $P = 0.01$ from those expected under the hypothesis of a female gametophyte-lethal phenotype (i.e. 1:1:0 segregation). [b] χ^2 values are not significantly different at a threshold of $P = 0.01$ from those expected under the hypothesis of wild-type male gametophyte transmission (i.e. 1:1 segregation).

normal female gametophytes corresponded to *agl61-1*. Wild-type female gametophytes at this stage have one egg cell, one central cell, and two synergid cells (Fig. 4A). In *agl61-1* female gametophytes at this stage, the egg cell and synergid cells were indistinguishable from those of the wild type (Fig. 4, B and C). By contrast, *agl61-1* central cells exhibited several defects including an overall reduction in size and a reduced (Fig. 4B) or absent (Fig. 4C) vacuole. In addition, the central cell nucleus (secondary nucleus) often was in an abnormal position in *agl61-1* central cells (Fig. 4C).

To determine whether *agl61-1* female gametophytes are affected at earlier developmental stages, we analyzed female gametophytes ($n = 59$) within stage 12c flowers, which contain embryo sacs at stages FG4 to FG6 (Christensen et al., 1997). In flowers at this stage, abnormal female gametophytes were not observed, suggesting that *agl61-1* female gametophytes do not exhibit defects at these earlier stages.

To characterize endosperm derived from fertilization of *agl61* central cells, we pollinated *agl61-1/AGL61* flowers with wild-type pollen, waited 24 h, and fixed seed tissue for confocal analysis. In the siliques resulting from this cross, approximately 50% (51/95) of the seeds were normal and approximately 50% (44/95) were abnormal, suggesting that that the abnormal seeds resulted from fertilization of *agl61-1* embryo sacs. In wild-type seeds at 24 h after pollination, one of the synergid cells is degenerated, the embryo is a single-celled zygote, and the endosperm typically consists of four to eight nuclei (Fig. 4D). In most (84%, 37/44) of the abnormal seeds, the embryo sac chamber was filled with highly autofluorescent material (Fig. 4E). A minority (16%, 7/44) of abnormal seeds had a few endosperm nuclei at abnormal positions (Fig. 4F) and a zygote-like structure (Fig. 4G).

To further characterize the defects in *agl61-1*, we used fluorescence microscopy to analyze development of GFP-marked central cells and endosperm. We analyzed plants heterozygous for the *agl61-1* mutation and hemizygous for *ProAGL61:GFP*, which is expressed in *agl61-1* central cells and endosperm (discussed below). In mature female gametophytes (stage FG7), defective central cells were readily apparent. Of the female gametophytes expressing GFP, approximately 50% (31/63) contained abnormal central cells that resembled those described above: the central cell vacuole was reduced in size or absent and the overall
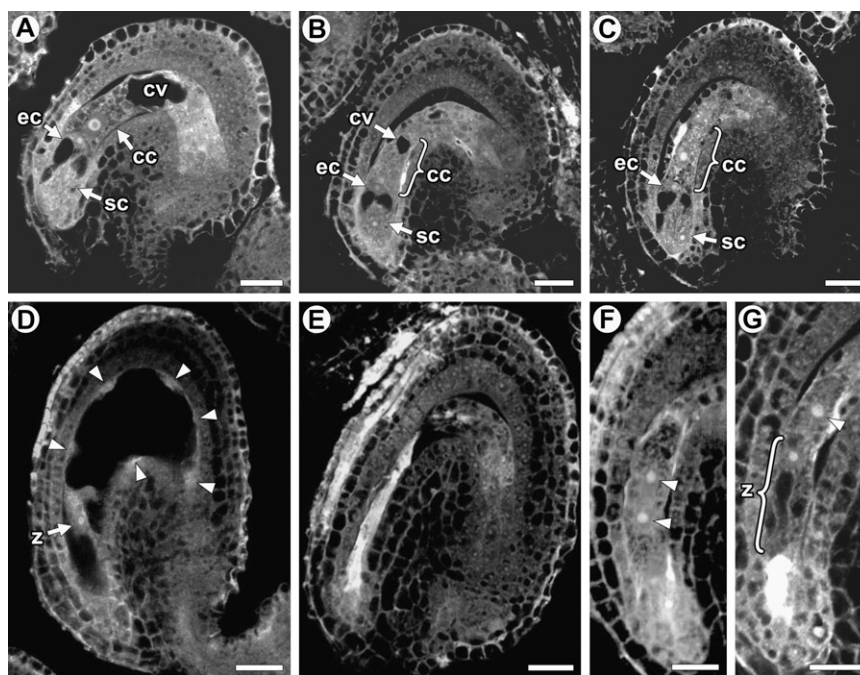

