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A pollen-specific calmodulin-binding protein, NPG1, interacts with putative pectate lyases

Sung-Bong Shin*, Maxim Golovkin† & Anireddy S. N. Reddy

Department of Biology, Program in Molecular Plant Biology, Program in Cell and Molecular Biology, Colorado State University, Fort Collins, CO 80523, USA.

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requests for materials
should be addressed to
A.S.N.R. (reddy@
colostate.edu)* Current Address:
United States
Department of
Agriculture – Tree Fruit
Research Laboratory,
Wenatchee, WA
98801, USA† Current Address:
Foundation for
Advancement of
Science, Technology
and Research,
Biotechnology Center,
PA 18902, USA

Previous genetic studies have revealed that a pollen-specific calmodulin-binding protein, *No Pollen Germination 1* (*NPG1*), is required for pollen germination. However, its mode of action is unknown. Here we report direct interaction of NPG1 with pectate lyase-like proteins (PLLs). A truncated form of AtNPG1 lacking the N-terminal tetratricopeptide repeat 1 (TPR1) failed to interact with PLLs, suggesting that it is essential for NPG1 interaction with PLLs. Localization studies with AtNPG1 fused to a fluorescent reporter driven by its native promoter revealed its presence in the cytosol and cell wall of the pollen grain and the growing pollen tube of plasmolyzed pollen. Together, our data suggest that the function of NPG1 in regulating pollen germination is mediated through its interaction with PLLs, which may modify the pollen cell wall and regulate pollen tube emergence and growth.

Pollination and fertilization are essential steps in sexual reproduction in flowering plants. Following pollination, pollen grains germinate and pollen tubes grow through the transmitting tract to reach the ovule and release the sperm cells in to the embryo sac and initiate double fertilization. One of the two sperm cells from a pollen tube fuses with the egg cell to produce the diploid zygote and the second one fuses with the central cell to produce the triploid endosperm¹. In the female tissues, the tube growth is guided through the extra-cellular matrix of the transmitting tract, which provides adhesion molecules and the nutrients required for tube growth^{1,2}. For successful pollen tube guidance, female tissues produce an assortment of chemotropic ions and molecules such as γ -amino butyric acid (GABA), D-serine, cysteine-rich peptides (CRPs), calcium and various transmitting tissue-specific proteins^{2–8}.

Successful fertilization depends on directed growth of pollen tube towards the synergids in the female tissue. Identification of signaling events, pathways and mechanisms that are involved in this process is critical to understanding pollen tube guidance⁹. Ca^{2+} , an important second messenger in plants, is implicated in regulating diverse cellular and developmental processes in response to external signals or developmental cues^{10,11}. Ca^{2+} is also one of the key regulators of pollen germination, tube growth, and tube guidance^{2,9,12–15}. A steep tip-focused Ca^{2+} gradient at the tube tip is observed in growing pollen tubes by ratiometric Ca^{2+} imaging and pollen tube growth can be inhibited when the apical Ca^{2+} gradient is disturbed by blocking calcium uptake^{13,16–19}. In plant species where pollen tube growth displays clear oscillations, it has been shown that Ca^{2+} oscillations at the tip are closely correlated with pollen tube growth rate and direction^{13,16,17,20}.

High levels of Ca^{2+} and its oscillations at the tube tip are likely recognized by Ca^{2+} sensors, which mediate Ca^{2+} signals^{10,21}. Ca^{2+} binding can cause conformational changes in its sensors, thus modulating the sensor's activity, and/or modulating its binding affinity to the target proteins, whose function or activity are dependent on the interaction with Ca^{2+} sensors¹⁰. Calmodulin (CaM) is one of the well-characterized Ca^{2+} sensors with four characteristic Ca^{2+} -binding EF-hand domains. The activated CaM (calcium bound CaM) interacts with many diverse proteins and regulates their activity/function^{10,21}. In recent years, many CaM target proteins have been identified and these proteins play key roles in diverse processes, such as ion transport, gene regulation, cytoskeleton organization, disease resistance, metabolism and stress tolerance^{11,21–23}.

Like Ca^{2+} , CaM is also involved in pollen germination and tube growth^{24–26}. Blocking of CaM by antiserum or CaM antagonists causes cessation of pollen tube growth, and exogenous CaM can enhance pollen germination and tube growth²⁷. In hydrated pollen, high levels of CaM is localized to the germinal apertures, plasma membrane of the germination bubble and to the cytoplasm near the bubble²⁴. Although CaM distributes uniformly in pollen tubes, a higher level of activated CaM is present in the tip region forming a tip-focused gradient, like Ca^{2+} in



the pollen apex^{24,28}. Moreover, activated CaM oscillates in growing pollen tubes, which correlates with Ca²⁺ oscillations²⁸. Although these studies implicate Ca²⁺/CaM-mediated signaling pathways in pollen germination and pollen tube growth, pathways downstream of Ca²⁺/CaM are not well understood.

We have previously isolated a Maize Pollen-specific CaM Binding Protein (MPCBP) and a homolog of this protein from Arabidopsis named No Pollen Germination 1 (AtNPG1)^{29,30}. AtNPG1 and MPCBP showed a very strong pollen-specific expression. These proteins contain several tetratricopeptide repeat (TPR) domains and a calmodulin-binding domain (CBD). Genetic studies have shown that AtNPG1 is required for pollen germination but not for pollen development³⁰. The amino acid sequence of AtNPG1 and the maize homolog share 56% identity and 70% similarity. Homologs of NPG1 are also identified in other plants including *Vitis vinifera* and *Oryza sativa*. However, no homologs of AtNPG1 are found in non-plant systems such as yeast, *Drosophila melanogaster*, *Caenorhabditis elegans*, and humans, suggesting that AtNPG1 represents a plant-specific CaM-binding protein.

