RESEARCH ARTICLE SUMMARY

PLANT SCIENCE

Cysteine-rich peptides promote interspecific genetic isolation in *Arabidopsis*

Sheng Zhong*, Meiling Liu*, Zhijuan Wang*, Qingpei Huang, Saiying Hou, Yong-Chao Xu, Zengxiang Ge, Zihan Song, Jiaying Huang, Xinyu Qiu, Yihao Shi, Junyu Xiao, Pei Liu, Ya-Long Guo, Juan Dong, Thomas Dresselhaus, Hongya Gu, Li-Jia Qu†

INTRODUCTION: Reproductive isolation is the inability of a species to breed with a related species and thus is key to the formation and evolution of a new plant species. Within the genus *Arabidopsis*, pistils of *A. thaliana* can be fertilized by pollen of related species, such as *A. lyrata*, but conspecific (self) pollen is preferred to maintain reproductive isolation. The molecular mechanisms by which the plant's own pollen tubes outperform heterospecific (alien) pollen tubes is largely unknown. In *A. thaliana*, maternal cysteine-rich peptides AtLURE1.1 to -1.5, secreted from the ovule, were reported to function through the male receptor PRK6 to mediate pollen tube attraction.

RATIONALE: Although AtLURE1s have been identified as species-specific pollen tube attractants in *A. thaliana*, down-regulation of *AtLURE1* genes and knockout of their receptor PRK6 did not disturb fertilization and seed set, indicating that the biological functions of AtLURE1s are not fully understood. We initially aimed to fully knock out genes for AtLURE1.1 to -1.5, as well as additional related *AtLURE1* genes, to understand their contribution to fertilization success and reproductive isolation.

RESULTS: In addition to the five reported AtLURE1 genes, we identified two further *A. thaliana*–specific *AtLURE1* genes and generated a loss-of-function *atlure1* null septuple mutant by knocking out the whole gene family. Although *atlure1* null mutants, resembling *prk6* receptor mutants, exhibited normal fertility, pollen tubes in *atlure1* null pistils, like *prk6* pollen tubes, displayed delayed emergence at the septum. These data collectively suggest that AtLURE1-PRK6 signaling accelerates pollen tubes to penetrate the septum and to grow toward ovules.

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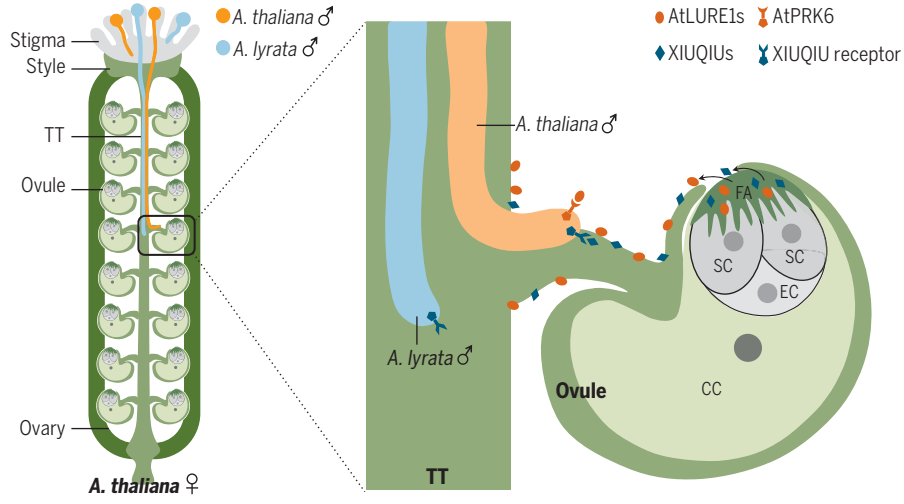
We further tested the function of AtLURE1-PRK6 signaling in reproductive isolation by depositing alien *A. lyrata* pollen onto *A. thaliana* pistils and showed that *A. lyrata* pollen tubes emerged much more slowly out of the septum. However, the emergence of *A. lyrata* pollen tubes in *A. thaliana* pistils was not further impaired in *atlure1* null pistils, suggesting that AtLURE1s promote reproductive isolation by accelerating their own pollen tubes, which thus outperform alien tubes.

This hypothesis requires the existence of more general, genus- and/or family-specific ovular guidance molecules. Therefore, we next investigated the functions of four AtLURE1-related Brassicaceae-conserved cysteine-rich peptides named XIUQIU1 to -4. We found that XIUQIU peptides attract pollen tubes in a non–species-specific manner and independently of the PRK6 receptor. This finding suggests that evolutionarily ancient XIUQIUs function as general pollen tube attractants in the Brassicaceae. Finally, after combining *atlure1* null with *xiuqiu* loss-of-function mutations, fertility in *A. thaliana* was reduced, supporting the biological importance of these cysteine-rich peptides in plant reproduction.

CONCLUSION: In *A. thaliana*, species-specific female AtLURE1 peptides and their male receptor PRK6 promote and maintain reproductive isolation by accelerating conspecific pollen tube growth to penetrate the septum. The AtLURE1-related cysteine-rich XIUQIU peptides are evolutionarily ancient and conserved attractants in the Brassicaceae and attract pollen tubes in a non–species-specific manner. XIUQIUs contribute to reproductive success and heterospecific fertilization. Their activity may lead to new genome combinations and thus to the formation of new species. ■



AtLURE1s, conspecific pollen tube attractants, promote reproductive isolation in *A. thaliana* and, together with the non–species-specific attractants XIUQIU1 to -4, contribute to fertility. AtLURE1s and XIUQIUs are cysteine-rich pollen tube attractants that are secreted by the synergid cells and diffuse from the micropylar region of the ovule toward the surfaces of the placenta and septum, respectively. Non–species-specific XIUQIUs occurring in diverse Brassicaceae species and their unknown receptor(s) are capable of attracting pollen tubes from *A. thaliana* (orange) and *A. lyrata* (blue) in similar manners. Signaling via species-specific AtLURE1s and their pollen tube receptor AtPRK6 functions to accelerate pollen tube emergence from the transmitting tract, giving precedence to the plant's own (*A. thaliana*) pollen tubes, which thereby outcompete *A. lyrata* pollen tubes at the emergence point. Cooperation between both attractants thus contributes to maintaining reproductive isolation while simultaneously allowing alien fertilization at low frequency. TT, transmitting tract; SC, synergid cell; FA, filiform apparatus; EC, egg cell; CC, central cell; male symbol, pollen tube; female symbol, pistil.

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RESEARCH ARTICLE

PLANT SCIENCE

Cysteine-rich peptides promote interspecific genetic isolation in *Arabidopsis*

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Reproductive isolation is a prerequisite for speciation. Failure of communication between female tissues of the pistil and paternal pollen tubes imposes hybridization barriers in flowering plants. *Arabidopsis thaliana* LURE1 (AtLURE1) peptides and their male receptor PRK6 aid attraction of the growing pollen tube to the ovule. Here, we report that the knockout of the entire *AtLURE1* gene family did not affect fertility, indicating that AtLURE1-PRK6–mediated signaling is not required for successful fertilization within one *Arabidopsis* species. AtLURE1s instead function as pollen tube emergence accelerators that favor conspecific pollen over pollen from other species and thus promote reproductive isolation. We also identified maternal peptides XIUQIU1 to -4, which attract pollen tubes regardless of species. Cooperation between ovule attraction and pollen tube growth acceleration favors conspecific fertilization and promotes reproductive isolation.