Figure 4. Microscopic analysis of wild-type and *agl61-1* female gametophytes and seeds. All panels are CLSM images. In these images, cytoplasm is gray, vacuoles are black, and nucleoli are white. A, Wild-type female gametophyte at the mature stage (stage FG7) containing one central cell, one egg cell, and two synergid cells. B, and C, *agl61-1* female gametophytes at the mature stage (stage FG7). The overall size of the central cell is reduced, the central cell vacuole is reduced (B) or absent (C), and the secondary nucleus occasionally is in the wrong position (C). D, Wild-type seed at 24 h after pollination. At this time point, the endosperm typically contains four to eight nuclei (arrowheads) and the embryo sac cavity is expanded. Only six of the eight endosperm nuclei are visible in this image. E to G, *agl61-1* seeds at 24 h after pollination. The predominant phenotype is shown (E); the embryo sac cavity is collapsed and is filled with highly autofluorescent material, and endosperm nuclei are not observed. A minority phenotype is shown (F and G); the embryo sac cavity is not fully expanded and contains a few endosperm nuclei in abnormal positions (arrowheads in F) and a zygote-like structure (G). Female gametophyte and endosperm stages are described in Christensen et al. (1997) and Boisnard-Lorig et al. (2001), respectively. cc, Central cell; cv, central cell vacuole; ec, egg cell; sc, synergid cell; z, zygote-like structure. Arrowheads point to endosperm nuclei. Scale bars = 20 μm.

size of the central cell was dramatically reduced (Fig. 3G). At 24 h after pollination with wild-type pollen, approximately 50% (51/108) of the seeds were defective and most of these had no endosperm (Fig. 3H).

In summary, *agl61* female gametophytes are defective in central cell development. *agl61* central cells are reduced in size and have collapsed vacuoles, but appear to be viable, based on expression of a central cell marker. Fertilization of *agl61* female gametophytes with wild-type sperm leads to aberrant endosperm development and eventually seed abortion.

agl61 Central Cells Express Synergid and Antipodal Markers

The CLSM analysis discussed above suggests that the egg cell, synergid cells, and antipodal cells are not affected in *agl61* female gametophytes. To investigate this issue further, we analyzed expression of markers for these cell types in *agl61* embryo sacs. We analyzed expression of *ProDD1:GFP*, which is expressed exclusively in the antipodal cells (Fig. 3I), and *ProDD3:GFP*, which is expressed strongly in the synergid cells and weakly in the egg cell and central cell (Fig. 3K; Steffen et al., 2007).

In *agl61-1* female gametophytes, *ProDD1:GFP* was expressed in the antipodal cells (Fig. 3J) and *ProDD3:GFP* was expressed strongly in the synergid cells and weakly in the egg cell (Fig. 3L). These results suggest that the antipodal, synergid, and egg cells are normal in *agl61-1* embryo sacs. However, in contrast to the wild type, *ProDD1:GFP* was also expressed in the central cell of *agl61-1* embryo sacs (Fig. 3J). Similarly, *ProDD3:GFP*, which was expressed weakly in wild-type central cells (Fig. 3K), was expressed strongly in *agl61-1* central cells (Fig. 3L). These data indicate that *AGL61* is required for suppression of *DD1* and *DD3* expression in the central cell and that an additional aspect of the *agl61* phenotype is misexpression of antipodal- and synergid-expressed genes.

agl61 Female Gametophytes Attract Pollen Tubes

Analysis of the *ccg* mutant suggests that the central cell is required for pollen tube guidance by the female gametophyte (Chen et al., 2007). However, the CLSM analysis of developing seeds discussed above suggests that *agl61-1* female gametophytes attract pollen tubes and become fertilized. To confirm these results, we analyzed pollen tube growth to *agl61* female gametophytes. We observed pollen tubes using pollen from transgenic plants containing the *ProLAT52:GFP* construct. *ProLAT52:GFP* is expressed in the vegetative cell of the elongating pollen tube and upon pollen tube discharge, a bolus of GFP is released into the degenerating synergid cell (Palanivelu and Preuss, 2006; Sandaklie-Nikolova et al., 2007; Fig. 3M).