Although genetic studies revealed a role for AtNPG1 in pollen germination³⁰, the mechanism by which it regulates pollen germination is unknown. To gain insights into the NPG1 mode of action and to identify NPG1 interacting proteins, we performed a yeast two-hybrid screen with a petunia pollen cDNA library. Here we report identification of putative pectate lyases (PLLs) as interacting partners of AtNPG1. Both *in vivo* and *in vitro* protein-protein interaction assays revealed the interaction of AtNPG1 with putative PLLs. The truncated form of AtNPG1 lacking the N-terminal TPR domain (TPR1) failed to interact with PLLs, suggesting that this region is essential for the interaction. Localization of AtNPG1 fused to either CFP or YFP driven by its native promoter revealed its presence in the cytosol and cell wall of the pollen grain and the growing pollen tube of plasmolyzed pollen. Together, these results suggest that the function of NPG1 in regulating pollen germination is mediated through its interaction with PLLs, which may modify the pollen cell wall and regulate pollen tube emergence and growth.

Results

AtNPG1 interacts with two putative pectate lyases from Petunia pollen. Previously we have shown that pollen lacking AtNPG1 do not germinate³⁰. However, its mode of action is unknown. In addition to a calmodulin-binding domain, AtNPG1 has several tetratricopeptide repeats that are implicated in protein-protein interaction^{31,32}. To identify AtNPG1 interacting proteins, we performed a yeast two-hybrid screen using AtNPG1 fused to the GAL4 DNA binding domain as a bait and a pollen cDNA library from petunia pollen as fusions to the GAL4 activation domain. Yeast colonies growing on selection plates (Trp-, Leu-, His- and 25 mM AT) were assayed for β -galactosidase activity. Two positives, named Pet #5 and Pet #30, showed strong β -galactosidase activity (Fig. 1), indicating interaction of these petunia proteins with AtNPG1. Sequence analysis of cDNAs from these positives has revealed that they encode two distinct putative pectate lyases (PLLs) (Fig. 2). Both cDNAs were partial and encoded different lengths of C-terminal regions of PLLs that showed very high sequence similarity to PLLs from Arabidopsis, tobacco and tomato (Fig. 2B). Petunia PLLs shared up to 93% similarity with tobacco and tomato PLLs.

AtNPG1 interacts with four pollen putative PLLs from Arabidopsis. In Arabidopsis 26 genes encode PLLs and all of them are expressed in flowers with 14 expressed in pollen³³. To test if Arabidopsis PLLs interact with AtNPG1, we cloned full-length cDNAs of four pollen expressed PLLs [PLL8 (At1g14420), PLL9 (At2g02720), PLL10 (At3g01270), and PLL11 (At5g15110)], that showed the highest sequence similarity at the amino acid level to petunia PLLs, into a yeast vector and used them in yeast one-on-

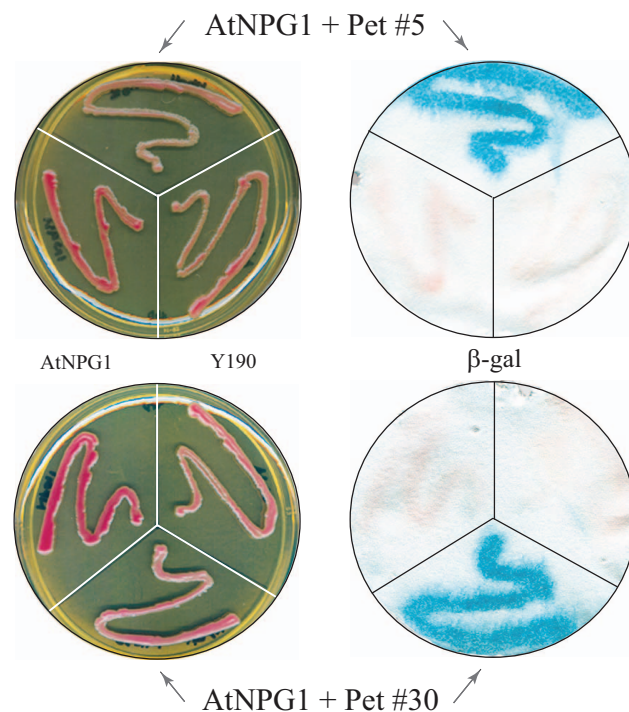


Figure 1 | Interaction of Arabidopsis NPG1 with proteins encoded by two positive clones from petunia pollen library. Y190 yeast, Y190 containing BD-AtNPG1 only (AtNPG1), and Y190 containing both BD-AtNPG1 and AD-Pet #5 (AtNPG1 + Pet#5) or AD-Pet #30 (AtNPG1 + Pet#30) were grown on YPD (left) and assayed for β -galactosidase (β -gal) activity (right).

one interaction assays with AtNPG1. The two original Petunia PLLs (#5 and #30) were used as positive controls. As shown in Fig. 3A, all four AtPLLs showed interaction with AtNPG1. Quantification of β -galactosidase activity showed the highest activity with AtPLL11 and the least activity was found with petunia #5. The expression of all four AtPLLs in yeast cells was confirmed by western blot analysis using antibodies to the GAL4 activation domain (Fig. 3B). To test the specificity of NPG1 interaction with PLLs, we performed a yeast two-hybrid assay with NPGR2, a protein related to NPG1 and is expressed in pollen³⁰, and PLLs from petunia and Arabidopsis. We did not observe any interaction of NPGR2 with neither petunia nor Arabidopsis PLLs (Suppl. Figure 1). These results suggest that the observed interaction of NPG1 with PLLs is specific.

