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Maternal ENODLs Are Required for Pollen Tube Reception in *Arabidopsis*

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SUMMARY

During the angiosperm (flowering-plant) life cycle, double fertilization represents the hallmark between diploid and haploid generations [1]. The success of double fertilization largely depends on compatible communication between the male gametophyte (pollen tube) and the maternal tissues of the flower, culminating in precise pollen tube guidance to the female gametophyte (embryo sac) and its rupture to release sperm cells. Several important factors involved in the pollen tube reception have been identified recently [2–6], but the underlying signaling pathways are far from being understood. Here, we report that a group of female-specific small proteins, early nodulin-like proteins (ENODLs, or ENs), are required for pollen tube reception. ENs are featured with a plastocyanin-like (PCNL) domain, an arabinogalactan (AG) glycomodule, and a predicted glycosylphosphatidylinositol (GPI) anchor motif. We show that ENs are asymmetrically distributed at the plasma membrane of the synergid cells and accumulate at the filiform apparatus, where arriving pollen tubes communicate with the embryo sac. EN14 strongly and specifically interacts with the extracellular domain of the receptor-like kinase FERONIA, localized at the synergid cell surface and known to critically control pollen tube reception [6]. Wild-type pollen tubes failed to arrest growth and to rupture after entering the ovules of quintuple loss-of-function *EN* mutants, indicating a central role of ENs in male-female communication and pollen tube

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.06.053.

AUTHOR CONTRIBUTIONS

L.-J.Q., T.D., J.D., and H.G. conceived and coordinated the study, with input from Y.H. and X.G. Y.H., X.G., L.C., and Y.L. conducted the genetic analyses, phenotypic observations, and yeast two-hybrid assays. Q.H. conducted bioinformatics analysis of the genes. P.C. and A.B. conducted in situ hybridization and pull-down assays. Y.Z. observed the protein localization. L.-J.Q., T.D., and J.D. wrote the paper, with input from other authors.

reception. Moreover, overexpression of *EN15* by the endogenous promoter caused disturbed pollen tube guidance and reduced fertility. These data suggest that female-derived GPI-anchored ENODLs play an essential role in male-female communication and fertilization.

RESULTS AND DISCUSSION

Isolation of Ovule-Enriched *AtENODLs*, or *ENs*

Phytocyanins, a plant-specific subfamily of copper-binding cu-predoxins, are small secretory proteins involved in electron transfer during redox reactions [7]. Previous studies have demonstrated that two members of phytocyanins—plantacyanin and chemocyanin—are female sporophytic signals for directional pollen tube growth in the pistils of *Arabidopsis* and lily, respectively [8, 9]. Early nodulin (ENOD) is a subclass of phytocyanins that are expressed very early in developing root nodules of legumes and play crucial roles in cell division and dedifferentiation [10]. A number of proteins have sequence similarity to ENOD, including the ENOD-like protein (ENODL, or EN) family, consisting of 22 members in *Arabidopsis*, 24 members in rice, and 52 members in Chinese cabbage [11]. Many *ENODL*s were expressed in reproductive organs, suggesting their possible functions in the reproduction process [11]. However, thus far, no specific function has been addressed to any of the ENODLs, likely due to genetic redundancy. According to our RNA-sequencing (RNA-seq)-based transcriptional profiling analysis [12], Clade A genes of the *Arabidopsis thaliana ENODL*s (*AtENODL*s, or *EN*s) showed strong expression in mature ovules but not in pollen tubes (Figure 1A). *EN15* and *EN14* were most abundantly expressed in mature ovules, while *EN11*, *EN12*, and *EN13* displayed lowered expression levels [12] (Figure 1A).

A glycosylphosphatidylinositol (GPI) anchor motif was predicted at the C-termini of EN11–EN15 (Figures 1A, 1B, and S1B) [13]. With the composition of a GPI anchor, a signal peptide, and an arabinogalactan (AG) glycomodule, EN11–EN15 are similar to “classical” AG proteins (AGPs) [14], which play multiple roles in plant reproductive processes [15, 16]. EN11–EN15 contain an additional plastocyanin-like (PCNL) domain present in sporophytic signaling molecules (discussed earlier). The GPI anchor likely localizes the modified protein at the plasma membrane [15]. After cleavage by phospholipase C, the anchor-free AGPs can be detached from the plasma membrane and released into the extracellular matrix [17, 18]. Both membrane-anchored ENDOLs and the soluble forms would allow them to mediate cell-cell communication between ovules and pollen tubes.