We pollinated wild-type and *agl61-1/AGL61* pistils with *ProLAT52:GFP* pollen and analyzed the resulting seeds at 24 h after pollination. In the wild-type polli-

nations, approximately 98% (112/114) of the seeds contained a pollen tube in the micropyle and a GFP bolus in the embryo sac. Similarly, in the *agl61-1/AGL61* pollinations, approximately 97% (115/119) of the seeds contained a pollen tube in the micropyle and a GFP bolus in the embryo sac, indicating that *agl61-1* female gametophytes can attract pollen tubes.

To verify these observations, we performed a similar analysis with central cells expressing *ProAGL61:GFP*, which allowed us to directly observe mutant embryo sacs (discussed above). At 24 h after pollination with *ProLAT52:GFP* pollen, 100% (35/35) of *agl61-1* female gametophytes had a pollen tube in its micropyle and a GFP bolus in the embryo sac (Fig. 3N). Together, these data indicate that *agl61* female gametophytes are not defective in pollen tube guidance.

AGL61 Is Not Autoregulated

Autoregulation is a common feature of MADS box genes (de Folter and Angenent, 2006). To determine whether *AGL61* regulates its own expression, we compared expression of *ProAGL61:GFP* in wild-type and *agl61-1* female gametophytes. We generated plants hemizygous for *ProAGL61:GFP* and heterozygous for *agl61-1* and scored the number of wild-type and *agl61-1* central cells expressing GFP. In these plants, the percentage of central cells expressing *ProAGL61:GFP* was approximately equal in wild-type (51%, 32/63) and *agl61-1* (49%, 31/63) female gametophytes. Furthermore, the intensity of the GFP signal was approximately equal in wild-type and *agl61-1* female gametophytes. Together, these data suggest that *AGL61* does not regulate its own expression.

AGL61 Interacts with AGL80

The phenotype of *agl61* female gametophytes resembles that of *agl80* female gametophytes and the two genes are expressed in a similar pattern (Portereiko et al., 2006), suggesting that AGL61 may interact with AGL80 in the central cell. To address this issue, we performed directed yeast two-hybrid assays using full-length AGL61 fused with the GAL4 DNA-binding domain (AGL61-BD) or the GAL4 activation domain (AGL61-AD) and full-length AGL80 fused with these domains (AGL80-BD and AGL80-AD). Figure 5 shows that AGL61-BD and AGL61-AD interacted with AGL80-AD and AGL80-BD, respectively, to stimulate transcription of the *HIS3* and *ADE2* reporter genes. By contrast, control cells containing constructs paired with empty vectors did not activate transcription of the reporter genes. These data indicate that AGL61 interacts with AGL80 in yeast.

DISCUSSION

AGL61 Encodes a Type I MADS Domain Protein

ALG61 is a Type I MADS domain protein (Parenicova et al., 2003). MADS box genes are subdivided into two types: Type I (approximately 61 genes in Arabidopsis)
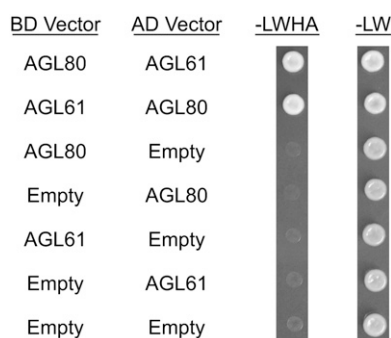
Figure 5. Yeast two-hybrid analysis of AGL61-AGL80 interaction. Growth occurs only when cells contain both AGL80-BD and AGL61-AD (row 1) or both AGL61-BD and AGL80-AD (row 2). Cells containing AGL80-BD only (row 3), AGL80-AD only (row 4), AGL61-BD only (row 5), AGL61-AD only (row 6), or neither AGL61 nor AGL80 (row 7) do not grow. AD, GAL4 activation domain; BD, GAL4 DNA-binding domain; -LW, growth medium lacking Leu and Trp; -LWHA, growth medium lacking Leu, Trp, His, and adenine.