Reproductive isolation is a key step in the formation and evolution of a new species (*1*, *2*). In angiosperms, reproductive isolation can occur at prezygotic or postzygotic phases. Prezygotic isolation includes environmental and ecological factors (*3*–*5*), as well as responses within the plant: The pistil is thought to control pollination success and thus to prevent hybridization beyond the species. Conspecific pollen precedence (*6*), in which pollen from plants of the same species is favored for fertilization (*1*, *7*, *8*), contributes to the divergence of subspecies.

In flowering plants, fertilization requires the delivery of immotile sperm cells as passive cargo through the pollen tube (*9*), which grows out of the pollen grain and through various pistil tissues toward the ovules (*10*). During this so-called progamic phase, the pollen tube needs to be properly attracted and guided to reach the ovule. Guidance cues provided by female pistil tissue include small peptides (*11*). The peptide EA1, for example, which is produced by the egg apparatus (egg and synergid cells) of maize (*Zea mays*), guides pollen tubes through the micropylar nucellus tissue inside the embryo sac (*12*). The cysteine-rich peptides LURE1 and LURE2, which are expressed in the synergid cells of *Torenia fournieri*, attract pollen tubes toward the egg apparatus (*13*, *14*). In *Arabidopsis thaliana*, the recently evolved cysteine-rich LURE1 peptides, which are unrelated to LUREs of *T. fournieri*, are also synergid cell–expressed attractants in micropylar guidance of pollen tubes (*15*). Thus, the pollen tube attractants identified so far seem to be species or genus specific and essential for successful fertilization (*13*, *15*). However, whereas the down-regulation of *Z. mays EA1* through RNA interference resulted in severe fertility defects (*12*), RNA interference that down-regulated *A. thaliana LURE1* (*AtLURE1*) genes and the knockout of their receptor PRK6 did not affect fertility in *Arabidopsis* (*15*, *16*). We studied the functions of AtLURE1s in fertilization and reproductive isolation.

AtLURE1-PRK6 is dispensable in *A. thaliana* reproduction

In *A. thaliana*, six species-specific *AtLURE1* genes, *AtLURE1.1* to *AtLURE1.6*, have been reported (*15*). In this study, we identified two additional *A. thaliana*–specific *AtLURE1* genes, *AtLURE1.7* (*At4g08869*) and *AtLURE1.8* (*At4g08875*) (Fig. 1A and fig. S1). The eight *A. thaliana*–specific AtLURE1s belong to the same clade, representing a group of recently and fast-evolving cysteine-rich peptides (Fig. 1A). Both *AtLURE1.7* and *AtLURE1.8* are expressed in synergid cells (Fig. 1B), as is *AtLURE1.2* (*15*). AtLURE1.7 and AtLURE1.8 proteins are localized to the filiform apparatus of synergid cells in *AtLURE1.7/1.8pro:AtLURE1.7/1.8-GFP* transgenic plants (Fig. 1C), suggesting that AtLURE1.7 and -1.8 likely share a biological function similar to those of AtLURE1.1 through -1.5. We expressed and purified AtLURE1.2, -1.7, and -1.8 peptides in insect cells (fig. S2) and conducted pollen tube attraction assays (*17*). AtLURE1.7 and AtLURE1.8 attracted *A. thaliana* wild-type (WT) (where "WT" refers only to *A. thaliana* hereafter) pollen tubes more effectively than *A. lyrata* pollen tubes [attracting frequencies, 41.4 ± 7.6% and 36.9 ± 7.1% versus 4.5 ± 4.0% ($P < 0.01$) and 3.0 ± 5.2% ($P < 0.01$), respectively] (Fig. 1, D, E, and G). In comparison, their activities were similar to but weaker than that of AtLURE1.2 (97.0 ± 5.2% for *A. thaliana* and 20.7 ± 5.3% for *A. lyrata* pollen tubes, respectively) (Fig. 1, D, E, and G). We found that, similar to AtLURE1.2, AtLURE1.7 and AtLURE1.8 showed significantly reduced attraction activity for *PRK6*-defective pollen tubes [i.e., 8.1 ± 7.3% ($P < 0.01$) and 14.8 ± 3.5% ($P < 0.01$), respectively] (Fig. 1, F and G) (*16*). Together with the genetic evidence described below, these results indicate that both peptides are *A. thaliana*–specific AtLURE1s and function as peptide ligands in the same PRK6-mediated signaling pathway.

To elucidate the biological function of the whole *A. thaliana*–specific *AtLURE1* family, we used the CRISPR-Cas9 technology to generate a septuple *atlure1* null mutant by simultaneously knocking out seven pollen tube attractants: *AtLURE1.1*, *-1.2*, *-1.3*, *-1.4*, *-1.5*, *-1.7*, and *-1.8* (Fig. 2A). Because *AtLURE1.6* is a pseudogene (*15*), we did not target it. By DNA sequencing, we confirmed that the CRISPR-Cas9–generated mutations existed, both in genomic DNA and in transcripts (cDNA) in *atlure1* null septuple mutants (figs. S3 and S4). Mutated peptides, both expressed in *Escherichia coli* (Fig. 2B) and chemically synthesized, did not exhibit pollen tube attraction activity in semi–in vivo pollen tube attraction assays (Fig. 2, C and D, and fig. S5). This indicated that the seven truncated or mutated AtLURE1s lost their pollen tube–attracting activities. The C-terminal region includes a cluster of basic amino acids (Arg^{66}, Arg^{67}, and Lys^{69}) (*16*), Arg^{83} (*18*), and the conserved three cysteines (Fig. 2, B and C) (*15*) that are essential for pollen tube–attracting activities. The dysfunctional mutation in *AtLURE1.6* as described in (*15*) was also confirmed by DNA sequencing (fig. S3F). However, despite the loss of function of the entire *AtLURE1* family, *atlure1* null mutants still exhibited normal fertility [a seed set ratio of 97.7 ± 1.9% ($P > 0.05$), comparable to that of 98.9 ± 1.3% in WT plants] (Fig. 2, E and F). Knocking out the AtLURE1 receptor gene *PRK6* abolished pollen tube responsiveness to attractants AtLURE1.2 (*16*), AtLURE1.7, and AtLURE1.8 in semi–in vivo assays (Fig. 1, F and G). Mutants null for *prk6-2* also showed normal fertilization (Fig. 2, E and F).

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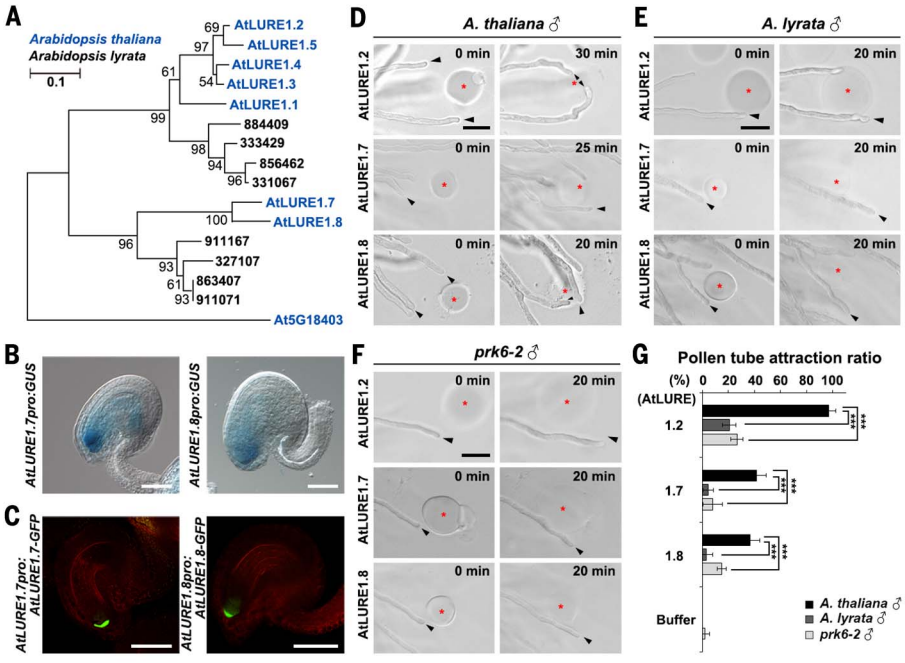