Interestingly, *EN11–EN15* appear in a separate clade exclusively composed of angiosperm species, distinct from other ENODL genes in gymnosperms, ferns (*Selaginella moellendorffii*), bryophytes (*Physcomitrella patens*), and green algae (Figure S1A). This indicates that functions of EN11–EN15 might be related to angiosperm-specific processes, e.g., double fertilization, which evolved from lower plants and is lost in the gymnosperm lineage.

ENs Are Expressed in the Ovules and the Funiculus

To elucidate their detailed expression profile in *Arabidopsis* flowers, we analyzed the promoter activity of *EN14* and *EN15* (strongest expression based on RNA-seq data) by

performing a β-glucuronidase (GUS) staining assay using *pEN::GUS* transgenic lines. The two promoters showed a largely overlapping expression pattern in inflorescences, and the highest GUS activity was found in mature pistils (Figures S2A and S2B). In ovules of stages FG3 to FG7 (FG, female gametophyte) (Figures 1I and 1K), the highest GUS activity was detected at the micropylar region and inner integument surrounding the female gametophyte (Figures 1C–1E for *EN14*; Figures 1F–1H for *EN15*). Whole-mount in situ hybridization (WISH) and fluorescent WISH (F-WISH) revealed the strongest expression in individual cells of the inner integument and weak expression at the micropylar region at stage FG7 (Figures 1L–1O). At 16 hr after pollination (HAP), about 7–10 hr after fertilization [19], GUS activity of the *EN15* promoter decreased dramatically in the ovules (Figure 1H). Egg apparatus expression of *EN11*, *EN12*, and *EN13* was previously reported [12, 20]. We also found that *EN12*, as a representative, was transcribed mainly in the micropylar region of the embryo sac (Figures S2F–S2H). These expression properties provide strong correlation of *EN* expression with the fertilization process.

To further investigate the expression patterns of *EN* genes, we transferred the *EN15* native promoter into an LhG4/pOp trans-activation system [21] (Figure S3A), in which the *GUS* reporter gene was activated synchronously with *EN15*, providing the convenience of visualizing the transcriptional pattern and activity of EN15 in planta. At later developmental stages, we observed an expression level shift of *EN15* from the micropyle toward the chalazal end (Figures 1P and 1Q), which nicely correlates with our WISH and F-WISH data. The qRT-PCR results confirmed that the expression levels of *EN15* were dramatically elevated in two transgenic lines: *EN15tcsoe-73* and *EN15tcsoe-45* (Figure 1R; Figures S3B–S3E). Additionally, we were able to observe GUS activity in the funiculus and at the junctions between the transmitting tissue and the funiculus (Figures 1S and 1T). The turning point of the funiculus along the transmitting tissue is a landmark where pollen tubes are guided to grow toward the ovules. The presence of EN15 at the funiculus junction suggests a potential role in pollen tube guidance.

EN Proteins Appear at the Surface of the Placenta and at the Micropylar Surface of the Synergid Cells

The localization of EN14 and EN15 proteins (EN14/EN15) was recently reported in the stomatal lineage cells [22]. We examined their YFP (yellow fluorescent protein)-fusion-protein expression in reproductive organs and found that EN15 localization was preferentially associated with cell walls (Figures 2A–2D). Notably, a strong signal was observed in the micropylar surface of the synergid cells (Figures 2B and 2C), i.e., the filiform apparatus, a highly thickened structure in the synergid cell wall closely associated with pollentubereception [23, 24]. The micropylar-enriched localization of EN15 suggested its potential function in fertilization, particularly in mediating male-female communication in the ovules.

