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A non-defensin peptide NPA1 attracts pollen tube in *Arabidopsis*

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Abstract

In angiosperms, female gametophyte secretes a range of attractants to entice the pollen tube for fertilization. In dicots, all the identified attractants are defensin-like cysteine-rich peptides (CRPs) family members, while monocots, like *Zea mays* in Gramineae utilize non-CRP-type Egg Apparatus 1-like peptides as pollen tube attractants. However, whether dicots have non-CRP attractants is still unclear. Here we characterize a non-defensin peptide attractant NON-DEFENSIN PEPTIDE ATTRACTANT 1 (NPA1) in *Arabidopsis*. *NPA1* is transcriptionally regulated by MYB98 in synergids. Besides the conspecific pollen tube, *At*NPA1 is also capable to attract pollen tubes of sister species *A. lyrata* and *C. rubella,* but not *E. salsugineum*. Furthermore, when NPA1 is introduced to complement *myb98*, it restores pollen tube attraction and fertility to a level comparable to the complementation with LUREs. Together, this study identifies a new type of peptide attractant in dicots and highlights the diversity of the attraction cues and signaling pathways.

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Introduction

The evolutionary journey of plants form ocean to terrestrial environment is accompanied with the shift of sexual reproduction manner from zoidogamy to siphonogamy. Angiosperm sperm have lost their mobility and are delivered to the female gametes through pollen tubes. When pollen grains are deposited on the stigma of flower, a tip-growing pollen tube forms and elongates into the style and then in the transmitting tract, which marks the initiation of pollen tube guidance[1]. Thereafter, in the extracellular matrix of transmitting tract, pollen tubes continuously perceive and respond to the guiding 'signals' from the female gametophytes to grow towards the ovule, which process is called pollen tube guidance. This polarized pollen tube growth, culminating in arriving at the receptive synergid and subsequent releasing two sperm cells. Over the last two decades, these guiding 'signals' are uncovered to be CRP-type peptides in dicots, including LUREs, XIUQIUs, TICKETs, as well as SALVAGERs[2–6]. Meanwhile, synergids and the central cell are validated to be the primary sources of these pollen tube attractants[7, 8].

CRPs are overrepresented in the plant peptide superfamily and play important roles in plant development, defense, symbiosis, and reproduction[4, 9, 10]. The hallmark of CRPs are the cysteines in their sequences, and intramolecular disulfide bonds between cysteine residues determines the three-dimensional structure of CRPs[4, 9]. CRPs are subdivided into subgroups with specific conserved cysteine-distribution patterns, including defensins, thionins, RALFs, and lipid-transfer proteins (LTPs), among others[11]. Moreover, CRPs among different subgroups or different species are highly polymorphic in sequences[11]. The reported defensin-like attractants in dicot plants are characterized with six cysteines that stabilize the CS$\alpha\beta$ and γ-core motifs by disulfide bonds, including LUREs in *Torenia fournieri* and LURE1s, XIUQIUs, TICKETs, SALVAGERs in *Arabidopsis* and other relatives[3, 5, 6, 8, 9]. *At*LURE1s are species-preferential attractants, which promote emergence of conspecific pollen tube from transmitting tract, while XIUQIUs, as AtLURE1-related CRPs entice pollen tube with no species preference[3]. By contrast, the LURE1-related TICKETs attract solely the conspecific pollen tubes[5]. *At*LURE1s, *At*XIUQIUs and *At*TICKETs belong to CRP_810 group and are transcriptionally regulated by transcription factor MYB98, the master regulator in synergids[12, 13]. The recently identified SALVAGER1 and SALVAGER2 are central cell-derived CRP-type attractants, which belong to the CRP_0300 group and CRP_0220 group, respectively, and mediate fertilization recovery in the case of fertilization failure with two synergid cells consumed[8]. However, a hendecuple mutants with lesions in all four *XIUQIUs* and seven *AtLURE1s* exhibited only a slight compromise in pollen tube guidance and fertility, suggesting the existence of other unknown attractants[3]. Unlike the case in dicot, the monocots utilize non-CRP-type pollen tube attractants[14, 15]. In *Zea mays*, the synergids and egg cell secrete Egg Apparatus1 (EA1) peptide to attract pollen tube into the embryo sac. Moreover, transgenic down-regulation of *EA1* induces severe ovule sterility [14, 15]. It appears that dicots and monocots employ different types of peptide attractants. Intriguingly, mammals apply diverse chemoattractants in guiding the sperm to the egg cell[16]. It is tempting to speculate that there are other types of attractant evolved in different angiosperm lineages.

To date, no non-defensin peptide attractant has been identified in *Arabidopsis*. In this study, a non-defensin peptide NPA1 is characterized to be a new pollen tube attractant in *Arabidopsis*. *NPA1* is expressed in the synergid cells and downstream of MYB98. Expression of *NPA1* in synergids increases the pollen tube attraction efficiency of *myb98* mutant ovules. Taken together, this study identifies a new type of peptide attractant, reveals the diversity of attractants in plants and opens the revenue for the further study of the signaling pathway.

Materials and methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* were sterilized and plated onto Murashige and Skoog media supplemented with 40 mg/L hygromycin for transgenic lines. The plants were grown at 22 °C with 16 h of light and 8 h of darkness. *Arabidopsis thaliana* Columbia-0 (Col-0) was used as the wild-type (WT) control. Mutant seeds of *myb98* (*SALK_020263*) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). *npa1 npa2* mutant were generated by CRISPR/Cas9 technology in Col-0 background.

RT-PCR

Total RNA was extracted from mature ovule of *myb98* and Col-0 using RNeasy Plant Mini Kit (QIAGEN), and reversely transcribed to cDNA after the contaminated genome DNA was removed. *ACTIN* was used as the internal control for quantitative normalization. Thirty PCR cycles were used for amplification. The specificity of the primers was confirmed by DNA sequencing of the products after electrophoresis. For primer information see Supplemental Table S1.

Vector construction and plant transformation

For the GFP reporter expression, genomic sequences containing 2-kb native promoters and genomic coding sequences for *NPA1* were fused to the GFP sequence to generate *pCAMBIA 1300-proNPA1:NPA1-GFP-TerNOS* and *pCAMBIA1300-proNPA2: NPA2-GFP-TerNOS*. For complementation assay, two *MYB98* promoters and *NOS* terminator were inserted into *pCAMBIA1300*, respectively. The genomic sequences of *NPA1/2*, *LURE1.2*, and *LURE1.5* containing the 3'-terminators were inserted after the first *MYB98* promoter, respectively. GFP sequence was inserted at the 3' end of the second *MYB98* promoter to generate *pCAMBIA1300-proMYB98:NPA1-ter-proMYB98:GFP-TerNOS*. For null mutant of *npa1 npa2* constructed by CRISPR/Cas9 technology, dual spacers were amplified from *pCBC-DT1T2* and cloned into vector *pHEE401E*[17]. For primer information see Supplemental Table S1.

