Transcriptional repression specifies the central cell for double fertilization

Meng-Xia Zhang[a,1], Shan-Shan Zhu[a,1], Yong-Chao Xu[b], Ya-Long Guo[b], Wei-Cai Yang[a,c,2], and Hong-Ju Li[a,2]

[a]State Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, 100101 Beijing, China; [b]Institute of Botany, Chinese Academy of Sciences, 100093 Beijing, China; and [c]College of Advanced Agricultural Sciences, University of the Chinese Academy of Sciences, 100049 Beijing, China

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Double fertilization is a key innovation for the evolutionary success of angiosperms by which the two fertilized female gametes, the egg cell and central cell, generate the embryo and endosperm, respectively. The female gametophyte (embryo sac) enclosed in the sporophyte is derived from a one-celled haploid cell lineage. It undergoes successive events of mitotic divisions, cellularization, and cell specification to give rise to the mature embryo sac, which contains the two female gametes accompanied by two types of accessory cells, namely synergids and antipodals. How the cell fate of the central cell is specified has long been equivocal and is further complicated by the structural diversity of female gametophyte across plant taxa. Here, MADS-box protein AGL80 was verified as a transcriptional repressor that directly suppresses the expression of accessory cell-specific genes to specify the central cell. Further genetic rescue and phylogenetic assay of the AGL80 orthologs revealed a possible conserved mechanism in the Brassicaceae family. Results from this study provide insight into the molecular determination of the second female gamete cell in Brassicaceae.

double fertilization | central cell specification | central cell evolution | transcriptional repression

A hallmark of flowering plant reproduction is double fertilization, characterized by fertilization events of the two female gametes—the egg cell and central cell—within the embryo sac (female gametophyte) with two sperm cells. Double fertilization initiates the development of the embryo and endosperm, which form the major part of the seed. The embryo sac is a multicellular specialized structure derived from the functional megaspore, which is the remaining meiosis product of the megaspore mother cell (1, 2). The functional megaspore undergoes several developmental processes to generate the mature embryo sac, including mitotic nuclear divisions, cellularization, nuclear reposition, and cell fate specification (2, 3).

The embryo sac shows structural variation in different plant taxa (4). Nevertheless, most of the mature embryo sac across taxa is composed of egg and central cells, as well as two types of accessory cells: synergid cells and antipodal cells. The central cell is found only in flowering plants among the extant species on earth and is the largest cell in the embryo sac (1–3). Its fertilization product forms the embryo-nurturing endosperm, which is also an important source of food for humans.

How the central cell is specified remains unclear. The histidine kinase CYTOKININ INDEPENDENT1 (CKI1) has been reported to be required for both embryo sac development and cell fate specification of both the central cell and antipodal cells in *Arabidopsis* (5, 6). In the *agl80* mutant that is null for the central cell-specific transcription factor-encoding gene *AGL80*, the central cell is smaller and fails to initiate endosperm (7). Thus, AGL80 has been suggested to be essential for central cell development, but its molecular mechanism and developmental program wired for normal central cell development are unknown.

In this study, we show that the central cell fate of *agl80* mutant is misspecified to the accessory cells through a series of cell type-specific markers and a fertilization assay. We also provide lines of evidence showing that AGL80 acts as a transcription repressor through the EAR motif to repress the expression of *MYB98*, which is specifically expressed in the synergid cells. Phylogenetic analysis and genetic rescue experiments provide clues that this molecular mechanism of AGL80 in the central cell might be conserved in Brassicaceae.

Results

Central Cell Function, but Not Pollen Tube Guidance, Is Disrupted in *agl80*. To verify the development of the *agl80* embryo sac, an embryo sac-specific nuclear marker, *BICE1pro:C2^{NLS}-3×GFP* (8), was introduced to the wild-type (WT) and *agl80*/+ heterozygous plants. Embryo sacs were then compared at the FG5 stage and thereafter, since *AGL80* expression commences in the central cell after cellularization (FG5) (7). The central cell in the mutant ovules is smaller (*SI Appendix*, Fig. S1 *A–D*), as reported previously (7). In addition, the two polar nuclei fuse normally as in the WT (*SI Appendix*, Fig. S1 *E* and *F*). This suggests that AGL80 is required for the later differentiation stages after polar nuclei fusion. A GFP reporter of six central cell-specific genes (9) was expressed in *agl80*/+ to verify the central cell identity. In contrast to the WT, in which all the ovules exhibit GFP fluorescence in the central cell, approximately one-half of the ovules that are characterized by smaller central cells in *agl80*/+ homozygous for the transgenes show no GFP (*SI Appendix*, Fig. S1 *G* and *H*). This result confirms the aberrant central cell development of *agl80* embryo sacs.