To validate the protein localization of mature EN14/EN15 in the filiform apparatus at the cellular level, we generated an antibody against specific regions in mature EN14/EN15 (Figure S2I). By using this antibody for immunofluorescence assays, we found that, in more than 70% of the immature ovules, EN14/EN15 exhibited a strikingly polarized localization

pattern and accumulated at the micropylar surface of the synergid cells (Figures 2E and 2F). In mature ovules, EN14/EN15 signals were found enriched in the filiform apparatus (Figures 2E and 2F). In the EN15 overexpression lines, the immunofluorescence assay detected enhanced signals and, thus, confirmed protein enrichment both at the flank region of the synergid cells and at the filiform apparatus (Figures 2G and 2H). Some signals appeared at the integuments in the micropylar region, recapitulating what we have observed from the trans-activation and in situ hybridization analyses (Figure 1). EN14/EN15 immunofluorescence signals were almost absent in *en14 en15* mutant ovules (Figures 2I and 2J). The expression pattern change from the integuments and flanking region of the synergid cells to the filiform apparatus suggests that the EN15 protein might have had its GPI anchor removed and then released from its original location and diffused toward the filiform apparatus to implement its function. Considering that EN11 and EN12 were also reported to be expressed in the egg apparatus [20], it is very likely that multiple ENs might be involved in the filiform apparatus function and pollen tube-ovule communication.

Loss-of-Function Mutations in *EN*s Cause Failures of Pollen Tube Burst

To study the function of polarly localized ENs, we made considerable effort to obtain loss-of-function mutants. However, although *en13*, *en14*, and *en15* were transfer DNA (T-DNA) insertional null mutations (Figure 3A and 3B), we did not observe any obvious phenotype in single, double, and triple mutants. As *EN11* and *EN12* clustered together with *EN13*, *EN14*, and *EN15* (Figure S1A), and they also showed detectable expression in ovules, we further constructed higher order mutants by introducing an RNAi silencing construct for *EN11/EN12* into the triple mutant *en13 en14 en15*. Finally, we created an *en-RNAi* mutant that contains the loss of function of *EN13 EN14 EN15* and lowered expression of *EN11* and *EN12*, respectively (Figure 3C). In these mutant plants, we observed a remarkably reduced seed set due to aborted ovules (52% and 27% in *en-RNAi-5* and *en-RNAi-7*, respectively) (Figures 3D and 3E). Aniline blue staining showed that pollen tubes in these ovules failed to rupture and release their sperm cell cargo (Figures 3F–3H). Two phenotypes might cause these failures: (1) pollen tube overgrowth in the embryo sac, a behavior similar to *feronia* and *lre* mutant ovules [4, 6, 25], where pollen tubes kept growing and failed to discharge in the ovule (Figure 3G); and (2) abnormal callose accumulation in the embryo sac (Figure 3H), which likely resulted as a defense reaction to pollen tube overgrowth or structural collapse after fertilization failure.

The receptor-like kinase FERONIA (FER) and the GPI-anchored protein (GPI-AP, GAP) LORELEI (LRE) were reported to be involved in pollen tube reception [4, 6]. Recently, both LRE and its homolog LLG1 were found to interact with the exJM domain of FER and to regulate the function of FER [26]. Because loss of *EN* function resulted in similar phenotypes of *fer* and *lre*, we speculated that ENs might work in the same signaling pathway. To test this hypothesis, we performed a split-ubiquitin yeast two-hybrid assay using the extracellular domain (ECD) of FER as bait and EN14/EN15 as prey [27]. We found that EN14 strongly interacted with the ECD of FER in yeast cells (Figure 3J). We further conducted in vitro pull-down assays to confirm the interaction and found that His-tagged EN14 (EN14-HisTag) was fully pulled down by glutathione S-transferase (GST)-tagged FER but only weakly pulled down by GST-tagged LRE (Figure 3K). GST-tagged

HERKULES (HERK), a closely related sister protein of FER, did not interact with EN14-HisTag (Figure 3K). These results support the hypothesis that the interaction between EN14 and the ECD of FER is strong and highly specific. We failed to detect the interaction between EN15 and the ECD of FER in in vitro pull-down assays, possibly due to differences between EN14 and EN15 in post-translational modifications in different organisms. It is worth noting that ENs harbor ECDs that are unrelated to those from LLG1 and LRE [4, 28]. Therefore, ovule-specific ENs may act as adaptors or tethering factors, capturing their specific interaction partner(s), like FER, via their glyco- and PCNL modules at the interface between the filiform apparatus and synergid cell surface for polar activation of the first steps in the FER signaling pathway [15].