and Type II (approximately 46 genes in Arabidopsis). Type II includes the MIKC genes and contains >20 well-characterized genes (Parenicova et al., 2003). By contrast, functional information is available for only five Type I genes: *AGL37/PHE1* (Kohler et al., 2003b), *AGL80* (Portereiko et al., 2006), *AGL62* (Kang et al., 2008), *AGL28* (Yoo et al., 2006), and *AGL23* (Colombo et al., 2008). Of these, loss-of-function information is available only for *AGL80*, *AGL62*, and *AGL23*. As discussed below, *AGL80* has an expression pattern and mutant phenotype similar to that of *AGL61*. *AGL62* is expressed in the endosperm, during the syncytial phase, and is required for suppression of cellularization during that time (Kang et al., 2008). *AGL23* is expressed during female gametophyte and embryo development and is required for development beyond the early stages of female gametophyte development and for chloroplast biogenesis during embryo development (Colombo et al., 2008). Mutations in *AGL37/PHE1* (Kohler et al., 2003b) and *AGL28* (Yoo et al., 2006) do not produce observable phenotypes. However, *AGL37/PHE1* is expressed during endosperm development and is overexpressed in *mea* endosperm, suggesting a role in endosperm development. *AGL28* is expressed in vegetative tissues and overexpression induces precocious flowering (Yoo et al., 2006). Thus, of six Type I MADS box genes now characterized, five play a role in female gametophyte and/or seed development, suggesting that other Type I genes may also function during these developmental stages.

AGL61 Is Required for Central Cell Development

During ovule development, *AGL61* is expressed exclusively in the central cell (Fig. 3, A to C). This expression pattern is consistent with the phenotype of *agl61* mutants. Based on both CLSM analysis of *agl61* female gametophytes (Fig. 4, A to C) and on analysis of antipodal-, egg-, and synergid-expressed genes in *agl61* embryo sacs (Fig. 3, I to L), *agl61* affects the

central cell but not the other cells of the embryo sac. Thus, the expression and phenotypic data suggest very strongly that *agl61* female gametophytes are affected in the central cell specifically.

The central cell defects include an overall reduction in size and a reduced or absent vacuole (Figs. 3G and 4, B and C). The vacuole in plant cells is known to generate turgor (Marty, 1999). Thus, it is likely that the central cell's reduced size results from the vacuole defect. Given that *AGL61* encodes a transcription factor, it is unlikely to directly influence vacuole morphology. More likely, *AGL61* regulates the expression of genes required for maintenance of vacuole integrity.

An additional aspect of the *agl61* central cell phenotype is ectopic expression of synergid- and antipodal-expressed genes (Fig. 3, J and L). These observations indicate that *AGL61* is required to suppress the expression of genes in the central cell. Of two genes tested, both are misexpressed, suggesting that additional genes are misexpressed in *agl61* central cells.

The expression of *AGL61-GFP* (Fig. 3D) and *ProAGL61:GFP* (Fig. 3F) in the endosperm suggests that *AGL61* plays a role during endosperm development. Consistent with this, an *agl61* homozygote was not identified despite a small percentage of transmission through the female gametophyte (Table I). Furthermore, seeds resulting from fertilization of *agl61* female gametophytes with wild-type pollen undergo abnormal endosperm development (Fig. 4, E to G). However, the failure of endosperm development in this cross may be attributable to defects earlier during central cell development.