***In vitro* confirmation of NPG1 interaction with PLLs.** To further confirm the observed interactions between AtNPG1 and putative pectate lyases we performed *in vitro* pull-down assays. AtNPG1 was expressed in *E. coli* as a T7-tag fusion whereas AtPLLs (8 to 11) were expressed as S-tag fusions. We were not able to obtain AtPLL8 and AtPLL9 protein in the soluble fraction (Fig. 4A), hence these were not used in pull-down assays. In the case of PLL10, very little protein was found in the soluble fraction whereas adequate amount of PLL11 was found (Fig. 4A). Interaction between AtNPG1 and AtPLL10 and AtPLL11 was analyzed by incubating the S-protein beads bound to AtPLL10 or AtPLL11 with bacterial extract (soluble fraction) containing AtNPG1. The unbound proteins were removed by washing and the protein bound to S proteins beads was analyzed by SDS-PAGE and probed for AtNPG1 with T7-tag antibody and for AtPLLs with S protein. PLL bound S beads effectively pulled down AtNPG1 (Fig. 4B) further confirming these interactions.

A maize pollen PLL was also identified as an AtNPG1 binding proteins. AtNPG1 binding proteins in maize pollen were isolated by

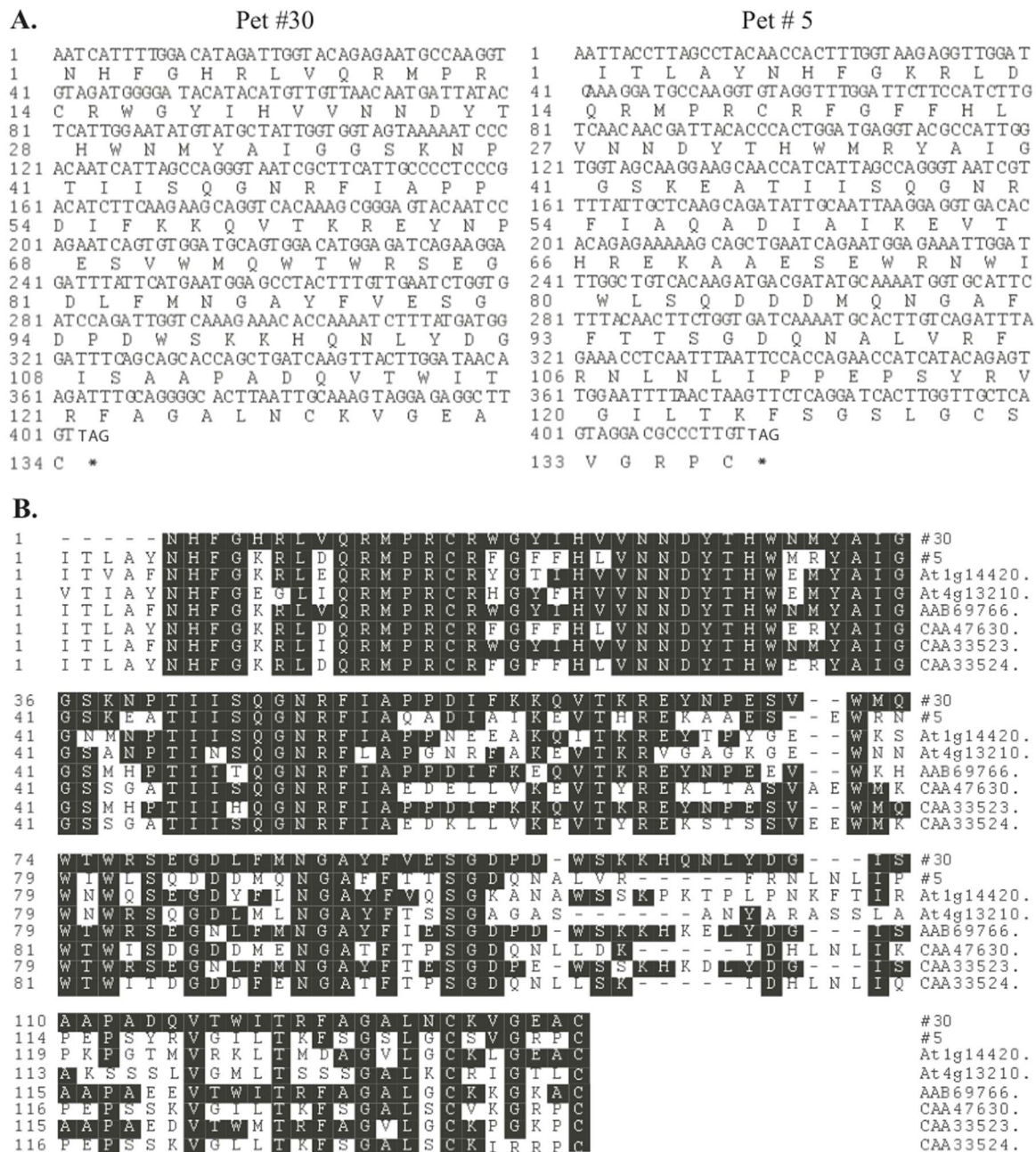


Figure 2 | Sequence analysis of two AtNPG1 interacting proteins from petunia pollen. (A.) Nucleotide and deduced amino acid sequence of Pet #30 and Pet #5. (B.) Alignment of deduced amino acid sequence of Pet #30 and Pet #5, with putative pectate lyases from Arabidopsis (At1g14420 and At4g13210), tobacco (AAB69766 Nt59 and CAA47630), and tomato (CAA33523, p59 and CAA33524, p56). Amino acids that are identical to the Pet#30 sequence are indicated by white letters on a black background.

applying maize pollen total soluble proteins to an AtNPG1 Sepharose 4B column. Following extensive washes, the bound proteins were eluted, subjected to trypsin digestion and the sequences of the peptides were identified by LC-MS/MS. The amino acid sequences from LC-MS/MS were identified by BLAST analysis. Interestingly, one of the proteins identified in this analysis is a pectate lyase (Fig. 5), further confirming the interaction of NPG1 with a PLL.