ENODLs Are Important for Pollen Tube Entrance into the Ovule, Growth Arrest, and Burst

To test whether the ovules of *en-RNAi* quintuple mutants are recognizable by normal pollen tubes, we performed a semi-in-vivo-fertilization assay [29] using the double marker line *pLat52::GFP pHTR10::RFP* as a pollen donor (labeling pollen tubes in green and both sperm cells in red). In wild-type (WT) ovules, 92.5% pollen tubes ruptured and released two sperm cells within about 70 min after entering the micropyle (Figures 4A–4H; Figure S4). However, a number of pollen tubes in *en-RNAi* mutant ovules were attracted but failed to release sperm cells within 120 min (Figures 4I–4P and S4). Moreover, we found that multiple pollen tubes gathered simultaneously at the micropylar opening of the ovule (Figures 4I–4P), which was also observed in vivo (Table S1). However, it is more likely that the observation of polytubey in *en-RNAi* mutant ovules was a side effect due to fertilization failure. In several ovules with overgrown pollen tubes (phenotype 1 described earlier), a second pollen tube was attracted by the same ovule. This phenomenon was also found in *feronia* [30] and other fertilization deficiency mutants such as *duo3*, *hap2*, *ec1*, and *gex2* [19, 30–33] and represents a fertilization recovery mechanism to enforce successful fertilization when the first pollen tube failed to fertilize [31, 32]. Altogether, based on the phenotype of *en-RNAi* plants, we hypothesize that the ovule-generated ENs represent mobile extracellular components essential in mediating female-male communication to induce pollen tube reception and burst in *Arabidopsis thaliana*.

Notably, *EN15tcsoe* plants also produced a severely reduced seed set (Figures S3F and S3G) with significantly aborted ovules (Table S2), which emphasized the critical function of ENs in female reproductive organs. Compromised pollen tube guidance was observed in *EN15tcsoe* ovules (Figures S3S–S3W), and pollen tubes frequently failed to grow onto the funiculus to approach *EN15tcsoe* ovules (Figures S3H–S3L). Meanwhile, the higher ratio of unfertilized ovules, although normally targeted by pollen tubes, suggested that *EN15tcsoe* ovules are not fully mature. Among the untargeted *EN15tcsoe* ovules, we often observed obvious callose accumulation (Figure S3J). In some *EN15tcsoe* ovules, only one nucleus could be observed in the embryo sac (Figures S3M–S3R). These observations suggest that over-accumulation of EN15 in some ovules interferes with mitosis in female gametophyte development and, as a consequence, with pollen tube guidance.

GAPs are secreted to the space between the plasma membrane and cell walls, which makes them ideal candidates in mediating cell-cell communication [17, 18]. ENODLs are a subset

of GPI-anchored secretory proteins that are enriched in the ovule [12], localized to the filiform apparatus [23, 24], and crucial for pollen tube reception. A similar localization was detected with an anti-AGP antibody and was hypothesized to correlate with ovule receptivity [16, 34]. Moreover, a methylated disaccharide 4Me-GlcA-Gal exposed on the side chains of ovular AGPs was recently reported to be essential for pollen tube competency to respond to female signals [35]. Whether ENs, a group of AGP-harboring AG glycomodules, also serve as a source for this competence signal remains to be investigated.

Disturbed expression of ENs, by loss of function or overexpression, led to partial failures in pollen tube reception and double fertilization. In addition, ENs directly interact with FER, the core signal receptor in ovules for pollen tube reception [6]. Thus, we propose that ENs, accumulating in the filiform apparatus from early stage to maturation of the female gametophyte, are used as female cues to facilitate polar anchoring of FER on the filiform apparatus and to mediate the crosstalk between male and female reproductive cells. Since EN14 interacts with the ECD of FER, and LLG1 binds to the juxtamembrane region of FER [26], it is very likely that these proteins form a large complex and cooperate with each other during FER-mediated signaling.