Pollen tube guidance is regulated by the central cell (9, 10), while it has been reported to be unaffected in the *agl80* mutant (7). To clarify this observation, pollen tube attraction was reinvestigated and found to be normal in *agl80* (*SI Appendix*, Fig.

Significance

Double fertilization is a key innovation of flowering plants. The central cell is the second female gamete, and its fertilization gives rise to the endosperm, the nurse of a seed. However, how this cell is specified and evolved in angiosperm remains unknown. This study reveals a transcriptional repression mechanism to specify the central cell in *Arabidopsis* and provides insight into the origination of this mechanism in Brassicaceae.

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[1]M.-X.Z. and S.-S.Z. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: wcyang@genetics.ac.cn or hjli@genetics.ac.cn.

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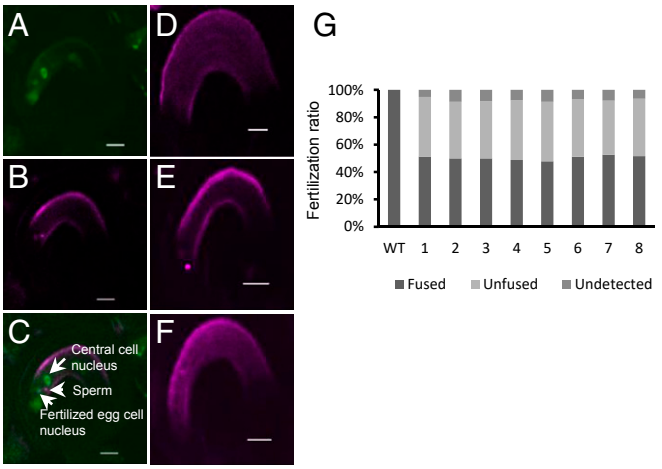

Fig. 1. The *agl80* central cell fails to be fertilized by the sperm cell. (*A–C*) Confocal images of an *agl80* ovule expressing *BICE1pro:C2^{NLS}-GFP* fertilized with *HTR10pro:HTR10-RFP* pollen at 15 HAP. (*A*) GFP channel. (*B*) RFP channel. (*C*) Merged. (*D–F*) Confocal images showing three different types of embryo sacs in *agl80*/+ carpel at 15 HAP. (*G*) Statistics of the three types of embryo sacs in nine siliques of eight independent *agl80*/+ plants at 15 HAP. $n > 35$ ovules from each silique were examined for the mutant; $n = 100$ ovules for WT. (Scale bars: 20 μm.)

S2*A*). Further expression of the synergid-specific pollen tube attractants *LURE1s* (11) was examined by native promoter-driven reporter GFP in *agl80* ovules. The results show that compared with the synergid-expression pattern in the WT, 46 to 55% ($n >$ 200) of the ovules in *agl80*/+ that are featured by the smaller central cell exhibit GFP fluorescence in both the synergids and the central cell (*SI Appendix*, Fig. S2*B*). Moreover, real-time qRT-PCR analysis with mature *agl80*/+ pistils shows comparable or even higher *LURE1* expression compared with the WT (*SI Appendix*, Fig. S2*C*). This explains why pollen tube guidance is normal even though central cell development is aberrant in the *agl80* mutant. Previously, the central cell-specific transcription factor CCG together with CBP1 were found to be important for pollen tube attraction (9). We found that expression of *CCGpro:GUS* was specific in the central cell of all of the ovules of *agl80*/+ at FG5 stage immediately after cellularization and in mature embryo sacs (*SI Appendix*, Fig. S2 *D* and *E*). These results indicate that *CCG* expression is not affected in *agl80*. Thus, AGL80 is essential for other aspects of central cell function but not for central cell-mediated pollen tube attraction.

The Central Cell Fails to Fuse with Sperm Cells in *agl80*. Next, whether *agl80* embryo sac could be fertilized remains to be determined. To this end, pollen of sperm cell marker *HTR10pro:HTR10-RFP* (12) was pollinated to the *agl80*/+;*BICE1pro:C2^{NLS}-3×GFP* pistils. At 15 h after pollination (HAP), the egg cell of *agl80* fused with one of the sperm cells, as shown by the merged fluorescence of GFP and RFP signals in the egg cell (Fig. 1 *A–C*), which is consistent with the previous report that a zygote is formed in *agl80* embryo sacs (7). However, the central cell of *agl80* failed to fuse with the other sperm cell, which is left free in the embryo sac (Fig. 1 *A–C*). To further confirm this result statistically, *agl80*/+ pistils were pollinated with pollen carrying RFP-labeled sperm cells. At 15 HAP, three different types of embryo sacs were observed: 52 to 57% showed enlarged embryo sacs as a result of fertilization similar to the WT (Fig. 1*D*), 43 to 48% contained unfused free sperm cell (Fig. 1 *E* and *G*), and 5 to 10% exhibited autofluorescence without detectable sperm cells (Fig. 1 *F* and *G* and *SI Appendix*, Table S1). This autofluorescence is possibly due to the cell death of the central cell. The ovules that showed autofluorescence or nonfusion are assumed to contain *agl80* embryo sacs. These results suggest that fusion of *agl80* central cell with the sperm is impaired, leading to the failure of initiation of endosperm development.