Phenotypic analysis

For seed set scoring, the silique of 10 d after pollination (DAP) were observed and recorded with a CCD camera (Leica, DFC450). For *in vivo* pollen tube growth and aniline blue staining, flowers at 12c stage were emasculated and left to grow for 12–24 h to achieve pistil maturation. Then, the pistils with mature stigmas were pollinated with wild-type pollen grains. After 3, 5, 8, 12, 24, 36, and 48 h, respectively, the pistils were excised and fixed in Carnoy's fixative (75% ethanol and 25% acetic acid) for at least 4 h. The pistils were washed three times with 50 mM PBS buffer ($NaHPO_4/NaH_2PO_4$, pH 7.0) and immersed in 1 M NaOH for 12 h. Then after three washes with PBS, the pistils were stained with 0.1% aniline blue (pH 8.0 in 0.1 M K_3PO_4) for 4 h. The stained pistils were observed with a fluorescence microscope (ZEISS, Axioskop2)[18–20].

Protein purification

The coding sequence of *NPA*s and *LURE1.2* lacking the putative N-terminal signal peptides (21, 20 and 19 amino acids, respectively) were inserted to the *pET28a* vector in fusion with the N-terminal His tag, and then transformed into *Escherichia coli* strain BL21 (DE3). Cells were grown to A_{600} = 0.6 at 37 °C and then induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 16 h at 20 °C. Then the peptides were purified from the *E. coli* lysate as described previously[21].

Semi-*in vitro* pollen germination and guidance assay

For semi-*in vitro* pollen germination, the wild-type pollen grains were pollinated on the emasculated Col-0 stigma for 20 min. Then, the stigmas were cut and put on the germination media (1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.01% H_3BO_4, 18% sucrose and 1% agarose) and cultured for 4 h at 28 °C in dark. For attraction activity assay, gelatin beads containing different peptides were prepared and placed beside the pollen tube tip using a micro-manipulator as previously reported[5]. The attraction of pollen tubes was monitored and recorded with a CCD camera (ZEISS, Axiocam 506 mono).

Immunofluorescence

For pollen tube binding assay, the wild type pollen grains were germinated in semi-*in vitro* conditions. The diluted His-tag fused peptides of NPA1, NPA2, LURE1.2 or TBS buffer were dropped on the pollen tubes for 2 or 5 min. Then, the samples were immediately fixed in 4% paraformaldehyde (50 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5% Sucrose) for 30 min, and then transferred to 3% bovine serum albumin (BSA) buffer for 2 h. Subsequently, the samples were incubated with the primary monoclonal anti-His antibody (1:500, Cwbio, CW0286) in PBS buffer containing 3% bovine serum albumin (BSA), overnight at 4 °C. Then, the samples were washed three times with PBS buffer before incubation with FITC-conjugated secondary antibodies (1:100, Abmart, M212308M) in 3% BSA buffer for 2 h in darkness at 4 °C. After washing at least three times, the fluorescence signal was observed using a confocal microscope (excitation/emission wavelength: 488 nm/505–530 nm).

Microscopy

For localization assay of NPA1 and NPA2, Zeiss 780 two-photon laser scanning confocal microscope and Zeiss 980 laser scanning confocal microscope (Carl Zeiss) were used to acquire the images with the excitation/emission of 488 nm/505–530 nm.

Bioinformatics and phylogenetic analysis

The dataset of RNA-seq was obtained from NCBI: SRP160651, NCBI: PRJNA527221 and GSE8392[3, 22, 23]. Phylogenetic tree was drawn with MEGA7 using the neighbor-joining method with bootstrapping of 1,000 replicates. The sequence alignment was performed using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2). Signal peptides were predicted by SignaP-4.1 Server (https://services.healthtech.dtu.dk/service.php?SignalP-4.1). Guide RNA design for gene editing by CRISPR/Cas9 gene editing was performed on CRISPOR (https://crispor.tefor.net/). The three-dimensional structure of NPA1 and NPA2 was predicted by AlphaFold Protein Structure Database (www.alphafold.ebi.ac.uk).

Results

NPA1 and *NPA2* are non-defensin peptides downstream of MYB98 in synergids

To investigate the existence of non-defensin peptides acting as pollen tube attractants in *Arabidopsis*, we searched for the candidate peptides in the reported transcriptome data[11, 22, 23]. Three gene clusters from published transcriptome datasets were selected to identify the synergid-derived non-CRPs, including the down-regulated genes in *myb98* mutant ovules, the synergids-expressed genes, and genes of non-CRPs expressed in the wild type *Arabidopsis*[11, 22, 23]. As a result, six genes were identified to be shared by these three gene groups (Fig. 1a). Among them, one gene candidate (*AT4G02655*) exhibits high expression level in the ovule and encodes a peptide with a signal peptide. *AT4G02655* was then named *NON-DEFENSIN PEPTIDE ATTRACTANT 1* (*NPA1*) based on motif analysis of protein sequences (Fig. 2). After screening, *AT1G18486* is the sole homolog of *NPA1* in *Arabidopsis* genome and was termed *NPA2* (*AT1G18486*). The mRNA of *NPA1* and *NPA2* were only detected in the pistil, instead in the root, leaf, or pollen by RT-PCR (Fig. 1b). These results are consistent with the transcriptome data, which showed *NPA1* and *NPA2* are expressed in ovules and early seeds[24]. Additionally, RT-PCR also validated the transcriptome data that transcription of *NPA1* and *NPA2* are hardly detectable in *myb98* ovules in comparison to the wild type (Fig. 1c, d). To confirm the expression pattern of *NPA1* and *NPA2*, plants expressing the genomic *NPA1* or *NPA2* fused with green fluorescent protein (GFP) under their native promoter were obtained. The fluorescent signal of NPA1-GFP and NPA2-GFP were initially detected in the synergids of immature ovules at FG5 stage after cellularization, and then dispersed in the filiform apparatus and the plasma membrane invagination facing the micropyle at synergid maturation stage (Fig. 1e). Moreover, the TAAC element was found to be present in the promoter of NPA1 at −925, −258, and −121 sites, and of NPA2 at −169, −56 and −34 sites, which has been reported to be the binding site of MYB98[13] (Fig. 1f).