The TPR1 domain in NPG1 is essential for its interaction with PLLs. TPR domains in diverse proteins are known to be involved in protein-protein interactions. To study the role of TPR domains in NPG1 interaction with PLLs, a truncated AtNPG1 lacking the TPR1 domain was tested for its interaction with Arabidopsis and petunia PLLs in a yeast two-hybrid assay (Fig. 6). No colonies were observed with the -TPR1 bait in all PLLs except for some colonies formed with

PLL9. Quantitative liquid culture β -galactosidase assay revealed very low activity with PLL9 in comparison with interactions with the full AtNPG1 (Fig. 6B).

In vivo localization of fluorescent AtNPG1 in pollen and pollen tube. Pollen specific expression of AtNPG1 was confirmed previously by RT-PCR, Northern analysis and by expressing a fluorescent reporter fused to AtNPG1 promoter³⁰. The putative PLLs have extracellular targeting signals at the N-terminus and are likely to be extracellular. However, NPG1 does not have any discernable extracellular targeting signals. In order for the observed interaction between NPG1 and PLLs to be physiologically significant we expect some or all of NPG1 in pollen to be localized to cell walls. To test the localization of NPG1 in the pollen grain and pollen tube, we generated an expression construct in which the AtNPG1

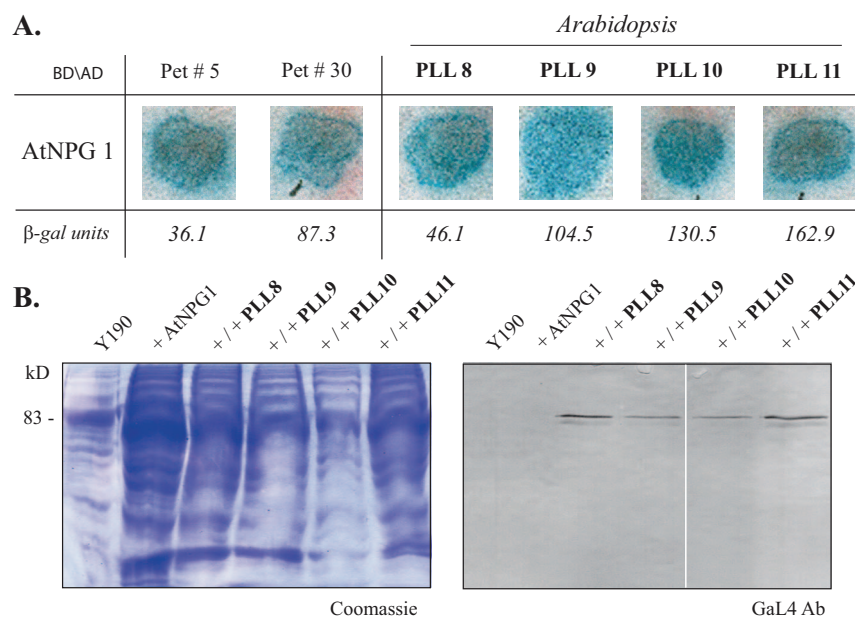


Figure 3 | AtNPG1 interacts with four Arabidopsis PLLs. (A.) β -galactosidase assay shows a strong interaction between AtNPG1 and four Arabidopsis pectate lyase-like (PLL) proteins. (B.) Immunoblot showing the expression of four Arabidopsis PLLs in the Y190 yeast cells. Gal4-AD antibody was used to detect PLLs. Lanes: 1, Y190; 2, Y190 with AtNPG1; 3, Y190 with AtNPG1 and AtPLL8; 4, Y190 with AtNPG1 and AtPLL9; 5, Y190 with AtNPG1 and AtPLL10; 6, Y190 with AtNPG1 and AtPLL11.