The Central Cell Switches to Accessory Cell Fate in *agl80*. Examining the expression of *LURE1* in *agl80*/+ revealed that the GFP signal expands from the synergid to the chalazal pole, likely to the central cell (*SI Appendix*, Fig. S2*B*). Given the impaired expression of the central cell markers and the full integrity of *agl80* embryo sacs (*SI Appendix*, Fig. S1*G*), the central cell is speculated to lose its cell fate and acquire the synergid fate. To test this hypothesis, synergid/antipodal cell-specific marker ET1811 and synergid-specific marker ET2632 were introduced into *agl80*/+. Both ET markers

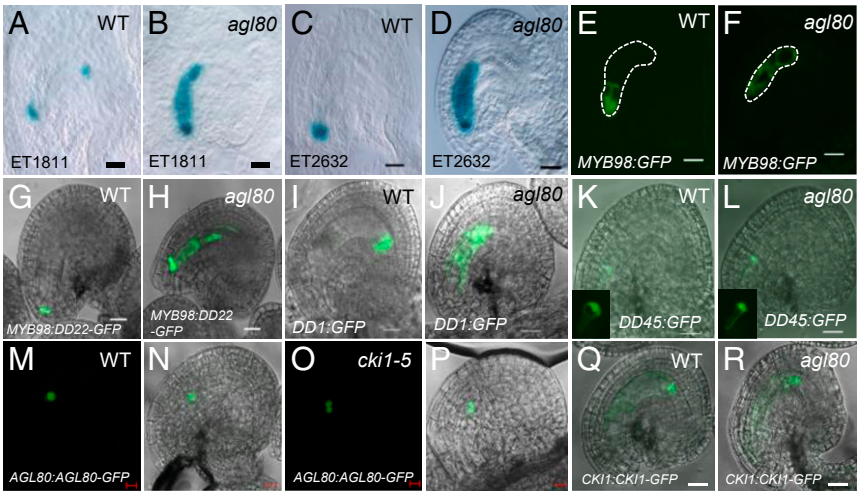

Fig. 2. The *agl80* central cell adopts the fate of accessory cells. (*A–D*) Micrographs showing the expression of synergid/antipodal cell (*A* and *B*) and synergid (*C* and *D*) markers in the mature WT (*A* and *C*) and *agl80* (*B* and *D*) ovules. (*E–H*) Confocal images of synergid-specific *MYB98pro:GFP* in WT (*E*) and *agl80* (*F*) ovules. Dashed lines demarcate the embryo sac. (*G* and *H*) Synergid-specific *MYB98pro:DD22-GFP* in WT (*G*) and *agl80* (*H*) ovules. (*I* and *J*) Antipodal cell-specific *DD1pro:GFP* marker in WT (*I*) and *agl80* (*J*) ovules. (*K* and *L*) Egg cell-specific marker *DD45pro:GFP* in WT (*K*) and *agl80* (*L*) ovules. (*M–P*) *AGL80:AGL80-GFP* in WT (*M* and *N*) and *cki1-5* (*O* and *P*) ovules. (*Q* and *R*) *CKI1:CKI1-GFP* in WT (*Q*) and *agl80* (*R*) ovules. For each marker, $n = 10$ siliques (>35 ovules were observed in each silique). The exact number of ovules for each marker is listed in *SI Appendix*, Table S1. (Scale bars: 20 μm.)

are expressed in the embryo sac after cellularization (*SI Appendix*, Fig. S3). In contrast to the GUS signal detected only in the synergids and/or antipodals of WT plants (Fig. 2 *A* and *C*), ~50.5% of the embryo sacs for ET1811 and 48.3% of those for ET2632 exhibited a similar GUS pattern as the WT, and 48.7% and 50.4%, respectively, show a GUS signal in the central cell corresponding to the smaller *agl80* embryo sac (Fig. 2 *B* and *D* and *SI Appendix*, Table S2).