Homologs of *NPA* were present in many close relatives of *Arabidopsis thaliana* in Brassicacea, such as *Arabidopsis lyrata*,

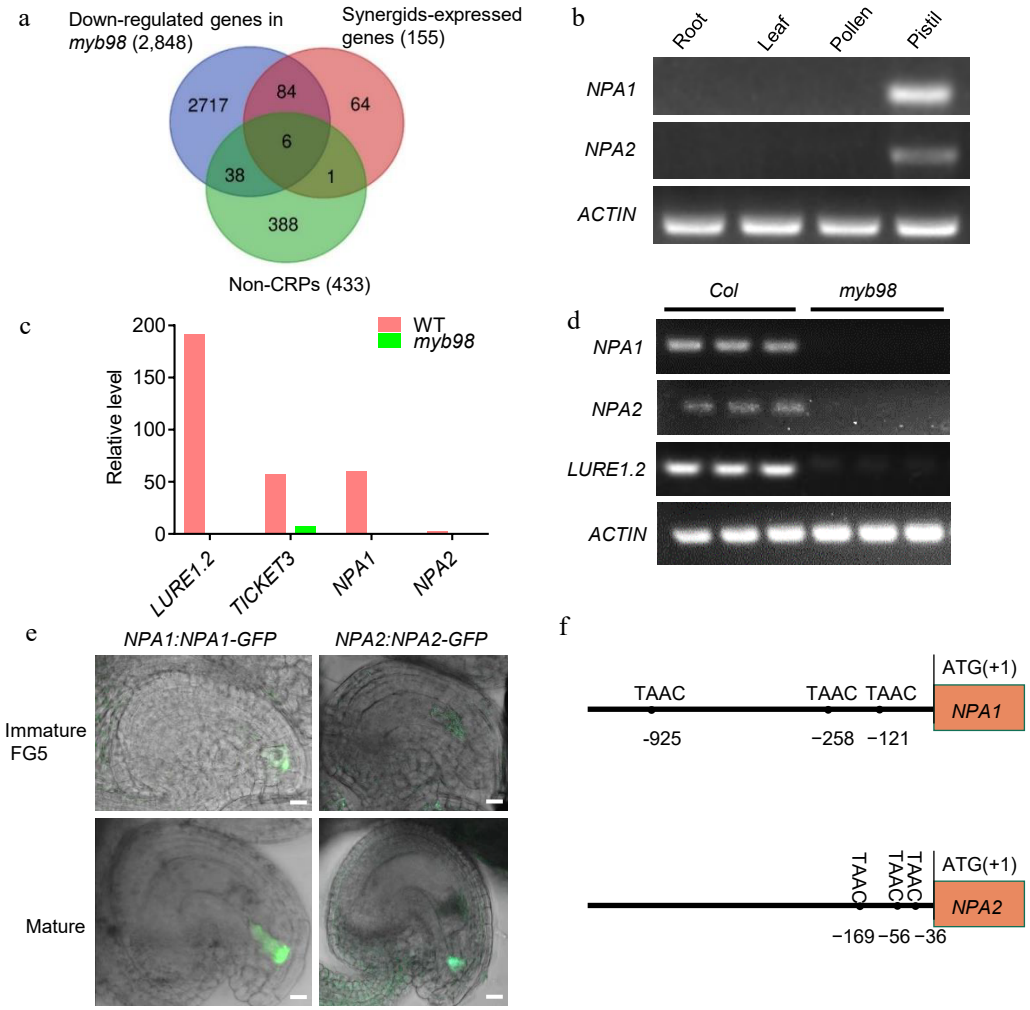

Fig. 1 (a) Venn diagram of the down-regulated genes in *myb98*, synergids-expressed genes, and non-CRPs. (b) RT-PCR showing the expression patterns of *NPA1* and *NPA2* in different tissues of *Arabidopsis thaliana*. *ACTIN*, internal control. (c) Relative level of transcripts of *LURE1.2*, *TICKET3*, *NPA1* and *NPA2* from the reported transcriptome data. (d) RT-PCR showing the mRNA level of *LURE1.2*, *ACTIN*, *NPA1* and *NPA2* in the WT and *myb98* mature ovules. (e) Expression of *NPA1* and *NPA2* in the synergids of immature and mature ovules. (f) The sites of TAAC element in the promoters of *NPA1* and *NPA2*.

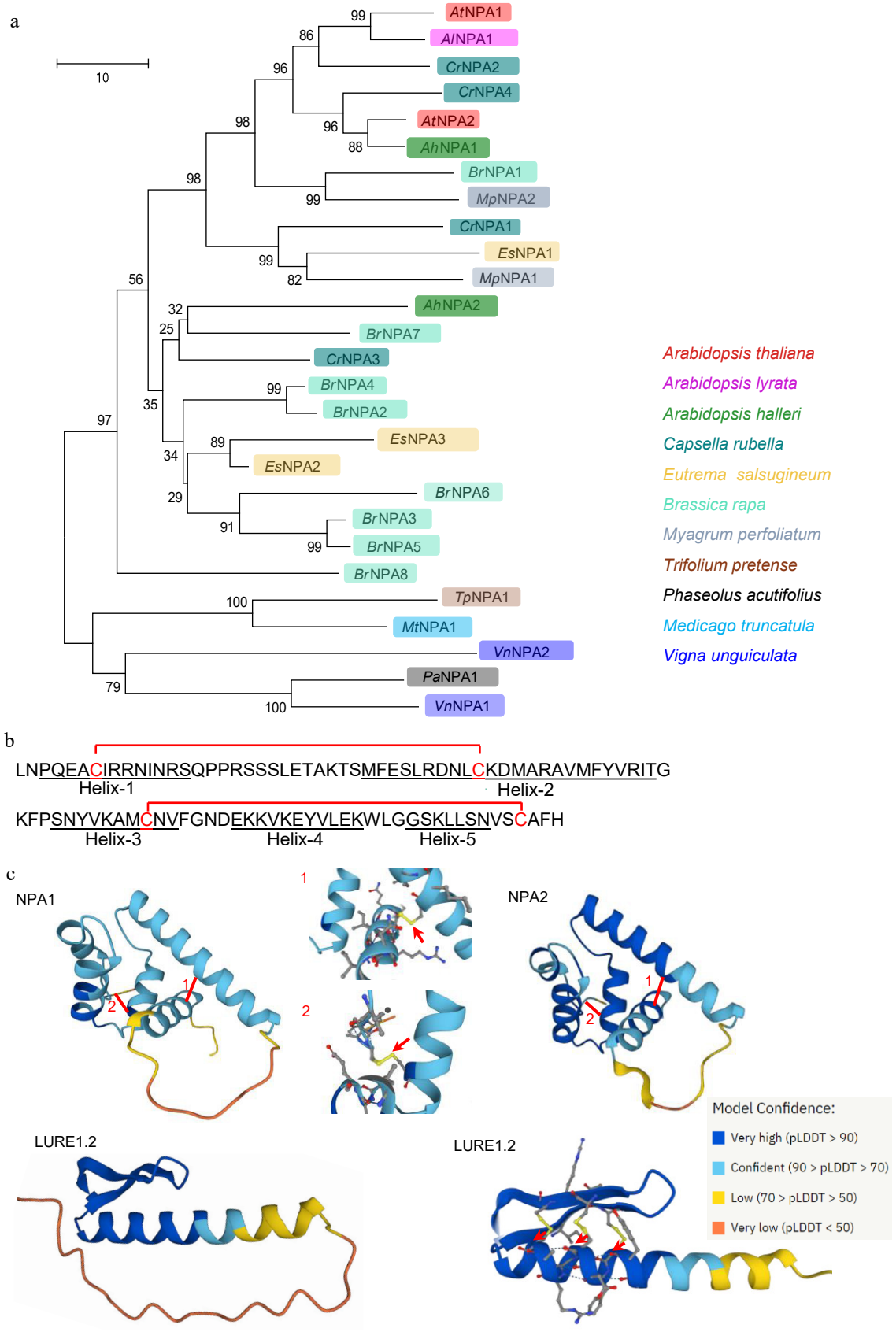

Fig. 2 The phylogeny and protein structure of NPAs. (a) Phylogenetic tree constructed with the protein sequences of NPA1 and NPA2 and their homologs. (b) Mature peptide sequence of NPA1 with five predicted helices and two disulfide bonds. (c) The structural modeling of NPA1, NPA2 and LURE1.2 by AlfaFold. Arrows indicate disulfide bonds.