Despite the strong morphological defects in the central cell, *agl61* female gametophytes are able to attract pollen tubes (Fig. 3N). This is also true of *agl80* female gametophytes (Portereiko et al., 2006). These observations are in contrast to those of the *ccg* mutant, which has subtle or no defects in the central cell but is defective in pollen tube guidance (Chen et al., 2007). These results suggest that the *agl61* mutation does not affect *CCG* expression and production of the central cell factors required for pollen tube guidance.

AGL61 Interacts with AGL80

MADS-domain proteins generally function as homodimers and/or as heterodimers with other MADS-domain proteins (de Folter and Angenent, 2006). Consistent with this, we have shown that AGL61 interacts with AGL80 in yeast. In a recent study, an interactome map of the Arabidopsis MADS-domain proteins was generated (de Folter et al., 2005). In this study, the AGL61-AGL80 interaction was not reported. In progress are experiments to verify that AGL61 and AGL80 interact in vivo.

Our results suggest that an AGL61-AGL80 heterodimer functions in the central cell. Consistent with this, *AGL61* and *AGL80* (Portereiko et al., 2006) are expressed in a similar pattern and exhibit a similar mutant phenotype. We previously showed that *AGL80*

is required for the expression of *DME* and *DD46* in the central cell (Portereiko et al., 2006). Together, these data suggest that an AGL61-AGL80 heterodimer is required for both the expression (*DME* and *DD46*) and suppression (*DD1* and *DD3*) of genes in the central cell. In progress are experiments to comprehensively assess the expression of female gametophyte-expressed genes (Steffen et al., 2007) in *agl61* and *agl80* central cells.

MATERIALS AND METHODS

Plant Material and Plasmids

agl61-1 (SALK_009008) was obtained from the Salk Institute Genomic Analysis Laboratory collection (Alonso et al., 2003). *agl61-2* (GABI-Kat 642H10) was obtained from the GABI-Kat collection (Rosso et al., 2003). The pBI-GFP(S65T) plasmid was provided by Ramin Yadegari. The transgenic line expressing *ProLAT52:GFP* was obtained from Ravi Palanivelu.

Plant Growth Conditions

Seeds were sterilized in chlorine gas and germinated on plates containing 0.5× Murashige and Skoog salts (M-9274; Sigma), 0.05% 2-(*N*-morpholino)-ethane-sulfonic acid, 0.5% Suc, and 0.8% Phytagar (Life Technologies). Ten-day-old seedlings were transferred to Sunshine Mix Number 2 and grown under 24-h illumination.

Plant Transformation

T-DNA constructs were introduced into Agrobacterium strain LBA4404 by electroporation. Arabidopsis (*Arabidopsis thaliana*) plants (ecotype Columbia) 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. Resistant seedlings were transplanted to soil after 10 d of growth.

Real-Time RT-PCR

For plant-wide real-time RT-PCR, we carried out the experiments and analysis as described in Steffen et al. (2007). Tissue was harvested from plants and placed immediately into liquid nitrogen. Ovaries were harvested from *ms1* and *dif1* at flower stages 12c (Christensen et al., 1997) and 13 (Smyth et al., 1990). Floral cluster tissue includes the inflorescence meristem and flowers at stages 1–10 (Smyth et al., 1990). Silique tissue includes siliques at 1 to 2 d after pollination. Leaf tissue includes leaves of sizes 5 to 12 mm. Roots were harvested from seedlings at 11 d after germination. Floral stem tissue includes internodes from 4-week-old plants. Anthers were collected from flowers at stages 11 to 13 (Smyth et al., 1990). RNA extractions, cDNA synthesis, and real-time RT-PCR were performed as described in Steffen et al. (2007). Each expression value is the result of three independent PCR reactions including technical and biological replicates. The PCR primers used were IHM41-F (5′-AGGCGGTCGATGATTAATTG-3′) and IHM41-R (5′-CCAGAAGGCATGTTCACGTA-3′). We calculated relative expression levels as follows. We first normalized *AGL61* transcript levels relative to a standard (*ACTIN2*) using the formula $\Delta C_T = C_T(AGL61) - C_T(ACTIN2)$. We next calculated an average ΔC_T value for each tissue. *ms1* pistil tissue with the highest relative expression (lowest ΔC_T value), was used as the standard for comparison of expression levels. We then calculated relative expression levels using the equation, $2^{-(\text{average }\Delta CT \text{ (tissue)} - \text{average }\Delta CT \text{ (}ms1\text{ pistil)})}$.