Fig. 1. AtLURE1.7 and -1.8 are newly identified members of the AtLURE1 family. (**A**) Phylogenetic tree of *A. thaliana* AtLURE1.1 to AtLURE1.8 (in blue, except for AtLURE1.6) and their *A. lyrata* orthologs (in black) on the basis of protein sequences. The closely related At5g18403 was used as an outgroup. The scale bar indicates the average number of amino acid substitutions per site. (**B**) GUS signals were predominantly concentrated in synergid cells. (**C**) Images of ovules with both AtLURE1.7–green fluorescent protein (GFP) and AtLURE1.8-GFP located in the filiform apparatus of synergid cells. Scale bars [(B) and (C)], 50 μm. (**D** to **F**) Semi–in vivo pollen tube attraction assay tracing guided pollen tube growth toward recombinant AtLURE1.2, AtLURE1.7, and AtLURE1.8 embedded in gelatin beads (indicated by asterisks). (D) *A. thaliana* WT pollen tubes; (E) *A. lyrata* pollen tubes; (F) *prk6-2* pollen tubes. Arrowheads indicate the direction of pollen tube growth. Asterisks indicate the center of the beads. Scale bars, 25 μm. (**G**) Statistical analysis of the pollen tube responding ratio shown in (D) to (F). Three repeats of the pollen tube attraction assays with 9 to 20 pollen tubes each were conducted for each assay. Data are mean values ± SD. ***$P < 0.01$ (Student's *t* test).

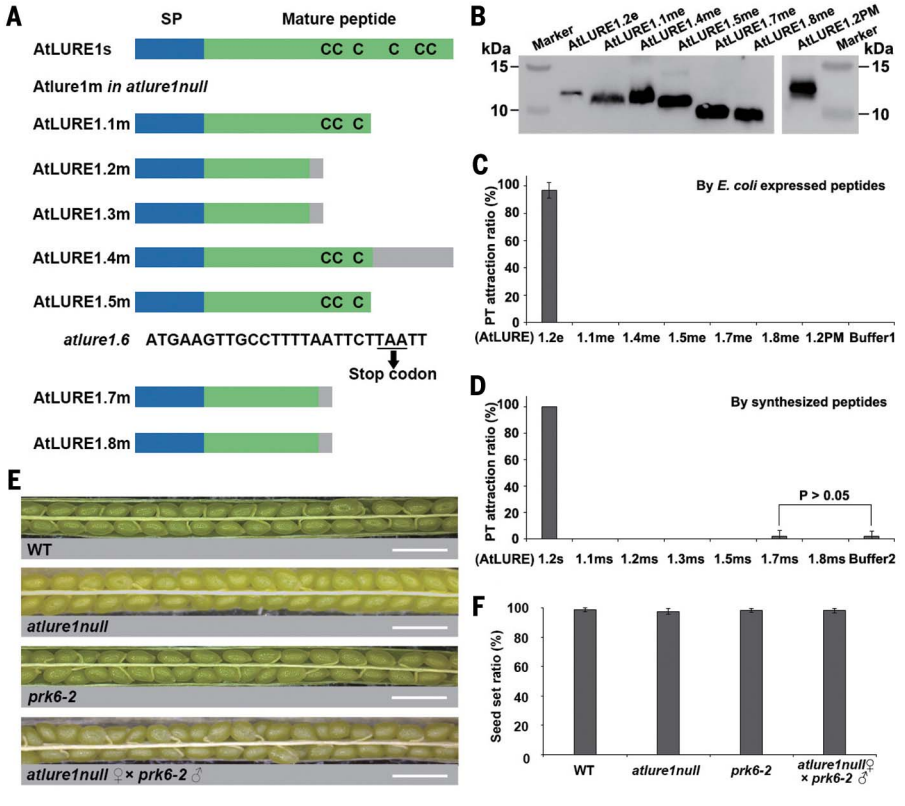

Fig. 2. *atlure1* null and *prk6-2* mutants show a full seed set. (**A**) Schematic diagram of AtLURE1 protein structures containing six conserved cysteine residues (C's). CRISPR-Cas9–edited mutant structures (m) for each AtLURE1 peptide in the *atlure1* null mutant are shown below the WT protein structure (s). *AtLURE1.6* is a pseudogene because of a premature stop codon. SP, signal peptides. Gray boxes indicate missense sequences due to frameshift mutations. (**B**) Western blots of AtLURE1.2 (designated AtLURE1.2e) and mutated AtLURE1 peptides with His tags (designated AtLURE1me) expressed in *E. coli*. AtLURE1.2PM represents *E. coli*–expressed mutated AtLURE1.2 with its C-terminal three conserved cysteines (C^{75}, C^{82}, and C^{84}) mutated to alanines (A^{75}, A^{82}, and A^{84}, respectively). (**C**) Statistical analysis of the pollen tube (PT) responding ratio with *E. coli*–expressed peptides. AtLURE1.2PM served as a negative control, and buffer 1 (tris-HCl, pH 8.0) as an empty control. Three repeats of the pollen tube attraction assays with 8 to 20 pollen tubes each for each expressed peptide were conducted. Six repeats of the pollen tube attraction assays with three to five pollen tubes each were conducted for positive, negative, and empty controls. Data are mean values ± SD. (**D**) Statistical analysis of the pollen tube responding ratio with chemically synthesized mutated peptides (designated AtLURE1ms). Pollen germination medium (buffer 2) served as the empty control. Three repeats of the pollen tube attraction assays with 8 to 20 pollen tubes each for each synthesized peptide were conducted. Six repeats of the pollen tube attraction assays with three to five pollen tubes each were conducted for controls. Data are mean values ± SD; *P* values are >0.05 (Student's *t* test). (**E**) Dissected siliques of WT, *atlure1* null, and *prk6-2* mutant plants, as well as products of a cross between *atlure1* null and *prk6-2* plants. Scale bars, 1 mm. (**F**) Statistical analysis of seed set in WT, *atlure1* null, and *prk6-2* self-fertilized siliques and siliques from a cross of *atlure1* null plants with *prk6-2* plants. Three or four repeats of silique examination were conducted, and 8 to 18 siliques were examined each time. Data are mean values ± SD; *P* values are >0.05 (Student's *t* test).