To further investigate these aberrant expression pattern, synergid-specific markers *MYB98pro:GFP* and *MYB98pro:DD22-GFP*, which drive the central cell-specific secreted peptide DD22 in synergids, were expressed in *agl80*/+. The results show that approximately one-half of the embryo sacs exhibit expression of *MYB98pro:GFP* in the synergids and the other half exhibit expression of this marker in both the synergids and central cell (Fig. 2 *E–H* and *SI Appendix*, Table S2). Similarly, in approximately one-half of the ovules, DD22-GFP is expressed in the synergid and secreted to the filiform apparatus as in the WT embryo sacs (Fig. 2*G*), but in the other half of the ovules with smaller central cells, it is also expressed in the central cell (Fig. 2*H* and *SI Appendix*, Table S2). These results confirm that the synergid cell-specific genes are expressed ectopically in the central cell of *agl80* embryo sacs.

Subsequently, an antipodal cell marker, *DD1pro:GFP*, was introduced into the WT and *agl80*/+ plants. *DD1* expression was found to be confined to antipodal cells in the WT (Fig. 2*I*) but extended to the central cell in one-half of the ovules in *agl80*/+ (Fig. 2*J* and *SI Appendix*, Table S2). However, when the egg cell-specific marker *DD45pro:GFP* was introduced into *agl80*/+, both the WT and the smaller *agl80* embryo sac exhibited specific GFP expression in the egg cell (Fig. 2 *K* and *L* and *SI Appendix*, Table S2). These data indicate that the central cell in *agl80* embryo sacs adopts the cell fate of accessory cells, and that AGL80 is required to determine the cell fate of central cell.

To examine the possible relationship between CKI1 and AGL80, the expression of *AGL80:AGL80-GFP* and *CKI1:CKI1-GFP* were examined in *cki1-5*/+ and *agl80*/+ mutants, respectively. In *cki1-5*/+, only 29.8% of the *cki1-5* embryo sacs can develop to the cellularization stage, and in most cases these mutant embryo sacs fail to undergo polar nuclei fusion (5). *AGL80* has been reported to be expressed immediately in the polar nuclei that are undergoing fusion in the central cell. Thus, we can only count the few *cki1-5* embryo sacs that are at the nuclear fusion stage or have completed nuclear fusion. Our results show that in WT, *AGL80-GFP* is expressed and localized in the central cell nucleus (Fig. 2*M*), and that in the T1 *cki1-5*/+*;AGL80-GFP* plants, among the 375 ovules analyzed, three types of ovules were observed. Twenty-nine ovules (7.7%) showed a GFP signal in the opposing polar nuclei, 96 ovules (25.6%) showed GFP in the central cell, and 250 ovules (66.7%) did not show a GFP signal (Fig. 2 *O* and *P*). In the T1 *agl80*/+*;CKI1-GFP* plants, among the 354 ovules examined, 49% showed a strong GFP signal in antipodals and a weak signal in the central cell, similar to that in the WT embryo sacs (Fig. 2*Q*) (13), and 48% showed the same GFP pattern but with a smaller embryo sac, indicative of the *agl80* embryo sac (Fig. 2*R*). These results suggest that *agl80* might not affect the expression of *CKI1* in the central cell, and that in the *cki1-5*/+ mutant, the small fraction