EN proteins appear as multifunctional proteins and may participate in male-female interaction on multiple levels, due to their multiple domains and expression patterns. EN15 preferentially associates with newly formed cell walls [22], raising the possibility that ENs are also involved in cell division of female gametophytes before fertilization. This is consistent with the observation that mitosis of megaspores was affected in some EN15 overexpressor lines. EN proteins also share a PCNL domain. Overexpression of PCN peptides was previously shown to disturb the penetration of pollen tubes into the stigma [9]. Therefore, the PCNL domains of ENs might also be involved in the interactions between pollen tubes and transmitting tissue/funiculus, e.g., during septum penetration and/or polytubey prevention.

GAPs, like ENs, also play key roles in gamete recognition in mammalians [36–38]. Although plants and animals belong to two kingdoms, it is interesting to note that both utilize GAPs for signaling during fertilization, such as COBL10 in pollen tubes, ENs in ovules, and LORELEI in the synergid cells of *Arabidopsis* [4, 25, 26, 39], while mammals express Juno at the surface of egg cells as a major sperm receptor [38]. Future work is now required to elucidate the detailed molecular interaction between mature ENs, FER, and LRE at the synergid cell surface in order to determine the structure and composition of the FER receptor complex, as well as its polar accumulation and activation.

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Highlights

- ENODL11–15 are GPI-anchored proteins, each with a plastocyanin-like domain
- ENODL11–15 are predominantly expressed in the ovules and the funiculus
- Loss of function or overexpression of *ENODL*s compromises pollen tube reception
- ENODL14 has physical interaction with the extracellular domain of FERONIA

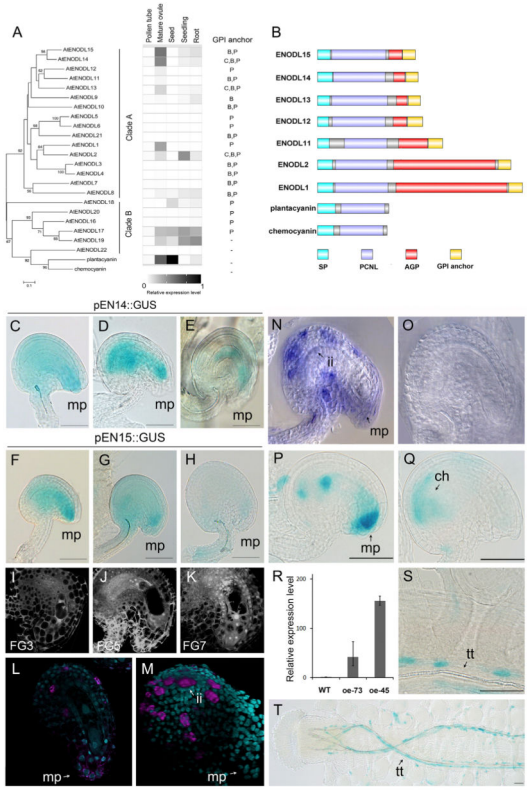

Figure 1. Phylogeny, Transcription Profile, and Structure of *A. thaliana* ENODL Family Proteins and the Detailed Expression Analysis of *EN14* and *EN15*

(A) Phylogenetic analysis and the transcription profile of all 22 *A. thaliana* ENODL (EN) family members. Related plantacyanin and lily chemocyanin were used as outgroups in the phylogram. Pollen tube: semi-in-vivo germinated pollen tubes. Lowest expression level is indicated in white, and highest expression level is indicated in black. GPI anchor signals were confirmed by Borner et al. [13] (C) or were predicted by big-PI predictor (B) and PredGPI (P) programs.

(B) Protein structure of selected ENODL proteins of Clade A expressed in mature ovules. Abbreviations: SP, signal peptide; PCNL, plastocyanin-like; AGP, arabinogalactan protein domain; GPI, glycophosphatidylinositol.

(C–K) Dynamic changes of transcription patterns of *EN14/EN15* during fertilization. *EN14* (C–E) and *EN15* (F–H) promoter activity of the GUS reporter before and after fertilization is shown. Scale bars, 50 μm. (Corresponding ovule development stages are shown in (I)–(K). (I) shows an ovule at stage FG3, as in (C) and (F). (J) shows an ovule at stage FG5, as in (D). An ovule at stage FG7 is shown in (G). (K) shows a fertilized ovule (16 HAP) comparable to the stages in (E) and (H). mp, micropyle. Scale bars, 10 μm.