gene containing a fluorescent reporter (CFP or YFP) as an internal tag was driven by the *AtNPG1* promoter (Fig. 7A). Internal tagging of a reporter was shown to eliminate localization artifacts, which happen when the reporter is fused to either the N- or C-terminus of a protein³⁴. Transient expression in tobacco pollen showed strong CFP or YFP expression in the whole pollen tube body (Fig. 7B). We then transformed Arabidopsis plants with this construct. Pollen from these transgenic lines were germinated *in vitro*, plasmolyzed and analyzed for NPG1 localization using confocal microscopy. Plasmolysis separates the cell wall from the cytoplasm and plasma membrane so that localization of protein to the cell wall can be easily visualized. NPG1 is localized in the cytoplasm as well as in the cell wall of the pollen grain and the growing tube in early and later stages of germination (Fig. 7C, Suppl. Figure 2, Suppl. Movies 1 and 2). We did not observe any autofluorescence from Arabidopsis wild type pollen grains and tubes neither in the YFP or CFP channel as shown in Supplemental Figure 3, suggesting that the observed CFP and YFP fluorescence in transgenic lines was due to introduced fusion proteins. Recently, immunoelectron microscopy with antibodies to rice NPG has also established the localization of NPG1 to the cell wall³⁵. Together, these studies suggest that some NPG1 is localized to the cell wall.

Discussion

The presence of TPR domains and absence of any known enzymatic domains in pollen-specific AtNPG1 prompted us to identify its putative interacting partners using a petunia pollen yeast two-hybrid library. Two AtNPG1-interacting proteins were identified in the screen. Sequence analysis of the cDNAs (Fig. 2) have shown that both are very similar to the late-anther development tomato proteins LAT56 and LAT59, from the PLL family of proteins that are preferentially expressed in anthers and pollen^{36,37}. Four close homologs to petunia PLL positives from Arabidopsis are well expressed in pollen³³ indicating that they may also interact with NPG1. One-on-one interaction studies between AtNPG1 and Arabidopsis PLLs (AtPLLs) using yeast two-hybrid (Fig. 3A) and pull-down assays confirmed that AtNPG1 interacts with AtPLLs.

A TPR motif of 34-amino acids was first described in cell cycle regulator CDC23 in yeast, and since then, the TPR motif was identified in a large number of proteins that perform many different functions³¹. A number of studies have shown that TPR domains function in protein-protein interactions and modulate different cellular processes including cell cycle regulation^{31,38}, transcription^{31,39}, protein transport across mitochondria and peroxisomes⁴⁰, and muscle development⁴¹. As the number of TPRs is variable in different proteins, it is thought that the TPR domain can function as a scaffold in binding to specific substrates, which is dependent upon the secondary structure assumed by the individual TPR, or the combination of TPRs^{31,41,42}. The loss of function in TPR domain-containing proteins is usually due to mutation or deletion in the TPR region, suggesting the importance of this domain in a protein's function^{31,41}. To test the function of TPR domain involvement in NPG1 and the PLLs interaction, a truncated version of AtNPG1 lacking the N-terminal TPR1 domain was prepared (Fig. 4A). Without this region, no interaction was observed between NPG1 and the PLLs (Fig. 6) suggesting that the TPR1 domain is essential for interaction.

Pectate lyases were initially characterized as pathogen-secreted extracellular enzymes that help pathogens to invade the host by degrading pectins. Pectate lyase activity from microorganisms has been well-characterized^{43–47}. Pectate lyases from various organisms have a high degree of similarity and there is a conserved Pec_Lyase_C domain in pectate lyases of all organisms⁴⁸. Enzymatic activity involves the cleavage of α -1,4-glycosidic linkages of galacturonosyl residues of demethylated pectin. The cleavage of glycosidic bonds occurs by a β -elimination reaction that finally produces 4,5 unsaturated oligogalacturonates^{49,50}. Calcium is required for the enzyme activity and was found to bind the enzyme⁵⁰. The pollen-specific pectate lyase activity is implicated in pollen tube growth emergence by initiating the loosening and breaking of the pollen cell wall and also in pollen tube penetration in transmitting tissue^{51,52}. Recent transcriptome studies showed cell wall modification enzymes, including PLLs, are highly up-regulated during pollen germination and tube growth⁵³. Pectate lyases like LAT56 and LAT59 are known to be preferentially expressed in pollen, though their function is not known^{36,37}. Surprisingly, LAT56 and LAT59 proteins expressed in a