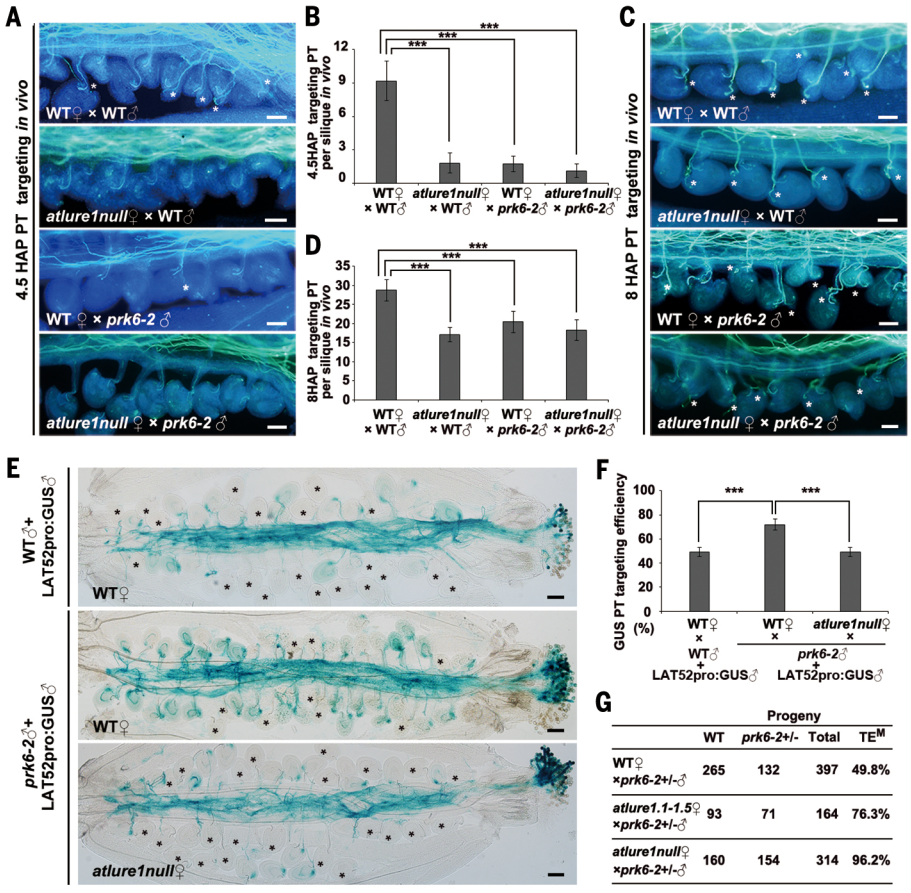

Fig. 3. Pollen tube emergence onto the septa and ovules of *atlure1* null mutants is strongly delayed compared with emergence in *A. thaliana* WT pistils. (**A** to **D**) Aniline blue staining and statistical analysis of *A. thaliana* WT and *prk6-2* mutant pollen tube (PT) emergence onto the septum and ovule in WT and *atlure1* null mutant pistils as indicated, (A and B) 4.5 HAP and (C and D) 8 HAP. Three to five repeats of the pollen tube targeting experiments were conducted for each combination, each with 6 to 12 siliques being examined. White asterisks indicate ovules targeted by pollen tubes. Data are mean values ± SD. ***$P < 0.01$ (Student's t test). Scale bars, 50 μm. (**E**) GUS staining of WT and *atlure1* null mutant pistils pollinated with either a mixture of WT and *LAT52pro:GUS* (WT) pollen grains or a mixture of *LAT52pro:GUS* (WT) and *prk6-2* mutant pollen grains, respectively. Asterisks indicate ovules that are targeted by pollen tubes without GUS staining. Scale bars, 50 μm. (**F**) Statistical analysis of (E). Three repeats of the pollen tube competition experiments were conducted for each combination, each with seven or eight siliques being examined. Data are mean values ± SD. ***$P < 0.01$ (Student's t test). (**G**) Genetic analysis of male (M) transmission efficiency (TE) of the *prk6-2* allele in WT, *atlure1.1* to *-1.5* quintuple mutants, and *atlure1* null septuple mutants.

This is independent from the usage of *atlure1* null pistils [98.6 ± 1.4% ($P > 0.05$) and 98.3 ± 1.4% ($P > 0.05$), respectively] (Fig. 2, E and F). Thus, the *A. thaliana*–specific AtLURE1-PRK6 signaling pathway is not required for successful fertilization in *A. thaliana*.

AtLURE1-PRK6–mediated signaling accelerates pollen tube emergence

We next examined the role of AtLURE1s in ovular pollen tube guidance by using the *atlure1* null mutant. We applied *A. thaliana* WT pollen grains to *atlure1* null pistils to investigate the pollen tube pathway in vivo. Overall pollen tube growth rates in the pistil were not affected (fig. S6, A to D), but defects were evident in the emergence of WT pollen tubes onto the septum surface. Further growth of pollen tubes onto the funiculus and ovule surface was slower in *atlure1* null pistils. At 4.5 hours after pollination (HAP), more WT pollen tubes had emerged onto the septum surface in WT pistils than in *atlure1* null pistils (9.2 ± 1.8 versus 1.8 ± 0.9; $P < 0.01$) (Fig. 3, A and B). The emergence of *prk6-2* pollen tubes was similarly reduced in both WT and *atlure1* null pistils [1.7 ± 0.7 pollen tubes per silique ($P < 0.01$) for WT pistils and 1.1 ± 0.6 ($P < 0.01$) for *atlure1* null pistils] (Fig. 3, A and B). At 8 HAP, the WT pollen tube emergence ratio (the number of targeting pollen tubes per silique) was 28.7 ± 2.7 in WT pistils and 17.1 ± 1.9 ($P < 0.01$) in *atlure1* null pistils, whereas *prk6-2* pollen tube emergence was still low, with ratios of 20.4 ± 2.9 ($P < 0.01$) for WT pistils and 18.3 ± 2.8 ($P < 0.01$) for *atlure1* null pistils (Fig. 3, C and D). In two independent T1 transgenic lines, the late pollen tube emergence defect phenotype was rescued by introducing a single *AtLURE1* gene (*AtLURE1.2pro*:*AtLURE1.2*) into an *atlure1* null mutant (fig. S7), indicating that the phenotype is caused by the loss of function of *AtLURE1* genes, consistent with the assumption that the related *AtLURE1* genes share similar and redundant functions. These findings indicate that the AtLURE1-PRK6–mediated signaling pathway accelerates pollen tube emergence at the septum and ovule surface. AtLURE1s can diffuse toward the funicular surface (*15*); their ability to diffuse toward the transmitting tract remains unknown.

To verify the AtLURE1-PRK6–mediated acceleration hypothesis in planta, we conducted a pollen tube competition assay on WT and *atlure1* null pistils by using mixtures of equal amounts of WT and *LAT52pro*:*GUS* pollen grains and equal amounts of *prk6-2* and *LAT52pro*:*GUS* pollen grains. The emergence of the first pollen tube onto the septum and ovule surface is a critical step for successful fertilization (*19*), and the first pollen tube to reach the ovule surface is the one most likely to fertilize the ovule (*20*) because of barriers to polytuby (*21*). To identify pollen tubes during their emergence onto the funiculus in the same pistil, we established a new method to stain the pollen tubes in a pistil for both β-glucuronidase (GUS) (*22*) and aniline blue (*23*) (fig. S8). In WT pistils, approximately 49.2 ± 3.7% of GUS-stained *LAT52pro*:*GUS* (WT) pollen tubes were successfully targeted toward ovules (Fig. 3, E and F, and fig. S9), indicating that WT and *LAT52pro*:*GUS* (i.e., WT) pollen tubes have similar ovule-targeting efficiencies. When *prk6-2* pollen was applied together with *LAT52pro*:*GUS* (WT) pollen, more GUS-stained *LAT52pro*:*GUS* (WT) pollen tubes (~72.0 ± 4.5%) were successfully targeted to the ovules (Fig. 3, E and F, and fig. S9), indicating that WT pollen tubes outcompeted mutants defective in the AtLURE1-PRK6–mediated signaling pathway. This is confirmed in *atlure1* null mutant pistils, where the targeting efficiency of *LAT52pro*:*GUS* (WT) pollen tubes (49.1 ± 3.8%) was comparable to that of *prk6-2* mutant pollen tubes (Fig. 3, E and F, and fig. S9). These observations are consistent with genetic analyses showing that the male transmission efficiency of the *prk6-2* mutant allele was ~50% in WT pistils, 76.3% in *atlure1.1* to *-1.5* quintuple-mutant pistils, and nearly 100% in *atlure1* null septuple-mutant pistils (Fig. 3G). Altogether, these results indicated that the loss of either the receptor PRK6 or the peptide ligand AtLURE1s reduced growth competitiveness and the time point of emergence onto the septum and ovule surfaces of the corresponding pollen tubes.