Cloning the *AGL61* cDNA

We identified the 5′ and 3′ untranslated sequences with RACE using the First Choice RLM-RACE kit (Ambion). For 5′ RACE, the gene-specific outer primer was AGL61raceR9 (5′-ATCTCTTCCATCGCTTGACCCT-3′) and the gene-specific inner primer was AGL61raceR8 (5′-TCAACACTTGGATGTCC-GAATGA-3′). For 3′ RACE, the gene-specific outer primer was 61-3RACEGSO1 (5′-TCAAGCGATGGAAGAGATGAGA-3′) and the gene-specific inner primer was 61-3RACEGSI1 (5′-AGCCAGTAGAGGAGATGAATATGG-3′). This analysis showed that *AGL61* contains 5′ and 3′ untranslated regions of 49 bp and 139 bp, respectively. To amplify a cDNA encompassing the entire

open reading frame of *AGL61*, we used the RLM-RACE kit outer primer and AGL61 cDNA R (5′-AATCAGAAACAACCATTTCCA-3′). The cDNA was cloned into the pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen) resulting in plasmid pCRII-cAGL61.

Sequence Analysis

We used PROSITE (http://ca.expasy.org/prosite) to identify predicted functional domains of AGL61 protein. This prediction tool identified the MADS domain but no other domains. We used PSORT (http://psort.nibb.ac.jp/form.html), WoLF PSORT (http://wolfpsort.org/), and PredictNLS (http://cubic.bioc.columbia.edu/predictNLS) to identify a nuclear localization signal (NLS) in AGL61 protein; no putative NLS was predicted.

Construction of *AGL61-GFP* and *ProAGL61:GFP*

The *AGL61-GFP* construct includes a 2,662-bp fragment containing 2,032 bp of sequence upstream of the predicted translational start codon and 630 bp of *AGL61* genomic coding sequence, excluding the stop codon. This genomic region was obtained by PCR amplification from genomic DNA using the primers 61ProtF (5′-TGATTACGCCGTCGACACTTCTTGGGTTCCGGGC-CGA-3′) and 61ProtR (5′-TGCTCACCATGGATCCGAAACAACCATTTC-CATTGGCAAAA-3′). These primers introduced *Sal*I and *Bam*HI sites at the 5′ and 3′ ends, respectively. The resulting PCR product was cloned into pBI-GFP(S65T) (Yadegari et al., 2000) using the *Sal*I and *Bam*HI sites, resulting in plasmid pBI-ProAGL61-GFP.

The *ProAGL61:GFP* construct includes 1,935 bp upstream of the predicted translational start codon. This genomic region was obtained by PCR amplification from genomic DNA using the primers AGL61Prom-F (5′-TGA-TTACGCCCTGCAGATGATTTTAGAGTCTCCCGC-3′) and AGL61Prom-R (5′-TGCTCACCATGGATCCTGTAACATACATTTGTAATTACTCG-3′). These primers introduced *Pst*I and *Bam*HI sites at the 5′ and 3′ ends, respectively. The resulting PCR product was cloned into pBI-GFP(S65T) (Yadegari et al., 2000) using the *Pst*I and *Bam*HI sites, resulting in the plasmid pBI-ProAGL61:GFP.

These constructs were introduced into Arabidopsis plants as described above and transformed plants were selected by germinating T1 seeds on growth medium containing 30 μg/mL kanamycin. The expression patterns reported in "Results" are derived from the analysis of at least 10 transgenic lines.