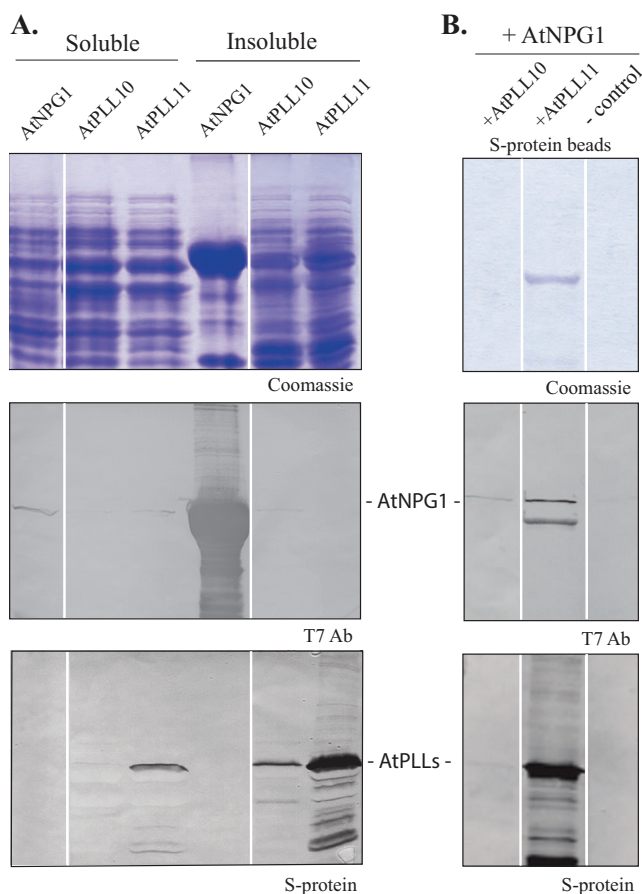


Figure 4 | *In vitro* confirmation of interaction between AtNPG1 and Arabidopsis PLLs. (A.) Soluble and insoluble proteins of bacterially expressed AtNPG1 and two AtPLLs were separated on SDS-PAGE gel and stained or blotted. The blot was probed with S-protein to detect AtPLL proteins that were used in pull-down assay. (B.) S-tag bead-bound proteins were separated on SDS-PAGE gels and identified by S-protein for AtPLLs and T7-tag antibody for AtNPG1.

baculovirus expression system did not show any pectate lyase activity *in vitro*⁵⁴. Negative results may be due to several reasons, which include i) they are not true pectate lyases, ii) they have stringent substrate specificity and/or iii) their activity may be modulated by other factors such as calcium/calmodulin through NPG1.

In Arabidopsis there are twenty-six pectate lyase-like proteins³³ forming a family, though the functions of most of them are not known. Four of them [encoded by *PLL8* (*At1g14420*), *PLL9* (*At2g02720*), *PLL10* (*At3g01270*), and *PLL11* (*At5g15110*)] are very similar to petunia PLLs and are highly expressed in pollen (Fig. 2)³³. Unlike other PLLs, only these four PLLs have a Pec_Lyase_N (Pfam04431) besides the Pec_Lyase_C domain⁵⁵, suggesting they may function on different substrates. The study of all Arabidopsis PLL promoter activity showed the activity is high at the cell separation area, which requires cell wall modification⁵⁵.

The length of PLLs varies greatly from 307 to 580 amino acids, suggesting that the members of the family may differ in their regulation and/or localizations. One of the Arabidopsis PLLs (*PMR6*) with a long C-terminal extension, as compared to the other PLLs, contains a plasma membrane anchoring domain and is shown to be indispensable for powdery mildew susceptibility in Arabidopsis⁵⁶. Mutation in *PMR6* can also alter leaf morphology and decrease the leaf size, all probably due to a decrease in cell expansion. Experimental confirmation of differential expression of PLL genes in different tissues was shown to be sensitive to various hormones and stresses³³.

The direct interaction of AtNPG1 with PLL proteins is rather intriguing and raises many questions. Pectate lyases are recognized as extracellular enzymes and thought to be extracellular or attached to the external surfaces of the plasma membrane. Twenty-six Arabidopsis PLLs can be localized to different cellular locations. Some putative pectate lyases on a plasma membrane could have their regulatory region facing the cytosol. Confocal imaging of AtNPG1 fused to a fluorescent reporter in the germinating pollen tube shows localization of AtNPG1 on the cell wall while also being present in the cytoplasm (Fig. 7).

An AtNPG1 homologous protein was identified in rice (*OsPCBP*, *Oryza sativa* pollen CaM-binding protein)³⁵. Like AtNPG1, *OsPCBP* is a pollen specific protein and a calmodulin binding protein³⁵. Using immunoelectron microscopy, it was shown that *OsPCBP* is localized

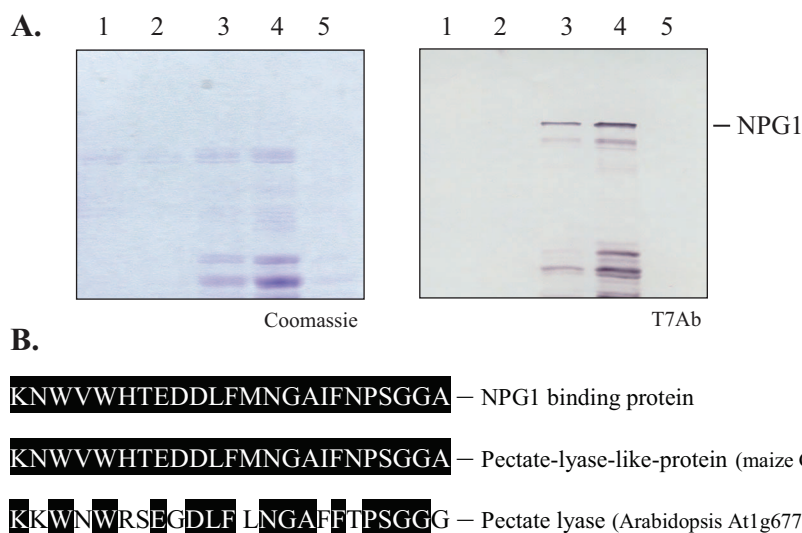


Figure 5 | Maize PLL bound to NPG1 affinity column. (A.) AtNPG1 protein was bacterially expressed and purified using a His-bind affinity column. Elution fractions (1 to 5) were separated on SDS pages gels and either stained (Coomassie) or blotted and probed with T7-tag antibody (T7Ab). This purified protein was used to prepare affinity column. (B.) Alignment of a maize pollen peptide identified in proteins bound to NPG1 affinity column with Arabidopsis and maize PLL. The identified NPG1 binding protein peptide (top sequence) is identical to a peptide from a pectate lyase-like protein from maize (middle sequence). The bottom sequence is a peptide from one of the Arabidopsis pectate lyases.