AtLURE1s promote conspecific pollination

As AtLURE1s are *A. thaliana* specific (Fig. 1, C to E, and fig. S1) (*15*), we speculated whether AtLURE1s promote pollen tube emergence in a

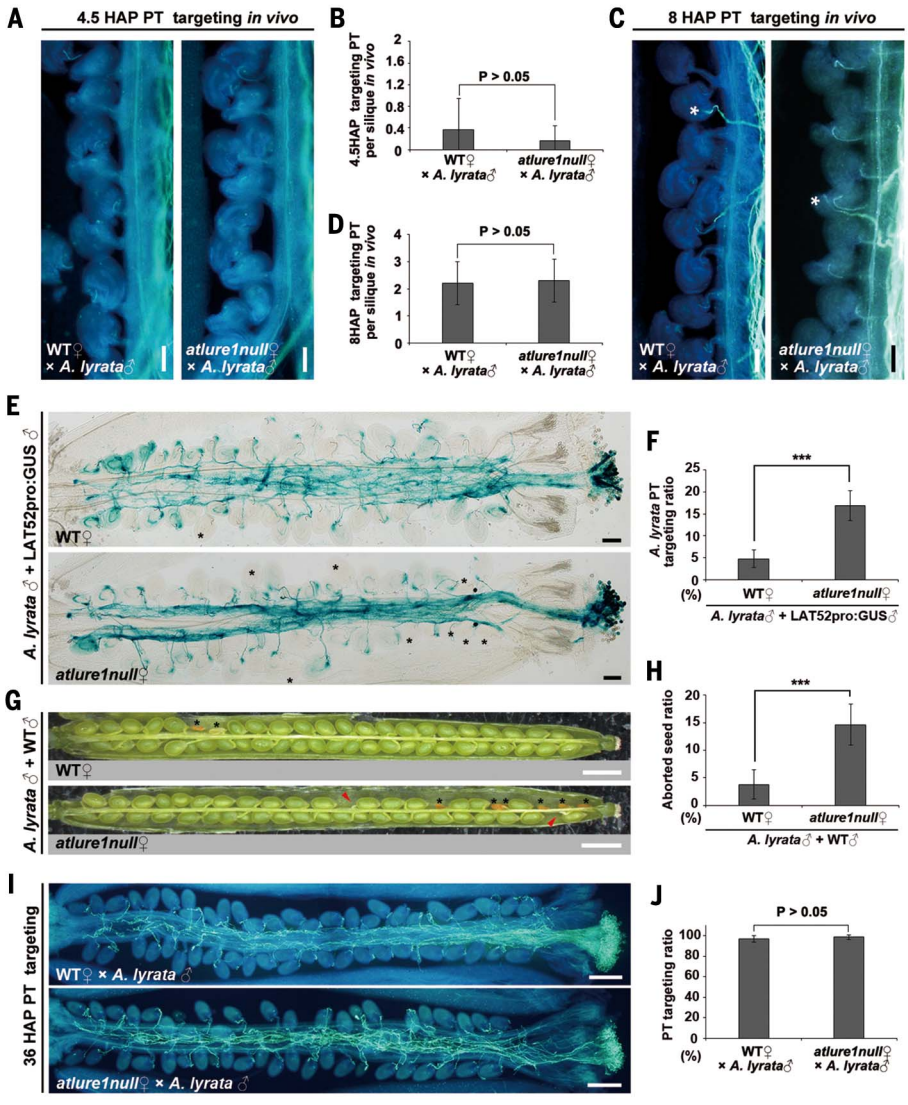

Fig. 4. Ovule targeting by pollen tubes after interspecific pollination between *A. thaliana* and *A. lyrata* is strongly delayed, resembling the *atlure1* null mutant phenotype. (**A** to **D**) Aniline blue staining and statistical analysis of the total numbers of *A. lyrata* pollen tubes (PT) emerging onto the septum and ovule in *A. thaliana* WT and *atlure1* null mutant pistils as indicated, (A and B) 4.5 HAP and (C and D) 8 HAP. Three or four repeats of the pollen tube targeting experiments were conducted for each combination, each with 7 to 11 siliques being examined. White asterisks indicate ovules targeted by pollen tubes. Data are mean values ± SD; *P* values were >0.05 (Student's *t* test). Scale bars, 50 μm. (**E**) GUS staining of *A. thaliana* WT and *atlure1* null mutant pistils pollinated with a mixture of *A. lyrata* and *LAT52pro:GUS* (*A. thaliana* WT) pollen grains at 24 HAP. Asterisks indicate ovules that were targeted by pollen tubes without GUS staining. Scale bars, 50 μm. (**F**) Statistical analysis of the percentage of ovules targeted by *A. lyrata* pollen tubes per silique shown in (E). Three repeats of the pollen tube competition experiments were conducted for each combination, each with seven or eight siliques being examined. Data are mean values ± SD. ***$P < 0.01$ (Student's *t* test). (**G**) Siliques of the WT or *atlure1* null mutant pistils pollinated with a mixture of *A. lyrata* and *A. thaliana* WT pollen grains. Asterisks indicate aborted seeds, and arrowheads indicate white unfertilized ovules, both of which are counted as aborted seeds. Scale bars, 50 μm. (**H**) Statistical analysis of aborted seeds in the siliques shown in (G). Seven to nine siliques were examined for each line for three repeats. Data are mean values ± SD. ***$P < 0.01$ (Student's *t* test). (**I**) Pollen tube targeting in vivo at 36 HAP in *A. thaliana* WT and *atlure1* null septuple-mutant pistils crossed with *A. lyrata* pollen. Scale bars, 200 μm. (**J**) Statistical analysis of the *A. lyrata* pollen tube targeting ratio shown in (I). Three repeats of the pollen tube targeting experiments were conducted for each combination, each with seven or eight siliques being examined. Data are mean values ± SD. The *P* value was >0.05 (Student's *t* test).

species-specific manner. We used *A. lyrata* pollen (*24*) to pollinate *A. thaliana* WT and *atlure1* null pistils. At 4.5 and 8 HAP, respectively, the lengths of *A. lyrata* pollen tubes in *A. thaliana* WT pistils were comparable to those in *atlure1* null pistils (fig. S10, A to D), demonstrating that AtLURE1s do not affect the *A. lyrata* pollen tube growth rate in the *A. thaliana* pistil. However, in contrast to the pollen tube emergence difference observed between self-pollinated *A. thaliana* WT and *atlure1* null mutant pistils, we observed no statistically significant difference between the *A. lyrata* pollen tube emergence ratios at 4.5 HAP in WT and *atlure1* null septuple-mutant pistils (0.37 ± 0.58 versus 0.16 ± 0.28; $P > 0.05$) (Fig. 4, A and B). Comparative *A. lyrata* pollen tube emergence ratios were also observed at 8 HAP in *A. thaliana* WT and *atlure1* null septuple-mutant pistils (2.2 ± 0.8 versus 2.3 ± 0.8; $P > 0.05$) (Fig. 4, C and D). Note that 9.2 ± 1.8 and 28.7 ± 2.7 *A. thaliana* WT pollen tubes had already emerged at 4.5 and 8 HAP in WT pistils (Fig. 3, A to D), respectively, which is 10 to 50 times as many as observed for interspecific pollination.