Analysis of GFP Expression Patterns

For analysis of mature female gametophytes, we emasculated flowers at stage 12c (Christensen et al., 1997), waited 24 h, and removed the flowers from the plants. We then removed the sepals, petals, and stamens, and dissected off the carpel walls using a 30-gauge syringe needle. For analysis of earlier developmental stages, we directly dissected the ovules from stage 12c flowers. For analysis of developing seeds, we emasculated flowers at stage 12c, waited 24 h, pollinated with self-pollen, waited 12 to 48 h, and then dissected the tissue as described above. In all cases, the dissected ovules/seeds were mounted on microscope slides in 10 mM phosphate buffer (pH 7.0) for microscopic analysis. GFP expression patterns were analyzed using a Zeiss Axioplan microscope. GFP was excited using a UV lamp and was detected using a 38 HE EGFP filter set. Images were captured using an AXIOCAM MRM REV2 camera with the AxioVision software package version 4.5 (Zeiss).

Characterization of the *agl61-1* and *agl61-2* Alleles

For both alleles, the left-border and right-border T-DNA junctions were determined by PCR using T-DNA-specific and genomic-specific primers. For *agl61-1*, the left-border junction was determined using the T-DNA primer pBinProK2-RB1 (5′-TCAGTTCCAAACGTAAAACGGC-3′) combined with the genomic primer AGL61-1LP (5′-GCCTAGGCTTGTAAGGTCCAG-3′) and the right-border junction was determined using T-DNA primer LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) and genomic primer AGL61-1RP (5′-CGTCCGATGCTTTCTTTCTTC-3′). For *agl61-2*, the left-border junction was determined using the T-DNA primer TDNA1 (5′-CCCATTTGGACGTGA-ATGTAGACAC-3′) combined with the genomic primer AGL61-2LP (5′-GCC-TCACACTCTCTTTTCTCATCT-3′) and the right-border junction was determined using the T-DNA primer TDNA3 (5′-CGCCAGGGTTTTCCCAGTCACG-ACG-3′) combined with the genomic primer AGL61-2RP CCTAGGCTTGT-AAGGTCCAGTTT (5′-CCTAGGCTTGTAAGGTCCAGTTT-3′).

The T-DNA in *agl61-1* is inserted 81 nucleotides upstream of the transcriptional start site, which is 130 nucleotides upstream of the predicted start codon, and is associated with a 17-nucleotide deletion (nucleotides −71 to −65 relative to the transcriptional start site deleted). The T-DNA in *agl61-2* is inserted 48 nucleotides downstream of the transcriptional start site, which is immediately upstream of the predicted start codon, and is associated with a 235-nucleotide insertion of unknown origin.

Segregation Analysis

For self-cross analysis, heterozygous plants were allowed to self-pollinate and progeny seed was collected. For reciprocal cross analysis, heterozygous plants were crossed with wild-type plants as outlined in Table I. In both cases, the progeny F₁ seed was germinated on growth medium containing no antibiotics and progeny seedlings were genotyped and scored using PCR. Plants segregating the *agl61-1* allele were genotyped using primers LBa1, AGL61-1LP, and AGL61-1RP (see above). Plants segregating the *agl61-2* allele were genotyped using primers TDNA1, AGL61-1LP, and AGL61-2RP (see above). Heterozygous plants, identified by PCR were used in the segregation analysis described below.

Table I shows that the *agl61* mutations transmit through the female gametophyte at low frequency. Based on the observed transmission frequencies, homozygotes should be present at a frequency of 1.1% to 2.5%. To identify homozygotes, for both mutants, we screened the siliques of >800 progeny from self-pollinated heterozygous plants. In addition, for both mutants, we genotyped >200 of these plants using PCR with primers LBa1, AGL61-1LP, and AGL61-1RP (see above). With both methods, plants homozygous for the *agl1-1* and *agl61-2* alleles were not identified.