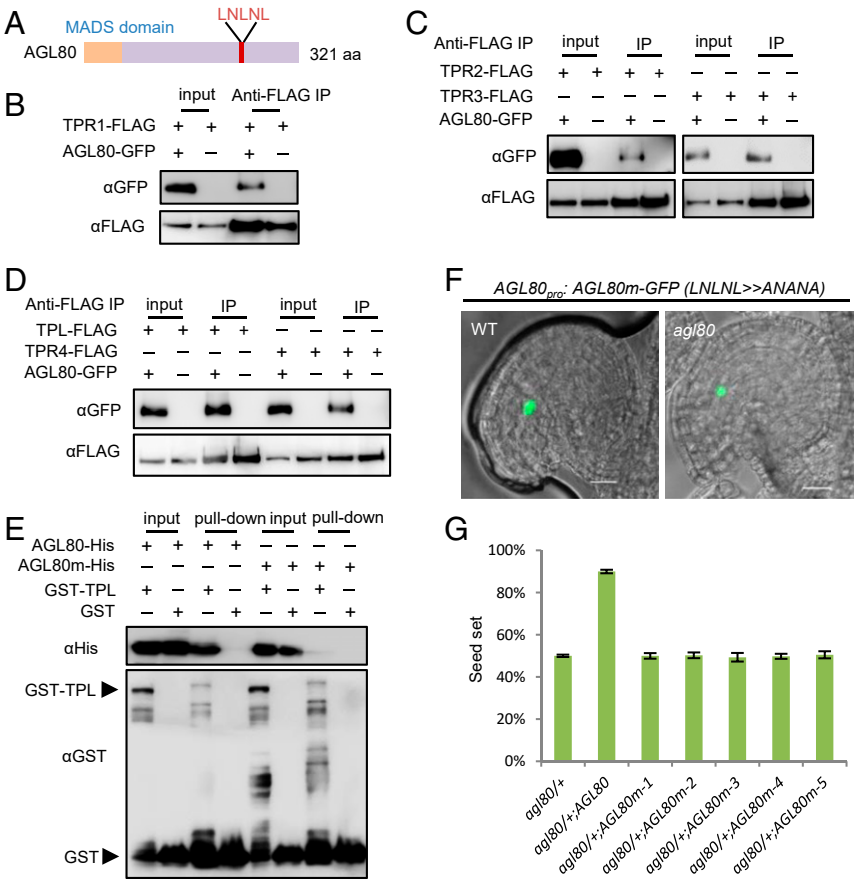

Fig. 3. AGL80 interacts with TOPLESS family proteins through the EAR motif. (*A*) Domain structure of AGL80. (*B–D*) Co-IP assay with *Arabidopsis* protoplasts showing the interaction of AGL80 with TOPLESS members. (*E*) GST pull-down assay showing that His-AGL80 interacts with GST-TPL, but not His-AGL80m. (*F*) Expression of *AGL80pro:AGL80m-GFP*. (Scale bars: 20 μm.) (*G*) Seed set at 3 d after fertilization. Five independent transgenic lines and five siliques for each genotype were analyzed.

of ovules that proceed to the polar nuclei opposition stage can initiate AGL80 expression.

AGL80 Interacts with Corepressor TOPLESS. To explore the mechanism of AGL80 in specifying the central cell identity, the protein sequence of AGL80 was analyzed, which revealed a putative EAR motif, LNLNL, in the C-terminal region (Fig. 3*A*). Transcription factors with EAR motifs often interact with TOPLESS (TPL) family proteins and other factors to transcriptionally repress downstream genes in diverse biological pathways (14). Therefore, the interaction between AGL80 and five TPL family proteins was tested by coimmunoprecipitation (co-IP) assays with *Arabidopsis* protoplasts. The results show that AGL80 can interact with all the TPL family members (Fig. 3 *B*–*D*), suggesting that AGL80 could possibly function as a transcription repressor.

To examine whether the interaction between AGL80 and TPL/TPRs depends on the EAR motif, the EAR motif LNLNL was mutated to ANANA to generate the mutated protein AGL80m, and then protein interaction was tested by an in vitro GST pull-down assay. The results show that His-AGL80 rather than His-AGL80m interacts with GST-TPL (Fig. 3*E*), indicating that the interaction between AGL80 and TPLs is dependent on the EAR motif. Furthermore, genetic complementation of *agl80*/+ by *AGL80pro:AGL80m-GFP* showed that the mutated AGL80 still localized in the central cell nuclei (Fig. 3*F*) but failed to rescue the seed set defect (Fig. 3*G*). This indicates that the EAR motif is essential for the AGL80 function.

AGL80 Represses *MYB98* Expression in the Central Cell. The next question is whether AGL80 can repress the expression of synergid-specific genes in the central cell. *AGL80-GFP* driven by the *MYB98* promoter was expressed in WT plants to ectopically express *AGL80* in the synergids (Fig. 4 *A* and *B*). The transgenic plants exhibited a variable seed abortion ratio caused by defective pollen tube guidance (Fig. 4 *C*–*F*), suggesting that the genes involved in pollen tube guidance might be affected. Next, synergid-specific *MYB98* and its downstream *LURE1* family genes positively involved in this process (15, 16) were investigated. As expected, the expression of *MYB98* was found to be down-regulated in mature siliques from eight independent homozygous T3 transgenic lines by qRT-PCR (Fig. 4*G*). Meanwhile, the percentage of pollen tube guidance phenotype was negatively correlated with the expression level of *MYB98* in those plants (Fig. 4 *F* and *G*). Thus, AGL80 appears to inhibit the expression of *MYB98*, affecting the expression of MYB98-regulated genes such as *LURE*s, *XIUQIU*s, and *TICKET*s (17, 18).