To compare pollen tube emergence and growth rates, we pollinated *A. thaliana* WT pistils and *atlure1* null pistils with a mixture of *A. thaliana LAT52pro:GUS* (WT) and *A. lyrata* pollen grains and examined in planta growth of the pollen tubes by using the dual-staining method. In WT pistils, *A. thaliana* pollen tubes (~95%) outcompeted *A. lyrata* pollen tubes (4.71 ± 2.0%) (Fig. 4, E and F, and fig. S11). However, a significant number of *A. lyrata* pollen tubes (~16.9 ± 3.4%; $P < 0.01$) successfully emerged, grew onto the funiculus surface, and targeted ovules in *atlure1* null pistils (Fig. 4, E and F, and fig. S11). Thus, the loss of AtLURE1s reduced the ability of *A. thaliana* pollen tubes to outcompete those of *A. lyrata*. We observed significantly more aborted seeds in mature siliques of *atlure1* null mutants (~14.7 ± 3.7%; $P < 0.01$) than in WT siliques (~3.8 ± 2.6%) (Fig. 4, G and H), which is consistent with the pollen tube competition results. In summary, these data demonstrated that AtLURE1s accelerate the emergence of pollen tubes onto the septum surface and that this effect is restricted to pollen tubes of the same species. Therefore, AtLURE1s appear to accelerate septum emergence of pollen tubes of their own plants. All *A. lyrata* pollen tubes reached the ovule both in *A. thaliana* WT and in *atlure1* null pistils by 36 HAP (Fig. 4, I and J), which suggests that *A. thaliana* pollen tube attractants can attract *A. lyrata* pollen tubes, albeit slowly, and that species-specific and non–species-specific attractants coordinately contribute to pollen tube guidance.

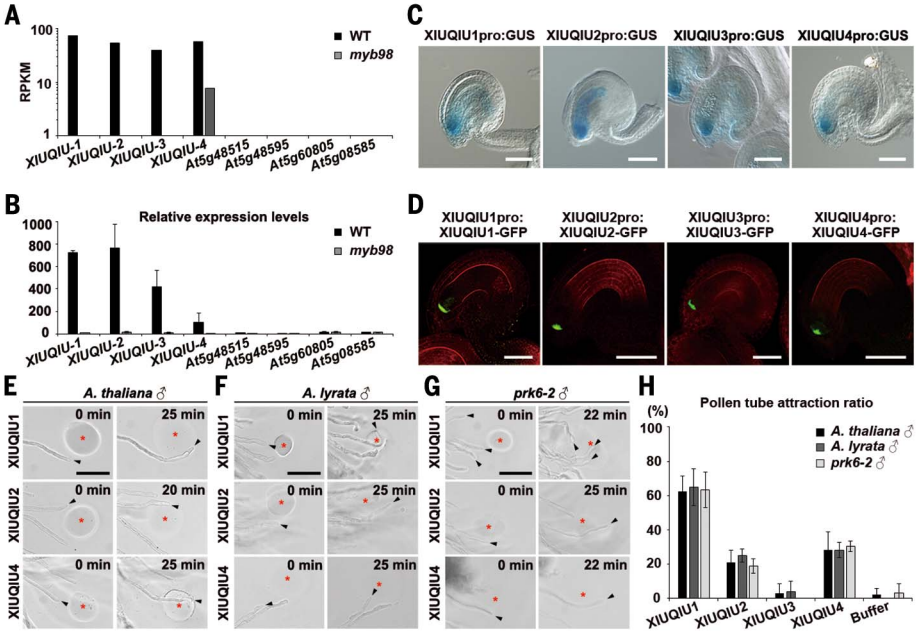

Fig. 5. XIUQIU peptides attract pollen tubes of *A. thaliana* and *A. lyrata* in similar manners and contribute to fertility. (**A**) Expression levels of *XIUQIU* genes and related genes in WT and *myb98* mature ovules. RNA-seq data are shown. RPKM, reads per kilobase per million reads. (**B**) Quantitative reverse transcription polymerase chain reaction analysis of *XIUQIU* genes and related genes in WT and *myb98* mature ovules. Three biological replicates, each with three technical repeats, were conducted for each gene in each sample. Data are mean values ± SD. (**C**) GUS signals from *XIUQIU1*, *-2*, *-3*, and *-4* promoters were predominantly concentrated in synergid cells. Scale bars, 50 μm. (**D**) Images of ovules with XIUQIU1-, -2–, -3–, and -4–GFP fusion proteins localized in the filiform apparatus of synergid cells. Scale bars, 50 μm. (**E** to **G**) Semi–in vivo pollen tube attraction assays revealed guided growth of *A. thaliana* (E), *A. lyrata* (F), and *prk6-2* (G) pollen tubes toward recombinant XIUQIU1, -2, and -4 embedded in gelatin beads (indicated by asterisks). Arrowheads indicate pollen tube tips. Scale bars, 50 μm. (**H**) Statistics of the ratio of attraction by XIUQIU1 to -4, comparing *A. thaliana*, *A. lyrata*, and *prk6-2* pollen tubes. For each assay, 8 to 24 pollen tubes were examined, and the assay was repeated three times for each condition. Data are mean values ± SD. The *P* value was >0.05 (Student's *t* test).

Cysteine-rich XIUQIU peptides are non–species-specific pollen tube attractants

The synergid cell–specific MYB transcription factor MYB98 is essential for pollen tube guidance; loss of *MYB98* function results in the failure of pollen tube entrance into the micropyle of the ovule and thus low fertility (*25*). *AtLURE1* genes depend on MYB98 activity (*26*) (see fig. S12, A and B). Our investigation showed that in the *myb98* mutant pistil, the WT pollen tube emergence ratio was reduced to 0.2 ± 0.5 at 4.5 HAP and 10.7 ± 1.9 at 8 HAP (fig. S12, C and D), similar to the ratio in the *atlure1* null septuple mutant. Therefore, we speculated that genes encoding the non–species-specific pollen tube attractants are likely downstream of MYB98. We used whole-genome RNA-sequencing (RNA-seq) analysis to identify cysteine-rich peptide–encoding genes that were down-regulated in ovules of *myb98* mutants. In addition to *AtLURE1* genes, we identified four AtLURE1-related cysteine-rich peptide genes with reduced expression and another four genes with undetectable expression (Fig. 5, A and B). The AtLURE1-related cysteine-rich peptides encoded by these genes are ~100 amino acids in length and contain six conserved cysteine residues (fig. S13). Phylogenetic analysis showed that, in contrast to AtLURE1s, each of the AtLURE1-related cysteine-rich peptides had ortholog(s) in other species of the Brassicaceae (fig. S1). These four cysteine-rich peptide genes, *At5g50423*, *At5g18403*, *At5g18407*, and *At5g48605*, were named *XIUQIU1*, *-2*, *-3*, and *-4*, respectively. XIUQIU represents a Chinese phrase naming the embroidered silk ball that was, in an ancient Chinese ceremony, traditionally thrown by a girl to attract and select her husband.