Arabidpsis helleri, *Capsella rubella*, *Eutrema salsugineum*, *Brassica rapa*, as well as some Leguminosae species (Fig. 2a). NPA1 (121 aa in full length) and NPA2 (118 aa in full length) were previously classified as non-CRPs[11, 25], although NPA1 and NPA2 indeed contain four cysteines that are conserved among Brassicacea species (Supplemental Fig. S1). As the classification of CRPs is based on a range of artificial criteria, such as the number of cysteine residues and sequence conservation[11, 25], NPAs were safely classified as non-defensin peptides in this study. Structural modeling using the AlphaFold program showed that both NPA1 and NPA2 possess five α-helixes. These helixes are connected by two disulfide bonds, which is distinct from the CRP_810 peptides that form an α-helix and a two-strand anti-parallel β-sheet connected by three disulfide bonds

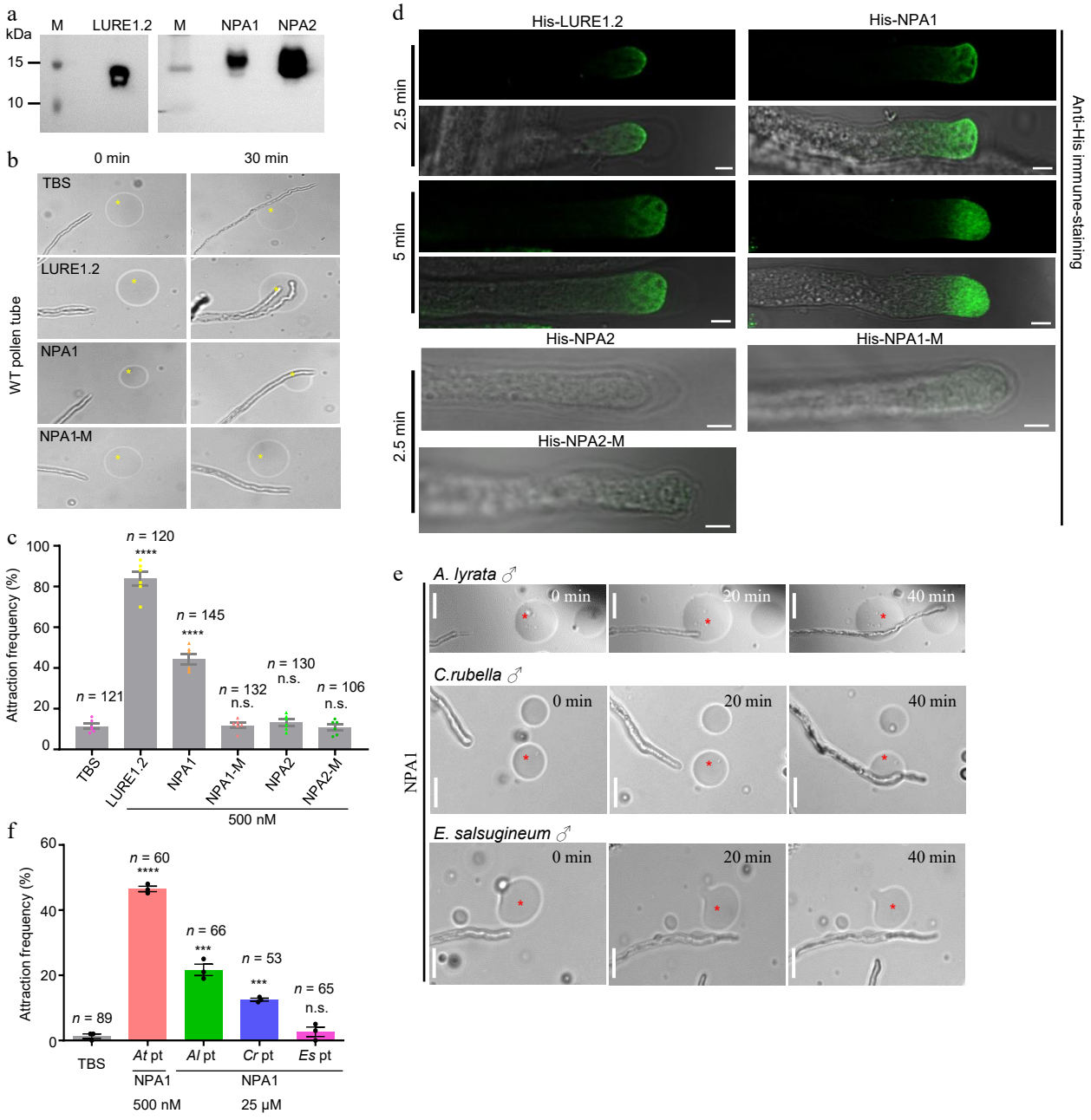

Fig. 3 NPA1 has pollen tube attraction activity. (a) Western blot of the purified His-tagged LURE1.2, NPA1 and NPA2 stained by His antibody. (b) Pollen tube attraction assay with gelatin beads containing the purified peptides, bar = 10 μm. (c) Statistics of (b), n = 121, 120, 145, 132, 130, 106 for each sample. The concentration for each assay is 500 nM. Two-tailed Student's *t*-test, **** $p < 0.0001$. Three independent biological experiments were repeated. (d) His-NPA1 binds to the pollen tube tip membrane. The LURE1.2 and NPA1 were incubated with the WT pollen tubes for 2.5 and 5 min, respectively. Endocytosis was observed at 5 min, n = 370 and 96 for each sample. NPA2, NPA1-M and NPA2-M were incubated with the WT pollen tubes for 2.5 min, n = 23, 19, 35, Bar = 5 μm. Three independent biological experiments were repeated. (e) Pollen tube attraction assay with gelatin beads containing the purified peptides. Pollen tubes of *A. lyrata*, *C. rubella* and *E. salsugineum* were used, bar = 10 μm. (f) Statistics of (e), n = 89, 60, 66, 53 and 65 for each sample. Two-tailed Student's *t*-test, **** $p < 0.0001$, *** $p < 0.001$. All data shown are the mean ± s.e.m. Three independent biological experiments were repeated.