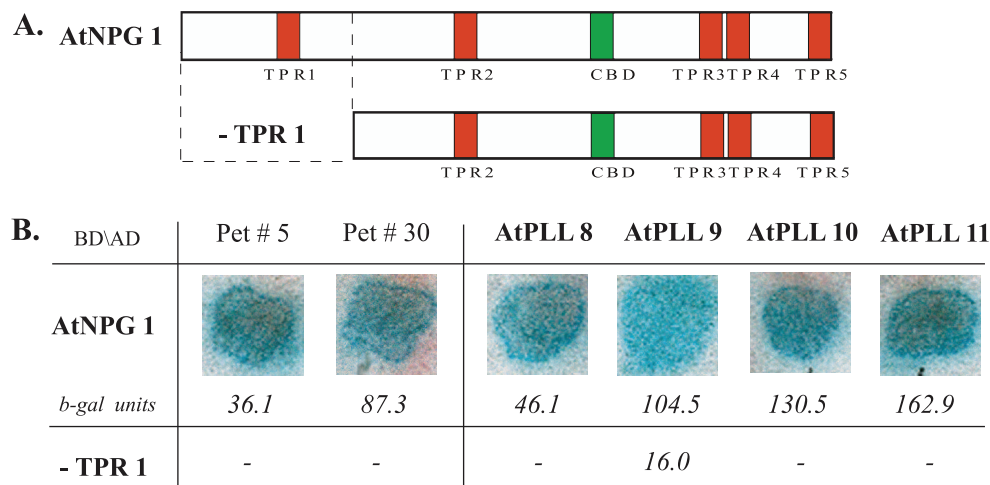


Figure 6 | TPR1 domain is essential for the interaction of AtNPG1 with PLLs. (A.) Schematic representation of AtNPG1 and truncated version of AtNPG1 lacking TPR1 (-TPR1). (B.) β -galactosidase assay shows interaction between AtNPG1 with petunia and Arabidopsis PLLs (upper panel) and lack of interaction between ANPG1 lacking TPR1 (-TPR1) with petunia and Arabidopsis PLLs (lower panel).

on the pollen tube cell wall and amyloplast³⁵. The RNAi down-regulation study also showed OsPCBP involvement in pollen development³⁷. These data from sub-cellular localization and protein-protein interaction analysis point to NPG1 involvement in modulating PLLs thereby modifying cell wall and pollen germination.

Methods

Yeast two-hybrid screening of a Petunia pollen cDNA library with AtNPG1. The coding region of *AtNPG1* was subcloned into the yeast expression vector pACT2J as a *NdeI/XhoI* fragment from a pET28a plasmid³⁰, and a *NdeI/SalI* fragment of *AtNPG1* was subcloned into the pAS1-CYH2 plasmid. These clones were verified by sequence analysis and introduced into Y190 yeast cells where the GAL4 DNA binding domain (BD)-carrying yeast colonies were selected on Trp-SD plates and used for transformation with the petunia pollen library prepared in a vector containing the GAL4 activation domain (AD). Library screening was performed with *AtNPG1*-pAS1-CYH2 as bait essentially as described in the Clontech manual PT1020-1. Selection of yeast cells for both BD and AD constructs was done on SD medium lacking Trp and Leu. The colonies were then tested on Trp-, Leu-, His- SD plates supplemented with 25 mM AT (3-aminotriazole). β -galactosidase filter and liquid culture assays were performed to confirm the interaction between proteins. Filter assay was performed as described earlier³⁸ and liquid culture assay with CPRG for quantifying the enzyme activity (Chlorophenol red- β -D-galactopyranoside) was done according to the procedure described in the Clontech MATCHMAKER library manual PT1020-1. Y190 yeast cells harboring no plasmids or individual BD or AD constructs were used as negative controls.

Generation of Petunia and Arabidopsis PLLs constructs for yeast two-hybrid assays. Full-length cDNAs encoding four Arabidopsis PLLs (*At1g14420*, *At2g02720*, *At3g01270* and *At5g15110*) showing the highest sequence similarity to petunia PLLs isolated as NPG1 interactors were amplified by RT-PCR using the gene-specific primers³³ and cloned into pACT2J. The 5' *NdeI-EcoRI* 3' sites were used for *At1g14420* and *At2g02720* cloning and 5' *XmaI-XhoI* 3' for the others. The constructs were confirmed by sequence analysis and used in one-on-one yeast two-hybrid interaction analysis.

Individual β -gal positive colonies were grown in liquid selection medium overnight at 30°C and used to inoculate larger volumes of non-selective YPD medium, grown at 30°C to OD₆₀₀ 0.5–0.8. Yeast cells were harvested in 50 ml polypropylene tubes, briefly pelleted at 5000 rpm and transferred to a flat-bottom (O-ring screw cap microcentrifuge tube) and re-suspended in 50 μ l Laemmle buffer supplemented with 500 μ l of acid washed beads (425–600 microns). After ~1 min of vortexing at a maximum speed the tubes were spun in an Eppendorf tube centrifuge at maximum speed for 5 min at 4°C, and clear supernatant was loaded onto SDS-PAGE gel for electrophoresis. The gel was blotted and probed with Gal4 AD antibody (Clontech). β -galactosidase activity was calculated using the following formula: (1000 \times OD₅₇₈) / (elapsed min \times 1.5 ml \times OD₆₀₀).