XIUQIU genes are all expressed in synergid cells (Fig. 5C). However, their encoded peptides are clearly localized to the filiform apparatus of synergid cells (Fig. 5D). All four recombinant XIUQIU peptides, expressed and purified from insect cells (fig. S14A), could attract pollen tubes of both *A. thaliana* (with pollen tube responsiveness of 62.2 ± 9.1%, 21.0 ± 7.2%, 3.0 ± 5.2%, and 28.3 ± 10.4%, respectively) (Fig. 5, E and H) and *A. lyrata* [64.8 ± 10.8%, 24.9 ± 3.7%, 3.7 ± 6.4%, and 28.2 ± 4.5% ($P > 0.05$), respectively] (Fig. 5, F and H) at similar levels in semi–in vivo pollen tube attraction assays (Fig. 5, E to G). XIUQIU1 was also able to attract pollen tubes of *A. halleri*, another member of the genus *Arabidopsis* (fig. S14, B and C). By contrast, AtLURE1s showed limited ability to attract *A. lyrata* pollen tubes (Fig. 1, D, E, and G). Pollen tubes carrying *prk6* mutations could also be attracted by XIUQIU peptides (Fig. 5, G and H), suggesting that XIUQIU peptides do not require signaling through PRK6. These data demonstrated that XIUQIU peptides equally attract pollen tubes of different *Arabidopsis* species and thus appear to function as non–species-specific attractants within the *Arabidopsis* genus.

AtLURE1-related cysteine-rich peptides contribute to fertility control

To study the biological function of these XIUQIU peptides, we used CRISPR-Cas9 to knock out the four genes *XIUQIU1* to *-4* in the *atlure1* null mutant background. We obtained two different allelic hendecuple mutants, designated *hendecuple-1* and *-2*, that generated the same defective peptides (Fig. 6A). Mutations were confirmed in both genomic DNA and transcripts (cDNA) of *hendecuple-1* (figs. S15 and S16). The truncated or mutated XIUQIUs were unable to attract pollen tubes (Fig. 6B and fig. S17). In hendecuple mutants, we also observed phenotypes of lower pollen tube emergence at 4.5 and 8 HAP (fig. S18), consistent with the observation in *atlure1* null mutants (Fig. 3, A to D). Moreover, we also observed reduced fertility and ovule abortion in siliques of hendecuple mutants (Fig. 6, C and D). The introduction of the *XIUQIU1* gene rescued the fertility defect but not the pollen tube emergence phenotype in the *hendecuple-1* mutant (Fig. 6, C and D, and fig. S18). In both hendecuple mutants, ~65 to 70% of ovule abortion is likely due to the failure of pollen tube emergence onto the funiculus and ~30 to 35% to misguided pollen tubes at the ovule surface (Fig. 6E). In conclusion, our findings suggest that the combined efforts of AtLURE1-PRK6 and XIUQIU signaling contribute to fertility and reproductive success. The observation that the loss of all AtLURE1s and XIUQIUs resulted in only ~20% reduction of fertility indicates that additional pathways guiding pollen tube attraction exist in *Arabidopsis*. Ethylene signaling may also guide pollen tube growth (*27*).

Discussion

Siphonogamy is an evolutionarily new way of fertilization for plants to transport male germ cells to female gametes in a nonaquatic environment. During this adaptation, sperm cells lost their mobility and pollen grains were developed to transport sperm cells from one plant to the other, ultimately delivering their cargo within an innovative tube to the female gametophyte for fertilization (*9*). Therefore, directional polar growth of pollen tubes mediated by extensive interactions with different female tissues, in particular the egg

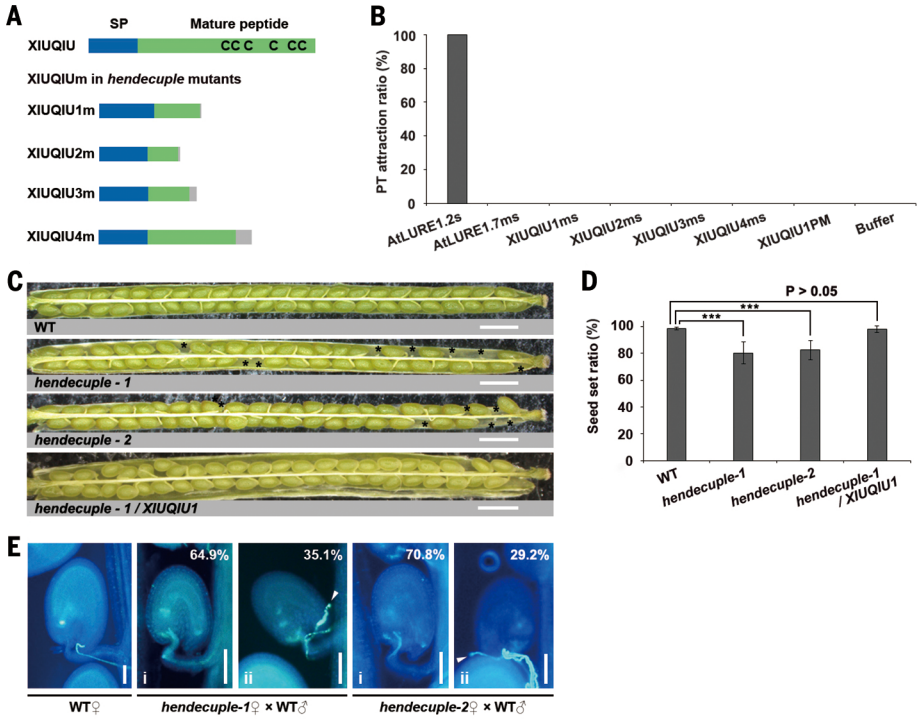

Fig. 6. Mutant generation and phenotypic analysis of hendecuple mutants. (**A**) Schematic diagram of protein structures of mutated (m) XIUQIU1 to -4 in *hendecuple-1* and *hendecuple-2* mutants. SP, signal peptide; C's, conserved cysteine residues; gray box, missense sequence due to frameshift mutation. (**B**) Statistical analysis of the pollen tube responding ratio with chemically synthesized mutated peptides (designated XIUQIUms). XIUQIU1PM represents *E. coli*–expressed mutated XIUQIU1 with its C-terminal three conserved cysteines (C^{85}, C^{90}, and C^{92}) mutated to alanines (A^{85}, A^{90}, and A^{92}, respectively) to serve as a negative control. Pollen germination medium (buffer) served as the empty control. Three repeats of the pollen tube attraction assays with 6 to 10 pollen tubes for each synthesized peptide were conducted. Six repeats of the pollen tube attraction assays with three to five pollen tubes each were conducted for positive, negative, and empty (buffer) controls. Data are mean values ± SD; *P* values were >0.05 (Student's *t* test). AtLURE1.2s, unmutated AtLURE1.2 peptide. (**C**) Dissected siliques of WT, *hendecuple-1*, *hendecuple-2*, and *XIUQIU1*-rescued *hendecuple-1* plants. Asterisks indicate the aborted ovules. Scale bars, 1 mm. (**D**) Statistical analysis of seed set in WT, *hendecuple-1*, *hendecuple-2*, and *XIUQIU1*-rescued *hendecuple-1* plants. Three repeats of silique examination were conducted, and seven to nine siliques were examined each time. Data are mean values ± SD. ***$P < 0.01$ (Student's *t* test). (**E**) Aniline blue staining showing pollen tube behavior at WT and aborted ovules in an unsuccessfully targeted *hendecuple-1* mutant at 48 HAP. The arrowhead indicates the tip of a pass-by pollen tube. Scale bars, 50 μm.