(Fig. 2b, c)[6]. These results suggest that NPA1 and NPA2 belong to a new non-defensin-type peptide family that are specifically expressed in the synergids under the control of MYB98.

NPA1 exhibits pollen tube attraction activity

To analyze whether NPA1 and NPA2 are capable to attract pollen tubes, mature NPA1 and NPA2 peptides, and AtLURE1.2 were synthesized using prokaryotic expression system, whereby the gene sequences of *NPA1*, *NPA2*, and *AtLURE1.2* without signal peptide were fused with the His-tag and expressed in *E. coli*, respectively (Supplemental Fig. S1). Western blot verified the successful purification of NPA1, NPA2, and LURE1.2 peptides with the desired protein sizes (Fig. 3a). Semi-*in vitro* pollen tube guidance assay was then performed with gelatin beads containing the purified peptides. The results showed that wild-type pollen tube can be attracted by LURE1.2 and NPA1, but not by NPA2 and negative control (Fig. 3b, c). Of note, the attraction activity of NPA1 appears weaker than LURE1.2 (Fig. 3c). Moreover, mutation versions of NPAs (NPA1-M and NPA2-M) with substitution of four conserved cysteines by alanine were also examined in the semi-*in vitro* pollen tube guidance assay. The results showed that pollen tube attraction ability is fully abolished for NPA1-M (Fig. 3b, c), suggesting the importance of these four cysteines for NPA1 in attracting pollen tube. To confirm whether NPA1 binds to the pollen tubes, we performed peptide binding assay by immunostaining with the

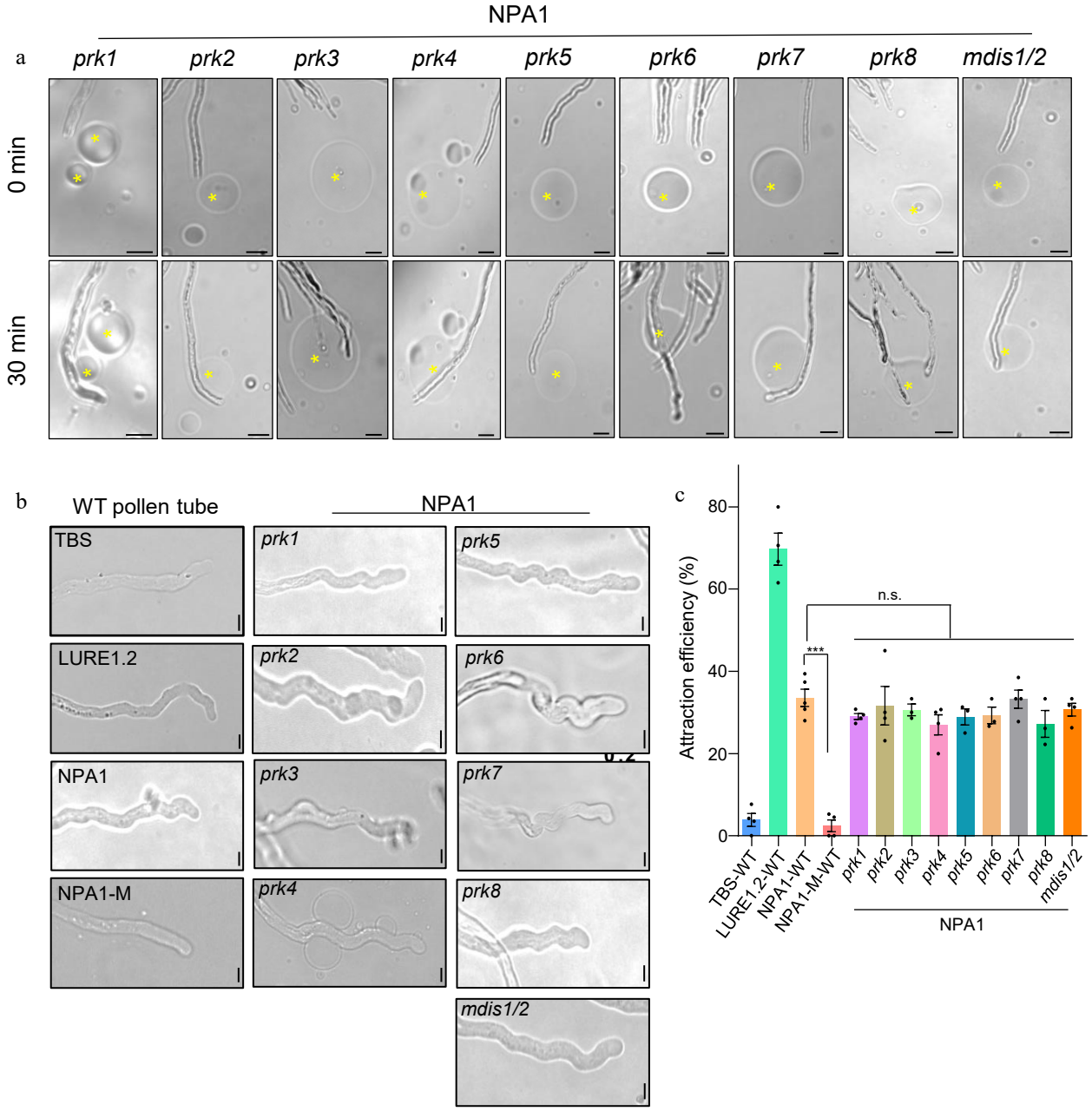

Fig. 4 NPA1 attracts the mutant pollen tubes of PRK family members and *mdis1/2*. (a) NPA1 attracted pollen tubes of different mutants, bar = 20 μm. (b) NPA1 induced wavy growth patterns of pollen tubes. The concentration of LURE1.2, NPA1 and NPA1-M was 500 nM, bar = 5 μm. (c) Statistics of (a), n = 82, 64, 145, 70, 55, 62, 48, 54, 51, 45, 55, 53, 69. Two-tailed Student's *t*-test, *** $p < 0.001$, n.s., no significance. The concentration of LURE1.2 was 500 nM, NPA1 and NPA1-M 500 nM. All data shown are the mean ± s.e.m.

purified His-tagged peptides. Once applied to the pollen tube, His-NPA1, like LURE1.2 bound to the plasma membrane of wild type pollen tube tips in 2.5 min and was further internalized by endocytosis in 5 min (Fig. 3d). In contrast, His-NPA2 and His-NPA1-M did not bind the pollen tube plasma membrane (Fig. 3d). These results suggest that NPA1, but not NPA2 is capable of attracting pollen tubes through directly binding to the pollen tube plasma membrane, in which process the four conserved cysteine residues are pivotal.