(L and M) F-WISH of *EN15* using an antisense probe against its 3′ UTR. Ovules are at stage FG5 (L) and FG7 (M). Nuclei are stained with DAPI. mp, micropyle; ii, inner integument.

(N and O) WISH of *EN14* using an antisense probe against its coding region (N). A sense probe is used as negative control (O).

(P and Q) Activity of the GUS reporter in EN15tcsoe trans-activation lines. An early stage of ovule development (P) and a mature ovule (Q) are shown. mp, micropyle; ch, chalaza. Scale bars, 50 μm.

(R) Relative overexpression levels of EN15 in ovules analyzed by qRT-PCR. The error bars represent the SD of three independent biological replicates of qRT-PCR experiments.

(S and T) Activity of the GUS reporter in EN15tcsoe trans-activation lines. Signals at the junction of transmitting tissue and funiculus (S) and an overview showing GUS signals in a mature pistil (T) are shown. The strongest signals are detected at the transmitting tract (tt), pollen tube exit region of the placenta and funiculus. Scale bars, 50 μm. See also Figures S1 and S2.

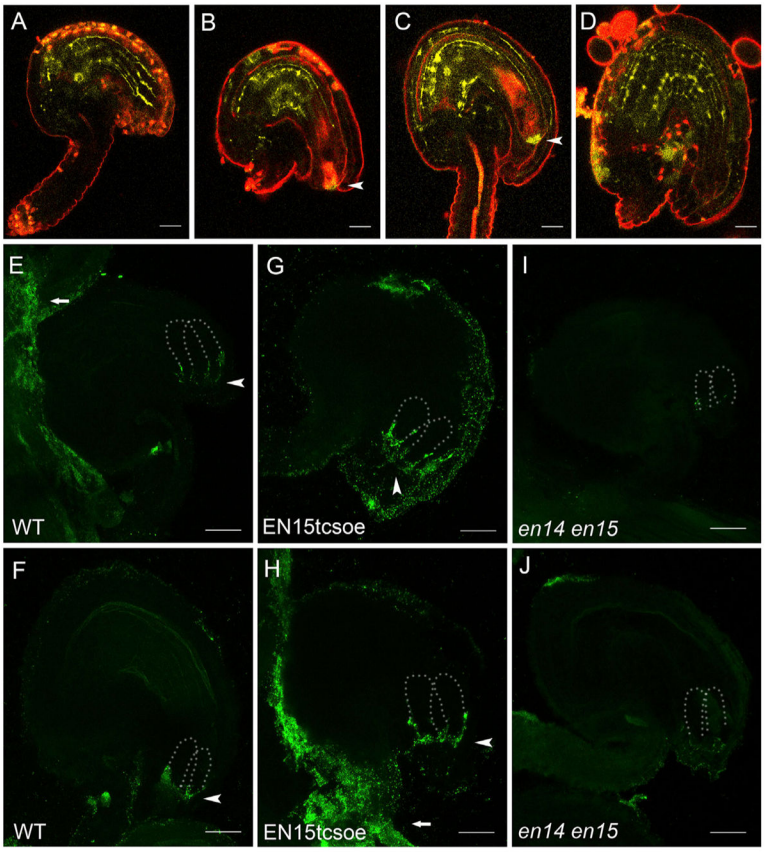

Figure 2. Protein Localization of EN14/EN15 in WT and EN15tcsoe Ovules

(A–D) Localization of EN15-YFP fusion protein at different developmental stages of WT ovules. FG3 (A), FG5 (B), FG7 (C), and a fertilized ovule (D) are shown. Arrowheads indicate signals detected at the filiform apparatus. More than 30 out of 50 ovules at each stage with similar expression patterns were observed. Scale bars, 20 μm.