apparatus (egg and synergid cells), is essential for fertilization and reproductive success (*11*, *28*–*31*). The finding that the genes involved in the regulation of these interactions are often species or genus specific implies that plants have evolved a number of prezygotic hybridization barriers to promote species isolation. The processes affected include pollen tube germination at the stigma, as well as polar and guided growth inside the pistil, which are mediated mainly through species-specific peptide signals. Conserved non–cysteine-rich peptides regulate many processes in vegetative plant development, including the differentiation of stem cells (*32*, *33*), vascular cell formation (*34*, *35*), root development (*36*), and stomata production (*37*), whereas polymorphic cysteine-rich peptide signaling was especially found in plant responses to pathogens (*38*–*40*) and during reproduction (*11*, *41*). In the past two decades, cysteine-rich peptide– and receptor-like kinase–mediated signaling was reported to control compatible male-female communication, as indicated, for instance, by the SCR/SP11 peptides regulating self-incompatibility determination in the Brassicaceae (*42*), RALF4/19/34 for pollen tube integrity and rupture in *A. thaliana* (*43*), LUREs for pollen tube guidance in *T. fournieri* and *A. thaliana* (*13*, *15*), ES1 to -4 for pollen tube burst in maize (*44*), and EC1 for sperm cell activation in *A. thaliana* (*45*). Moreover, in this study we elucidated a new role of the *A. thaliana* cysteine-rich LURE1 peptides as conspecific pollen tube precedence factors, and the AtLURE1-related XIUQIUs were identified as a conserved class of Brassicaceae-specific peptides capable of attracting pollen tubes of different *Arabidopsis* species.

The role of AtLURE1s in reproductive isolation occurs at the septum emergence point in the pistil, which represents a critical step in the long journey of the pollen tube, as pollen tube emergence from the transmitting tract toward the funiculus surface usually results in successful sperm delivery. Almost without exception, only a single pollen tube—although there are always excessive numbers of pollen tubes in the style—outcompetes other tubes to successfully emerge from the transmitting tract and enter the micropyle of the ovule for fertilization (*20*). Therefore, by contrast to micropylar pollen tube guidance, pollen tube emergence ultimately determines the identity of the pollen tube that delivers its sperm cell cargo for fertilization. The observation that polytubey (the arrival of multiple pollen tubes at the ovule surface) is very rare (*20*) indicates the existence of emergence refusal molecules for future studies, which may interfere with AtLURE1 and/or XIUQIU signaling.

The working distance of the AtLURE1-XIUQIU attraction signaling in vivo is also not yet clear. The semi–in vivo pollen tube attraction activity of AtLURE1 peptides was effective within a range of ~20 μm (*16*). However, the center of the transmitting tract is ~100 μm away from the micropylar opening of the ovule. Therefore, the above-described cysteine-rich peptides, after being secreted from the synergid cells, will most likely diffuse over some distance to the septum to execute their major function. Although a few general small molecules have been reported as ovule-derived long-distance attraction signals, such as NO or carbohydrates such as AMOR (ovular methyl-glucuronosyl arabinogalactan) (*46*, *47*), their general effects on cell functions exclude their possible contribution to a highly regulated process, for example, how the plant's own pollen tubes are promoted and alien ones are outcompeted. We therefore hypothesize that polymorphic AtLURE1 and XIUQIU peptides diffuse to reach the transmitting tract, so that reproduction isolation can be precisely controlled by species-specific acceleration of pollen tube emergence.

In summary, we have shown here that the recently evolved *A. thaliana*–specific AtLURE1s accelerate the growth of conspecific pollen tubes from the transmitting tract toward the septum and ovule, representing a critical step during the pollen tube journey. The speed of pollen tube emergence at the septum surface between conspecific and heterospecific pollen tubes thus establishes a genetic isolation barrier between *A. thaliana* and closely related species. We identified the evolutionarily ancient AtLURE1-related cysteine-rich XIUQIU peptides as non–species-specific pollen tube attractants. XIUQIUs contribute to reproductive success but likely also support heterospecific fertilization. Our study identified molecular signaling mechanisms underlying intra- and interspecies reproductive isolation barriers in plants. Disabling conspecific pollen tube accelerators

in crops may facilitate interspecific fertilization in future plant breeding efforts to increase the gene pool by adding desired agronomic traits to crop plants.

Materials and methods summary

Plant growth conditions

All plants were grown in the greenhouse with LED lights (GPL production modules DR/W and DR/B/FR, Philips) under long-day conditions (16 hours light/8 hours dark) at 22° ± 2°C.

Phylogenetic analysis

The genomic sequences of seven species, including *A. thaliana*, *A. lyrata* (*24*), *Capsella rubella* (*48*), *Capsella grandiflora* (*48*), *A. halleri* (*49*), *Boechera stricta*, and *Brassica rapa* (*50*), downloaded from Phytozome (*51*), were used (table S1). A gene family calculation study was conducted as described (*52*). The neighbor-joining trees were constructed by using MEGA6 with 1000 replicates of bootstrap (*53*).

Generation of high-order mutants

We used a YAO-based CRISPR-Cas9 system (*54*) to obtain *AtLURE1.1* to *-1.5* quintuple mutants, followed by the egg cell–targeting CRISPR-Cas9 system (*43*, *55*, *56*) to knock out *AtLURE1.7* and *-1.8* and *AtLURE1.7* and *-1.8* and *XIUQIU1* to *-4* to generate *atlure1* null septuple and *atlure1* null *xiuqiu1* to *-4* hendecuple mutants, respectively.

To generate *atlure1* null septuple mutants, the dual spacer was amplified from pCBC-DT1T2 and cloned into binary vector pHEE401E (*55*–*57*), generating pHEE401E-AtLURE1.7-1.8, which was then transformed into Cas9-free *atlure1.1* to *-1.5* quintuple mutants. To generate hendecuple mutations in seven *AtLURE1* and four *XIUQIU* genes, three dual spacers were amplified from pCBC-DT1T2 and first cloned into pENTR-MSR (*43*) and then into pHEE401E (*55*–*57*).

In vivo and semi–in vivo pollen tube assays

In general, flowers at floral stage 12 were emasculated, followed by pollination in 36 hours. For quantitative pollination, pollen grains were manually collected by a homemade eyelash pen and deposited onto the stigma. Typically, the pollinated pistils were collected at 4.5 and 8 HAP for phenotypic examination. Three biological repeats were performed for each group. For dual staining with GUS and aniline blue, the pistils were first treated for GUS staining as described (*22*), followed by soaking in 8 M NaOH overnight before aniline blue staining (*23*).

To perform the pollen tube competition assay, comparable numbers of pollen grains from different combinations of different species and/or genotypes were applied to the same emasculated stigma and scored for their pollination efficiency. Pollinated pistils were collected at 24 HAP for phenotypic analysis.

To conduct the semi–in vivo pollen tube attraction assay, styles of the emasculated WT pistils were cut and placed on the pollen germination medium in a culture dish with a cover glass in the bottom center. The culture dish was placed into a 22°C incubator for pollen germination. The insect cell– or *E. coli*–expressed and purified peptides (50 μM) were tested for pollen tube attraction as described (*58*).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S18
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Science

Cysteine-rich peptides promote interspecific genetic isolation in *Arabidopsis*

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Racing to fertilization

Pollen tubes, which carry plant sperm, need to grow from where they land in the flower to where the ovule is. Zhong *et al.* now show how pollen from related plant species race to reach the ovule first. One set of fast-evolving peptide signals is tuned to speed up growth of conspecific pollen tubes. A related set of evolutionarily ancient peptides is tuned to attract all pollen tubes. Thus, fertilization is more likely to happen through conspecific pollen tubes, but a fail-safe system encourages even the laggards to get where they need to go.

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