AGL80 belongs to the type I MADS transcription factor family (19). The MADS box domain of this family binds to the *cis*-acting elements CArG boxes in the promoter of downstream

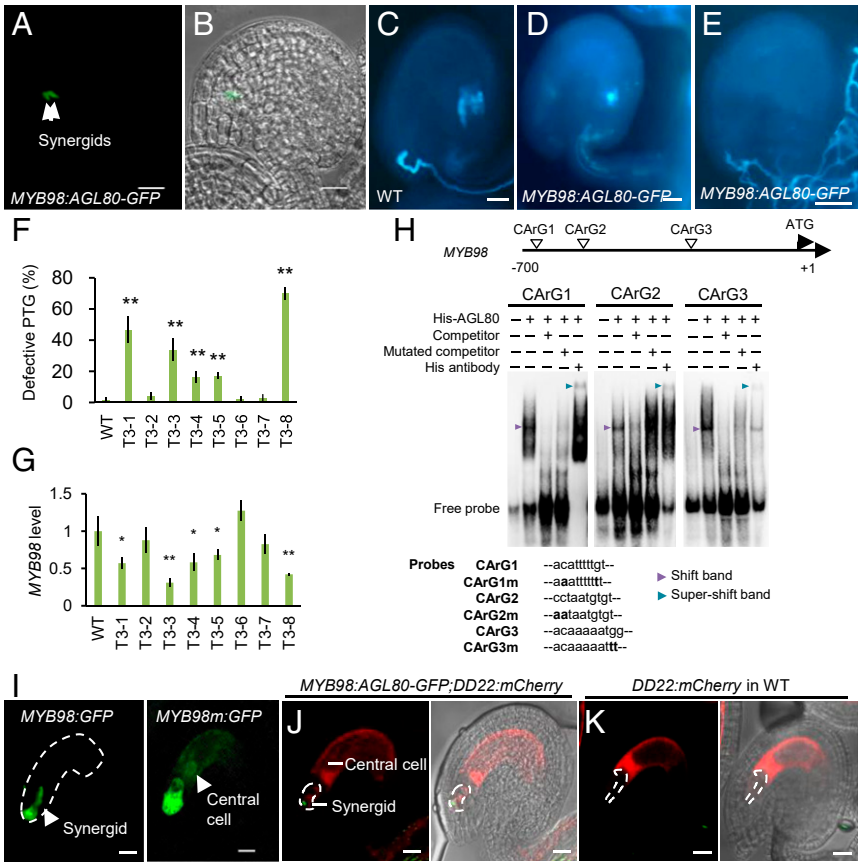

Fig. 4. AGL80 directly represses the expression of *MYB98*. (*A* and *B*) Confocal images showing ectopic expression of AGL80 in *MYB98pro:AGL80-GFP* transgenic ovule. (*C*) Aniline blue staining showing WT pollen tube in WT ovule at 24 HAP. (*D* and *E*) Defective pollen tube guidance in the T1 transgenic ovules at 24 HAP by WT pollen. (*F*) Statistics of pollen tube guidance defect in WT and eight independent T3 homozygous transgenic plants. $n = 10$ siliques for each genotype. $^{**}P < 0.01$, Student's *t* test. (*G*) Real-time qRT-PCR result showing *MYB98* level in pistils of the WT and eight transgenic plants in *F*. Data are presented as mean ± SD for three replicates. $^{**}P < 0.01$; $^{*}P < 0.05$, Student's *t* test. (*H*) EMSA assay showing the binding of His-AGL80 with three candidate probes of the intact and mutated CArG boxes of *MYB98* promoter. CArG1, CArG2, and CArG3 refer to the three probes, and CArG1m, CArG2m, and CArG3m refer to the mutated probes. (*I*) Confocal images showing expression of *MYB98:GFP* (*Left*) and *MYB98m:GFP* in which the three CArG boxes were mutated (*Right*). (*J*) Confocal image showing DD22-mCherry and AGL80-GFP in the synergids. (*K*) Confocal image showing DD22-mCherry in the central cell of the WT ovule. More than 20 siliques (>35 ovules per silique) were examined. (Scale bars: 20 μm.)

genes. To examine whether *MYB98* is a direct target of AGL80, electrophoretic mobility shift assay (EMSA) was performed with purified His-AGL80 protein and three predicted CArG box sequences in the *MYB98* promoter. The results show that His-AGL80 can bind to all three predicted CArG boxes but not to the mutated CArG box (Fig. 4*H*). Furthermore, the *MYB98* promoter with all these three CArG boxes mutated was used to drive the expression of *GFP* reporter in the WT plants. In 12 T1 transgenic lines, the GFP signal was detected not only in synergids, but also in the central cell (Fig. 4*I*), indicating that these CArG boxes are required for the transcriptional repression of *MYB98* in the central cell. Furthermore, the central cell marker construct *DD22:mCherry* was transformed into the T3 plants of *MYB98:AGL80-GFP-1*. In the T1 generation, nine siliques from four independent T1 transgenic lines were analyzed. At least 35 ovules from each silique were observed under confocal microscopy. Among 305 ovules checked, 36 ovules (12%) were seen to carry visible signals of both AGL80-GFP and mCherry. In all these ovules, mCherry showed ectopic synergid expression in addition to the central cell expression (Fig. 4 *J* and *K*). Forty ovules carried a visible mCherry signal in only the central cell, 163 ovules (53%) exhibited a GFP signal only in the synergids, and 66 ovules (22%) showed no visible signal. This implies that AGL80 alone can trigger the cell fate rewiring in the synergid. Nevertheless, the mCherry signal in the synergid was weak compared with that in the central cell, possibly because other central cell components, such as other AGLs, are also required for the cell specification.