(E and F) Immunofluorescence assay of EN14/EN15 (using an EN14/EN15-specific antibody) in WT ovules. In FG4 ovules, signals were detected at the surface of the placenta (arrow) and accumulate at flank region of synergid cells (arrowhead) (E). In mature ovules, signals were detected at the filiform apparatus (arrowhead) (F). Dotted lines define synergid cells. Out of 14 well-stained ovules, at least ten ovules with the same or similar patterns were observed. Scale bars, 20 μm.

(G and H) Immunofluorescence assay of EN14/EN15 in FG5 EN15tcsoe ovules. In FG4 ovules, signals were detected at the flank region of synergid cells (arrowhead) (G). In FG5 ovules, signals were detected at the filiform apparatus (arrowhead) and surface of the placenta (arrow) (H). Out of 20 well-stained ovules, at least 14 ovules with the same or similar patterns were observed. Scale bars, 20 μm.

(I and J) Immunofluorescence of EN15 in *en14 en15* ovules. Signals were almost undetectable in mutant ovules of FG3 (I) and FG5 (J). Out of 15 well-stained ovules, at least 11 ovules with the same or similar patterns were observed. Scale bars, 20 μm. See also Figures S2 and S3 and Table S2.

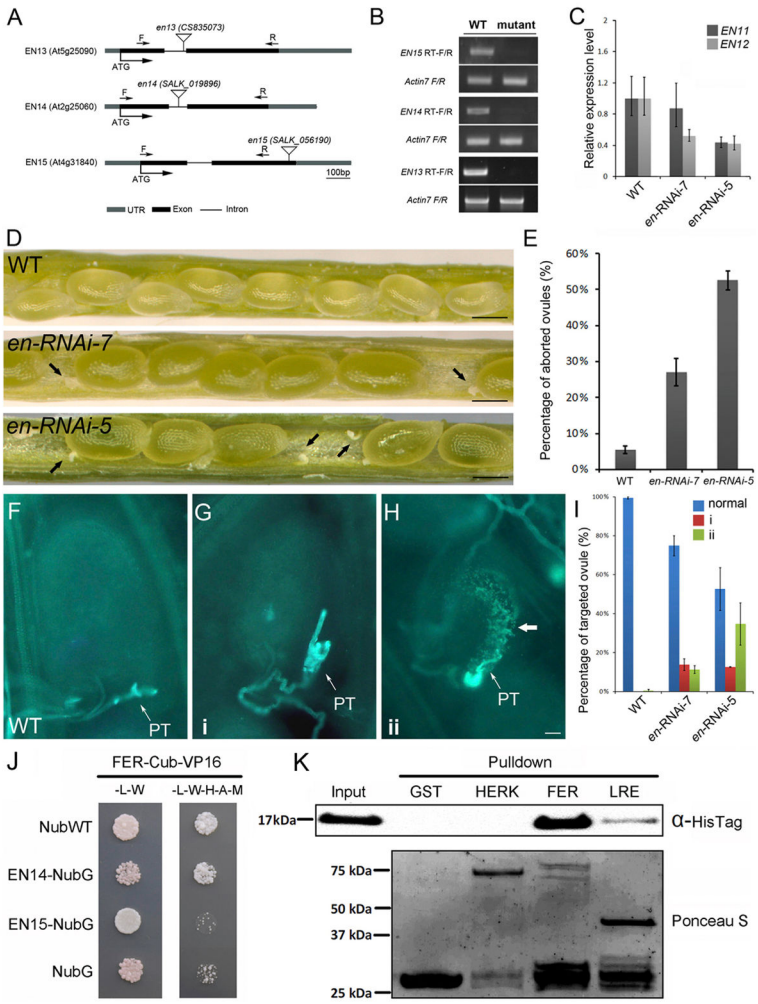

Figure 3. Pollen Tube Rupture Phenotype in *en-RNAi* Mutants and Interaction between ENODLs and FERONIA

(A) Diagram showing T-DNA insertion sites in *en13*, *en14*, and *en15* mutants.

(B) Semi-qRT-PCR analysis to show the expression levels of *EN13*, *EN14*, and *EN15* in the corresponding SALK T-DNA mutant lines.

(C) Expression levels of *EN11* and *EN12* in *en-RNAi* mutants detected by qRT-PCR assays. The error bars represent the SD of three independent biological replicates of qRT-PCR experiments.