To investigate the role of NPA1 in reproductive isolation among the close relatives in Brassicaceae, we examined the attraction activity of NPA1 to pollen tubes of different species. As the pollen tubes of *A. lyrata* and *C. rubella* germinate and grow well within the transmitting tract of *A. thaliana* pistil[5], the pollen grains of *A. lyrata*, *C. rubella*, and *E. salsugineum* were pollinated to the pistil of *A. thaliana* in semi-*in vitro* pollen tube guidance assay. NPA1 attracted the pollen tubes of both *A. lyrata* and *C. rubella* with a relative lower efficiency than that of *A. thaliana* but showed no attraction to *E. salsugineum* pollen tubes (Fig. 3e, f). This result suggests that NPA1 is a species-preferential attractant, preferring conspecific pollen tube over pollen tubes of other species.

To investigate whether NPA1 is perceived by the known receptors on pollen tube plasma membrane, we performed pollen tube attraction assay with the mutants of LURE receptors and their homologous genes. The result showed that the mutants of the *Pollen Receptor Kinase* (*PRK*) family members, MALE DISCOVERER 1 (MDIS1) and MDIS2 exhibited normal attraction response to NPA1 (Fig. 4a, c). In addition, NPA1 induced wavy growth pattern of the WT and these mutant pollen tubes, like the effect of LURE1.2, while NPA1-M did not (Fig. 4b). These results suggest that NPA1 acts through a new signaling pathway.

Expression of *NPA1* in the synergids alleviates the phenotype of *myb98* mutant

To investigate the biological function of *NPA1* and *NPA2 in vivo*, three independent *npa1 npa2* null mutants (*npa1 npa2-1*,

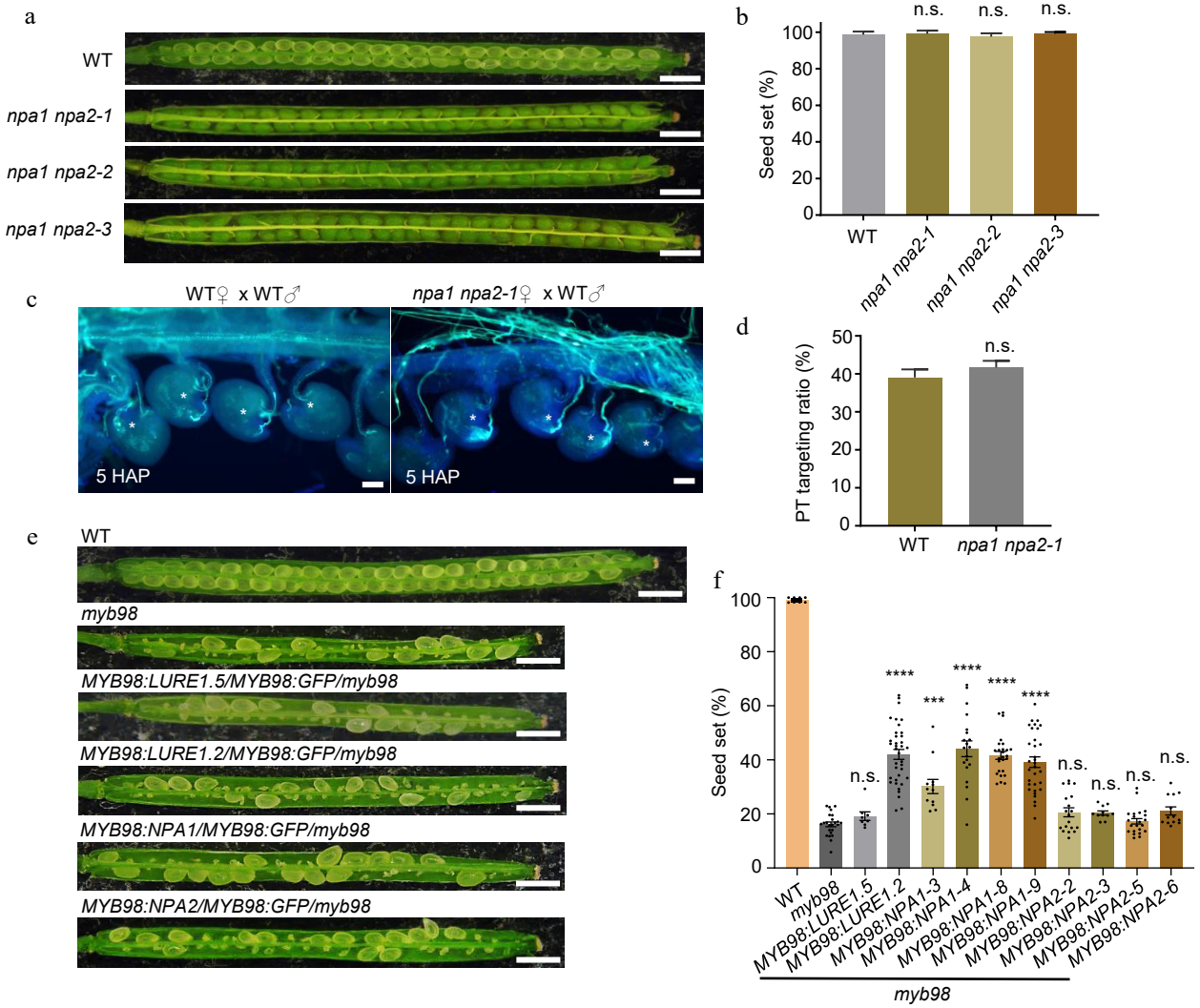

Fig. 5 The function of NPA1 in seed production. (a) *npa1 npa2* null mutant shows full seed set, bar = 1 mm. (b) Statistics of (a), n = 1,190, 865, 907, 905 for each sample. Two-tailed Student's *t*-test. (c) The pollen tube attraction efficiency at 5 HAP, bar = 50 μm. (d) Statistics of (c), n = 736, 1007 for each sample. Two-tailed Student's *t*-test. (e) Seed set of *myb98* was increased by the expression of *NPA1* in the synergids, bar = 1 mm. (f) Statistics of (e), n = 1,068, 1,294, 1,401, 587, 832, 1,043, 1,264, 927, 606, 942, 971 for each sample. Two-tailed Student's *t*-test, **** $p < 0.0001$. All data shown are the mean ± s.e.m. Three independent biological experiments were repeated.

npa1 npa2-2, and *npa1 npa2-3*) were created by knocking out these two genes in the wild type plants using CRISPR/Cas9 gene-editing (Supplemental Fig. S2). These three *npa1 npa2* null mutants exhibited no seed set defect (Fig. 5a, b). Moreover, there was no difference observed between wild type and these double mutants regarding pollen tube attraction at 5 h after pollination (HAP), 8 and 12 HAP (Fig. 5c, d; Supplemental Figs S3, S4). The normal seed set and pollen tube guidance of these *npa1 npa2* mutants are expectable as more than ten CRP attractants are still present in the synergids[3]. MYB98 is the predominant regulator in synergids that governs the transcription of multiple pollen tube attractants[12, 13]. To test the *in vivo* function of *NPAs*, *NPA1* and *NPA2*, together with *LURE1.2* and *LURE1.5* were expressed in the *myb98* mutant under *MYB98* promoter, respectively. In this context, the transcription of *MYB98* promoter was not affected by the knockout of *MYB98*[13]. The results showed that the seed set defect of *myb98* was significantly alleviated in the transgenic lines expressing *NPA1* or *LURE1.2*, but not in the lines expressing *NPA2* or *LURE1.5* (Fig. 5e, f).