Molecular Complementation

Molecular complementation was performed using a 3,662-bp DNA fragment containing the *AGL61* coding sequence (633 bp) along with 2,084 bp of sequence upstream of the predicted translational start codon and 945 bp of sequence downstream of the stop codon. This DNA fragment was amplified by PCR from genomic DNA using the primers AGL61-ResF GATG-ATTTTAGAGTCTCCCGC (5′-CCATGATTACGAATTCGATGATTTTAGAG-TCTCCCGC-3′) and AGL61-ResR (5′-ATGCCTGCAGGTCGACAAAATTC-CTTCAAGTATTTTC-3′). These primers introduced *Eco*RI and *Sal*I sites at each end, respectively. The resulting PCR product was cloned into pCAMBIA1300 (CAMBIA, Canberra, Australia) using the *Eco*RI and *Sal*I sites, producing plasmid pCAMBIA1300:AGL61-Res. pCAMBIA1300 contains a marker gene conferring resistance to hygromycin. pCAMBIA1300:AGL61-Res was introduced into Arabidopsis plants as described above and transformed plants were selected by germinating seeds on growth medium containing 15 μg/mL hygromycin. Hygromycin-resistant plants also containing the *agl61-1* allele were identified by PCR by using primers LBa1 and AGL61-1RP (see above). To verify that hygromycin-resistant plants had the rescue construct, we performed PCR using primers pCAMLacZR (5′-CCAGCTGGCGAAAGGGGGAT-3′) and AGL61ATG800R (5′-CCGCATCGTTTATAACAAAGTGTTAACAGTG-3′). These plants had 25% aborted seeds. Four T1 plants identified above were allowed to self-pollinate. In the T2 generation, plants containing the *agl61-1* allele (either heterozygous or homozygous) and the rescue construct (either hemizygous or homozygous) were identified by PCR using primers LBa1, AGL61-1RP, pCAMLacZR, and AGL61ATG800R (see above). These plants were screened for siliques containing full seed set. Plants with full seed set putatively were homozygous for the rescue construct; to verify this, we collected seed from these plants and scored progeny seedlings for the presence of the rescue construct by PCR using primers pCAMLacZR, and AGL61ATG800R (see above).

Yeast Two-Hybrid Analysis

We used the CLONTECH Matchmaker GAL4 Two-Hybrid System 3 for the yeast (*Saccharomyces cerevisiae*) two-hybrid analysis. The AGL80 and AGL61 open reading frames (without introns) were fused to the GAL4 activation domain and GAL4 DNA-binding domain in pGAD-T7 and pGBK-T7. Yeast strain AH109 was cotransformed with combinations of pGAD-T7 and pGBK-T7 constructs (AGL80 plus AGL61 or controls containing one or both empty vectors) and selected on synthetic dropout (SD) medium lacking Leu and Trp (SD-LW). Cotransformants were then assayed for interaction and activation of the His and adenine reporter genes on SD medium lacking Leu, Trp, His,

and adenine (SD-LWHA). For this, fresh colonies were grown in SD-LW at 30°C overnight to an OD of 1 to 2, the cells were pelleted and resuspended in 0.5 M sorbitol to an OD of 0.5, and 3 μL of each cell suspension was spotted on SD-LWHA plates using a multichannel pipetor and grown at 30°C for 2 to 3 d. In this analysis, the second ATG (at position +49 relative to the transcriptional start site) was used as the start codon.

Analysis of Expression of Promoter:Reporter Constructs in *agl61* Female Gametophytes

agl61-1/AGL61 plants were crossed as males with plants homozygous for the promoter:reporter constructs. To identify F₁ plants containing the *agl61-1* T-DNA allele, PCR was performed with primers LBa1 and AGL61-1RP (see above). F₁ seed was plated on growth medium containing 30 μg/mL kanamycin to identify seedlings containing the promoter:reporter constructs. Plants heterozygous for the *agl61-1* mutation and hemizygous for the promoter: reporter construct were allowed to self-cross. Progeny from the self-cross were then scored for the *agl61-1* T-DNA insertion by PCR, as described above. One-quarter of these plants should also be homozygous for the promoter:reporter construct. To identify these plants, we made use of the fact that all promoter: reporter constructs were inserted into vectors conferring kanamycin resistance. Offspring containing the *agl61-1* T-DNA allele were then allowed to self-cross. Seed from this cross was plated on media containing 30 μg/mL kanamycin and the ratio of kanamycin-sensitive to kanamycin-resistant seedlings was scored. Plants that produced 100% kanamycin-resistant progeny were determined to be homozygous for the reporter construct.

The GenBank accession number for the *AGL61* mRNA sequence is EU836691.

Supplemental Data

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

Supplemental Figure S1. Silique phenotype of *agl61-1* mutants.

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