(D) Ovule abortion in *en-RNAi* siliques. Arrowheads indicate aborted ovules. Scale bars, 200 μm.

(E) Percentage of aborted ovules in two independent quintuple mutant lines compared with the wild-type (WT). Error bars are mean ± SD of three biological replicates; more than 50 siliques from each line were examined.

(F) Fertilized WT ovules. Aniline blue stains a single pollen tube (PT).

(G) Pollen tube failed to rupture and showed overgrowth in an *en-RNAi* mutant ovule (phenotype 1; i).

(H) Pollen tube failed to rupture and showed callose deposition in ovules of an *en-RNAi* mutant line (phenotype 2; ii). Arrow, callose deposition. Scale bar, 20 μm.
(I) Histogram to show quantification of both phenotypes. N > 120. Error bars are mean ± SD of three biological replicates; more than 50 siliques from each line were examined.
(J) ENODL14 strongly interacts with FERONIA (FER) in a split-ubiquitin yeast-two-hybrid assay. Cub-VP16, the C-terminal half of an ubiquitin fused with the transcription factor (LexA-VP16) to activate the reporter genes; NubWT, the N-terminal half of a wild-type ubiquitin; NubG, the N-terminal half of a mutated ubiquitin as a negative control; -L, medium without Leu; W, medium without Trp; H, medium without His; A, medium without Ade; M, medium without Met.
(K) ENODL14 strongly and specifically interacts with FER in a pull-down assay. ENODL14-HisTag (top) is pulled with GST-tagged extracellular domains (ECDs) of CrRLK-like receptor kinase FER, but not with its family member HERKULES (HERK). GST-LRE is weakly pulled down with ENODL14-His. The bottom panel shows GST pull-downs with Ponceau S staining.
See also Figure S4.

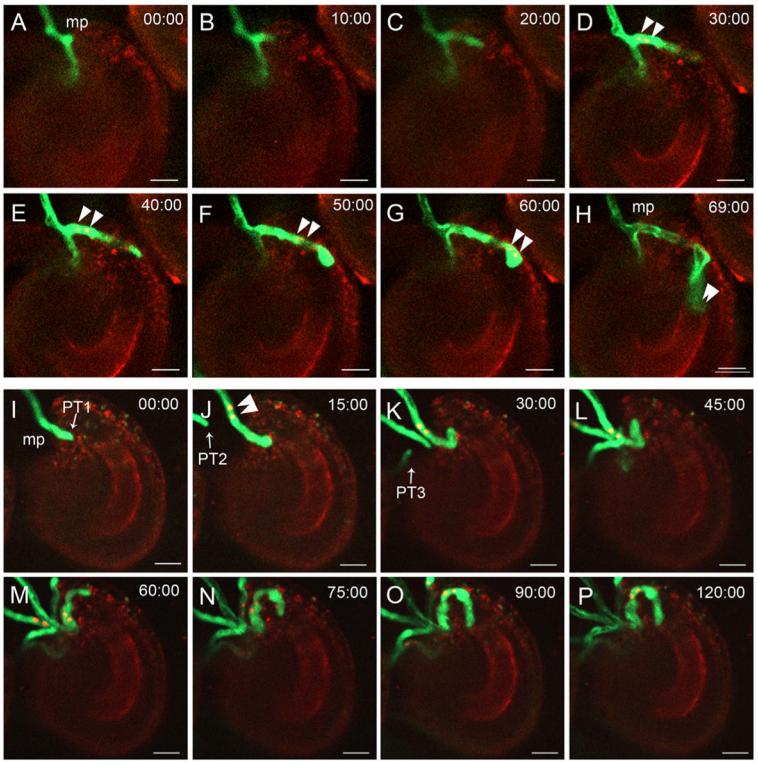

Figure 4. Confocal Live-Cell Imaging of the Fertilization Process in WT and *en-RNAi* Ovules

(A–H) Imaging of the fertilization process of wild-type ovules (recording for 69 min).

(I–P) Imaging of the fertilization process of a representative *en-RNAi* ovule (recording for 120 min).

Arrowheads point toward sperm cells, and arrows point at pollen tubes (PTs). mp, micropyle. Scale bars, 10 μm. See also Table S1.