To confirm whether NPA1 increases the seed set rate of *myb98* by alleviating the pollen tube guidance defect, pollen tube attraction efficiency of these transgenic plants was evaluated. The results showed that pollen tube attraction efficiency of the transgenic ovules expressing *NPA1* or *LURE1.2* was significantly higher than that of *myb98* mutant ovules at 12, 24, and 36 HAP (Fig. 6). These results indicate that NPA1 can compensate the loss of pollen tube guidance ability in *myb98*, thereby increasing the seed set rate of *myb98*.

NPA1 does not alleviate the defective pollen tube reception and seed development of *myb98* mutant

During analyzing the fertility rescuing effect of *NPA1* in *myb98*, we noticed that the expression of *NPA1* increased the pollen tube targeting rate of *myb98* from 30% to more than 50% at 36 HAP (Fig. 6d). However, the seed setting rate of *myb98* was only rescued to 40% in *MYB98:NPA1* and *MYB98:LURE1.2* plants. This percentage discrepancy between targeted-ovule and seed set implies that *myb98* has fertility defect post pollen tube targeting.

To confirm this speculation, we carefully examined and quantified the pollen tube behaviors and seed development in *myb98* and the rescued lines. Among them, pollen tube overgrowth is a sign of failed pollen tube reception post pollen tube guidance. The results showed that *myb98* plants exhibited pollen tube overgrowth within the embryo sac and aberrant seed development (Fig. 7a–c). The statistical data showed that expression of *NPA1* and *LURE1.2* in *myb98* mutant only decreased the percentage of pollen tube guidance defect, but not the pollen tube overgrowth and seed development (Fig. 7d). Together, a new type of secreted peptide NPA1 expressed in the synergid functions as a pollen tube attractant *in planta*.

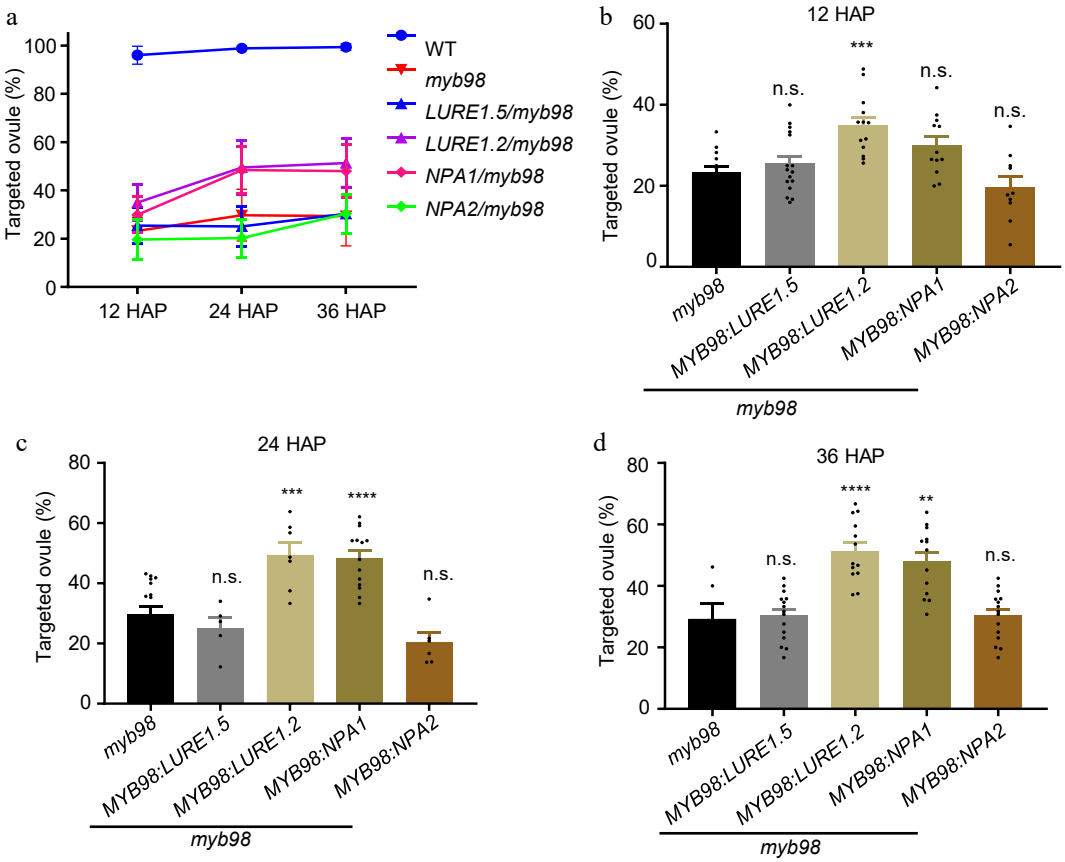

Fig. 6 Expression of *NPA1* in the synergids alleviates the defective pollen tube attraction of *myb98* mutant. (a) The percentage of targeted ovules. Statistics of the pollen tube attraction efficiency at (b) 12, (c) 24, (d) 36 HAP. Data in (b), n = 604, 595, 794, 477 for each sample. Two-tailed Student's *t*-test, *** $p = 0.0002$. Data in (c), n = 723, 241, 406, 660, 361 for each sample. Two-tailed Student's t-test, *** $p = 0.0005$, **** $p < 0.0001$. Data in (d), n = 249, 699, 544, 495, 701 for each sample. Two-tailed Student's *t*-test, **** $p < 0.0001$, ** $p = 0.0040$. All data shown are the mean ± s.e.m. Three independent biological experiments were repeated.