MADS box proteins often function in the form of dimers in transcription regulation, and AGL80 possibly forms a heterodimer with AGL61 in the central cell specification (20, 21). To explore whether AGL80 itself as a homodimer is sufficient to regulate the synergid gene expression, co-IP was performed with *Arabidopsis* protoplasts. The results show that indeed the tagged proteins AGL80-GFP and AGL80-HA associate with each other (*SI Appendix*, Fig. S4). These data suggest that AGL80 alone can repress *MYB98* expression in the central cell by directly binding to the CArG box motifs, and that the ectopically expressed AGL80 can to some extent switch the cell fate of synergids to the central cell.

Function of AGL80 Is Conserved in Brassicaceae. The central cell emerges in the angiosperm, and its evolutionary history is unknown. Blast analysis and homologous comparison of AGL80 protein sequences across 94 sequenced species, including 93 species on the Phytozome 12 database and *Thellungiella parvula* (a salt-tolerant species relative to *Arabidopsis thaliana)* revealed that homologous sequences can be identified from 65 sequenced species, including liverwort, moss, fern, gymnosperm (Norway spruce), and angiosperms. The homologs in nine Brassicaceae species—*Arabidopsis halleri*, *Arabidopsis lyrata*, *A. thaliana*, *Boechera stricta*, *Brassica oleracea*, *Brassica rapa*, *Conradina grandiflora*, *Eutrema salsugineum*, and *T. parvula* —are highly similar, and all have the N-terminal

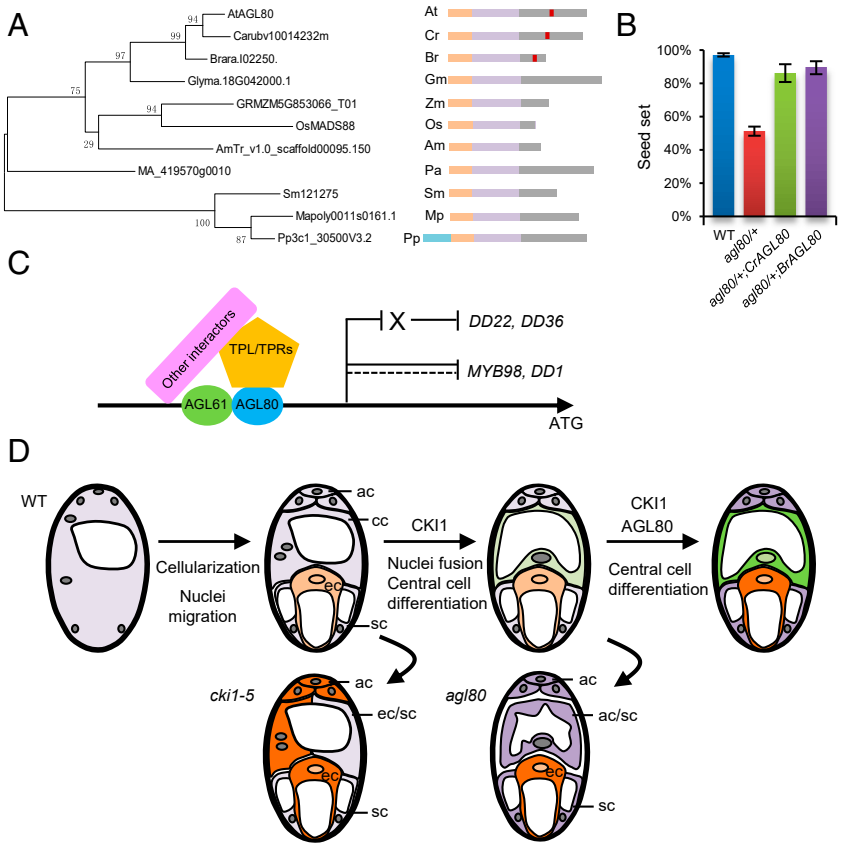

Fig. 5. Evolution of AGL80 and a working model of central cell specification. (*A*) The phylogenetic tree and domain arrangement of the representative AGL80 orthologs. The AGL80 orthologs used in the alignment are from *Amborella trichopoda, Brassica rapa, Glycine max, Zea mays, Oryza sativa*, *Capsella rubella*, Norway spruce, *Marchantia polymorpha*, *Physcomitrella patens*, and *Selaginella moellendorffii.* Yellow, MADS box domain; purple, unknown domain between the MADS box and C-terminal domain; gray, C-terminal domain; blue, domain specific in *P. patens*; red, EAR motif. (*B*) The seed set of the transgenic *agl80*/+ expressing the corresponding orthologous genes. Ten siliques were examined for each genotype. (*C*) AGL80 functions as a transcription repressor to directly and indirectly regulate the genes specifically expressed in the accessory cells and central cell. X is an unknown factor that is repressed by AGL80. (*D*) CKI1 and AGL80 specify the central cell sequentially. ac, antipodal cell; cc, central cell; sc, synergid cell; ec, egg cell.

MADS domain, a C-terminal domain containing the LxLxL EAR motif (LxLxP in *T. parvula* and LxLxQ in *E. salsugineum*, another salt-tolerant species), and a middle, unknown domain (Fig. 5*A*). The homologs in species outside of the Brassicaceae family share relatively high similarity with AtAGL80 only at the MADS box domain and the central domain, but have lower homology at the C-terminal domain (*SI Appendix*, Fig. S5). In addition, species in taxa outside of the Brassicaceae family do not contain the canonical EAR motif (*SI Appendix*, Fig. S5). To investigate the functional conservation of the homologs with canonical EAR motif, homologs in *Capsella rubella* and *B. rapa* in the Brassicaceae family were cloned and driven by the *AGL80* promoter to be expressed in *agl80*/+ plants. The results show that *CrAGL80* and *BrAGL80* can rescue the seed set defect of *agl80*/+ in T1 generation (Fig. 5*B*). These results imply that an AGL80-mediated transcriptional repression mechanism through the canonical EAR motif in the central cell specification is derived in Brassicaceae family.

Discussion

Here we report the phenotypic and functional analysis of AGL80 as a transcription repressor in specifying central cell fate. AGL80 and AGL61 form a heterodimer and most likely function together in the central cell (20, 21). *DD1* as well as *DD3*, which is expressed mainly in accessory cells, also are expressed in the central cell of the *agl61* mutant (20). Based on these findings, we propose that AGL80, possibly together with AGL61, represses the accessory cell-specific genes through TPL/TPRs (Fig. 5*C*). In addition, AGL80 may possibly repress another transcription factor that inhibits the expression of central cell-specific genes before central cell establishment (Fig. 5*C*). The cytokinin signaling component *CKI1* is expressed in and specifies the central cell and antipodals (5). In the *cki1* mutant, the central cell and antipodals acquire the egg cell fate, but in *agl80* they acquire the accessory cell fate. Thus, it appears that before final specification, the central cell has the potential to develop into the egg or into accessory cells. Expression of *CKI1* in embryo sacs begins at the functional megaspore stage and is retained in the antipodals and central cell at maturity (5, 6, 13, 22), while *AGL80* expression is initiated at polar nuclei fusion and present only in the central cell (7), suggesting that *CKI1* and *AGL80* possibly sequentially specify the central cell (Fig. 5*D*). After cellularization, *CKI1* promotes polar nuclei fusion and inhibits the transcriptional switch of chalazal gametophytic cells (central cell and antipodals) to micropylar gametophytic cells (egg cell and synergids). Thus, before fusion, the polar nuclei may still carry the transcriptional program from the two poles, and the immature central cell has the capability of differentiating to all four cell types. At fusion, expression of *AGL80* further represses the transcriptional setting for the accessory cells and facilitates the central cell transcriptional program.

The central cell is present in all flowering plants, and no transition species are found in extant species or fossils, so the origin of the central cell remains a mystery (23). Two major hypotheses of the origin of the central cell have been proposed: that it is derived from a companion altruistic egg cell, and that it is derived from the sexualized gymnosperm female gametophyte (24). From the gene expression aspects, *agl80* embryo sac contains only one gametic cell, the egg cell, surrounded by accessory cells, which resemble the gymnosperm. Distinct from *agl80*, a range of genetic studies suggest that loss of specific genes can switch other embryo sac cells to egg cells (25–27). Our results support the assumption that the accessory cells can be transcriptionally rewired to a central cell. On the other hand, exploring whether transcriptional repression by AGLs with an uncanonical EAR motif or other transcription factors are involved in the central cell specification in other non-Brassicaceae taxa will provide further insight into the evolution of the central cell.

Materials and Methods

cki1-5 was in the *Ws-2* background, and *agl80* was in the *Col* background, as reported previously (5–7). The materials, methods, and protocols used in this study are described in detail in *SI Appendix, Materials and Methods*.

Data Availability Statement. All data discussed in the paper are available within the manuscript and *SI Appendix*.

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