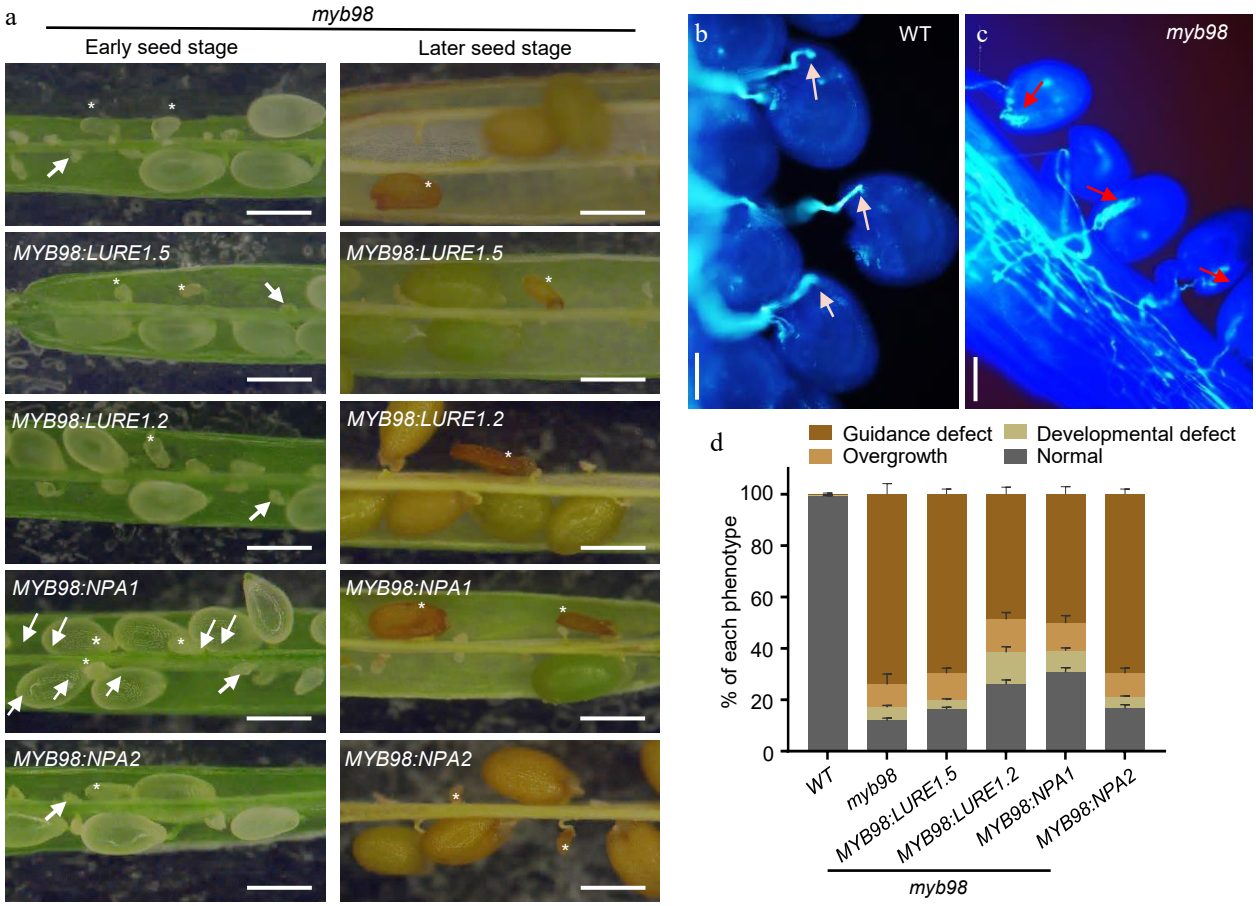

Fig. 7 *NPA1* does not rescue the defective pollen tube reception and seed development of *myb98* mutant. (a) The seed set of *myb98* and the rescued plants at the early and late developmental stages. Asterisks, aborted seeds. Arrow, aborted ovules, bar = 1 mm. (b) and (c) Pollen tube overgrowth in *myb98* ovules, bar = 20 μm. White arrow, normal pollen tubes. Red arrow, overgrowing pollen tubes. (d) Statistics of pollen tube guidance defect, pollen tube overgrowth, seed development defect, and normal seeds. Data shown are the mean ± s.e.m. Three independent biological experiments were repeated.

Discussion

Secreted peptides constitute a large gene family and function in diverse cell-to-cell communication in plants. In the model plant *Arabidopsis*, > 1,000 putative peptides have been predicted[26]. However, partially due to the functional redundancy caused by gene duplication, only a small fraction of them have been functionally studied[4, 27]. To date CRPs, like defensin-like peptides and RALFs, and cleaved small peptides, like CLE and phytosulfokine (PSK), constitute the most characterized secreted peptides[4, 27]. NPA1 as a new type of peptide, its characterization would expand our knowledge on the function of the large repertoires of secreted peptides.

The identification of NPA1 suggests diversity of the pollen tube attractants and its underlying biological relevance. Distinct from the synergid-derived CRP_810 group defensin-like attractants and the central cell-derived CRP-type attractants[8], NPA1 has fewer cysteines and a totally different conformation, which is consistent with its new perception mechanism. Due to the high functional redundancy of the more than a dozen attractants, selection constraint could be alleviated to allow the accumulation of mutations that contribute to species-specificity and gene loss with high efficiency. For instance, in the CRP_810 subgroup, LUREs and TICKETs are species-specifically active, while LURE1.5 and TICKET1 lose the pollen tube attraction activity[3, 5, 6]. Compared to NPA1, NPA2 does not have attraction activity and shows much lower expression level. *At*NPA1 shows attraction activity to pollen tubes of *Arabidopsis* and its close relatives *A. lyrata* and *C. rubella*, which may contribute to possible interspecific gene flow and the consequent speciation of new species. It is of note that *At*NPA1 shows preferential attraction of the conspecific pollen tubes than the pollen tubes of sister species and no attraction activity to the *E. salsugineum,* suggesting that *At*NPA1 may also contribute to the reproductive isolation between *Arabidopsis* and the distantly related species. NPA1 is supposed to function redundantly with the other pollen tube attractants, like LUREs, XIUQIUs and TICKETs in the CRP_810 group. This conjecture is based on two facts that CRP_810 members have functional redundancy in enticing pollen tubes to the micropyle[3], and NPA1 also function in this process. The presence of a large amounts of attractant-encoding genes ensures reproductive success in a plant species by rendering precise and timely pollen tube targeting into the ovule and reduces the risk of fertilization by alien pollen. The diversity of these attractants raises an intriguing question of how the different types of attractant peptides were evolved to regulate a single process. Peptides evolve rapidly compared to other genes via duplications and mutations emerging during

evolution, leading to their abundance among species. Most duplicated peptides including CRPs arised from whole genome duplication or segmental duplication with 22%−39% of CRPs in most species exhibit clustering patterns with frequent tandem duplications[28−30]. The aforementioned evolutionary pattern results in the repeats of peptide genes in plants, thereby serving as an intrinsic driving force behind the abundance of pollen tube attractants. And the abundance of these peptides could alleviate the selection constraint to allow accumulations of mutations that contribute to species-specificity and gene loss. The function of the homologs of NPAs encoded in Leguminosae species, such as *Trifolium pretense*, *Phaseolus acutifolius*, *Medicago truncatula*, and *Vigna unguiculata* still need further experimentation. It can be expected that other types of signals would be identified in plants in the future and would further help to explain the signal diversity and species isolation.

Author contributions

The authors confirm contribution to the paper as follows: data analysis and interpretation of results: Wang WQ, Meng JG, Yang F, Xu YJ; study conception and design, Supervision, draft manuscript preparation: Li HJ; manuscript revision: Li SZ. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated and analyzed during this study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare no competing interest. Li HJ is the Editorial Board member of *Seed Biology* who was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of this Editorial Board member and the research groups.

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