Verification of NPG1 and PLL interactions using pull-down assay. For S-tagged proteins expression of PLLs in bacterial pET32b vector (Novagen) was used. *BglII-EcoRI* fragments of *At1g14420* (PLL8) and *At2g02720* (PLL9) were cloned into the corresponding sites of the vector. In the case of *At3g01270* (PLL10) the *BglII-XhoI* cloning sites were used and the *EcoRI-XhoI* sites were used for *At5g15110* (PLL11). All *AtPLLs* constructs were verified by sequencing and electroporated into an *E. coli* BL21

(DE3) strain containing a chaperone plasmid pG-KJE8 (Takara). The T7-tagged AtNPG1 construct for bacterial expression was prepared in pET28a using *EcoRI*. These constructs were introduced into BL21 (DE3) *E. coli* strain for protein induction (Novagen) and detection with a T7-tag antibody. Arabidopsis pollen mRNA was used for initial cloning of AtNPG1 cDNAs into the pGEM-T Easy (Promega) as described earlier³⁰.

Soluble forms of AtPLL10 or AtPLL11 were bound to S-protein agarose beads (Novagen) at 4°C for 3 hours. Beads (~200 μ l volume) were washed according to the manufacturer's recommendations and immediately incubated with the soluble fraction of bacterially expressed AtNPG1 at 4°C for 3 hours. Beads were washed again and the proteins were eluted in 75 μ l of 1 \times SDS loading buffer (0.0625 M Tris pH 6.8, 2% SDS, 5% mercaptoethanol, 10% glycerol, 0.0075% bromophenol blue) and separated on three SDS-PAGE gels. One was used for Coomassie staining and two were blotted and probed with the S-protein to detect PLLs and T7-tag antibody to detect AtNPG1.

Purification and identification of maize pollen AtNPG1 binding protein. An AtNPG1 affinity column was prepared by coupling purified AtNPG1 to CNBr-activated Sepharose 4B (GE Healthcare). Two grams of CNBr-activated Sepharose 4B powder was suspended in 10 ml of 1 mM HCl. The beads were washed for 15 minutes with 1 mM HCl on a sintered glass filter (porosity G3) and kept in the coupling buffer (0.1 M NaHCO₃ pH 8.3, 0.5 M NaCl). AtNPG1 and CNBr-activated Sepharose 4B were mixed and incubated, rotating the mixture end-over-end overnight at 4°C. Excess ligand was washed away with 5 volumes of coupling buffer on a sintered glass filter (porosity G3). Remaining active groups were blocked by incubating in 0.1 M Tris-HCl buffer pH 8.0 for 2 hours. The beads were washed with buffer (0.1 M acetic acid/sodium acetate pH 4.0 containing 0.5 M NaCl and 0.1 M Tris-HCl pH 8 containing 0.5 M NaCl).

One gram of maize pollen and 1.2 g of sand were added to a chilled mortar and ground for 1 hour. Ten ml of cold extraction buffer (50 mM TrisHCl pH 7.5, 250 mM sucrose, 5 mM DTT, 1 \times protease inhibitor cocktail from Roche) was added. The sample was ground one more hour and centrifuged at 17,000 rpm in a Sorval SS-34 for 30 min. The supernatant was centrifuged in an ultracentrifuge at 50,000 rpm for 1 hour. The supernatant was filtered with a 0.22-micron filter. Maize pollen protein extract was applied to the NPG1 affinity column and washed with binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). The column was washed with 10 mM sodium phosphate buffer pH 7.2. The proteins bound to the column were eluted using elution buffer (3.5 M MgCl₂, 10 mM sodium phosphate pH 7.2). The eluted proteins were digested by trypsin and the amino acid sequences of digested fragments were identified by LC-MS/MS at W. M. Keck foundation biotechnology resource laboratory at Yale University. The amino acid sequences of identified fragments were used for BLAST search against the Arabidopsis database.

Generation of AtNPG1-YFP or -CFP expression cassettes for *in vivo* localization studies. Arabidopsis genomic DNA was used to synthesize the fusion constructs for *AtNPG1-CFP/YFP* and it was prepared as a series of PCR reactions following the protocol described in Tian et al., 2004³⁴. Primers used for these reactions are as follows: AtNPG1 P1: 5'-GCT CGA TCC ACC TAG GCT GTA TGA GTC GAG TGT CTG ACT T-3', AtNPG1 P2: 5'-CAC AGC TCC ACC TCC ACC TCC AGG CCG GCC GAG AGT TGG CTG ATG ATC TTT CCC TCT T-3', AtNPG1 P3: 5'-TGC TGG TGC TGC TGC GGC CGC TGG GGC CCC CGT GGC TAG AAG TTT GCT CTC TGA TG-3', AtNPG1 P4: 5'-CGT AGC GAG ACC ACA GGA TAG AGA AGA GAC TCT GTT TTG-3', CFP/YFP forward: 5'-GGC CGG CCT GGA GGT GGA GGT GGA GCT GTG AGC A-3', CFP/YFP reverse: 5'-GCC CCC AGC GGC CGA

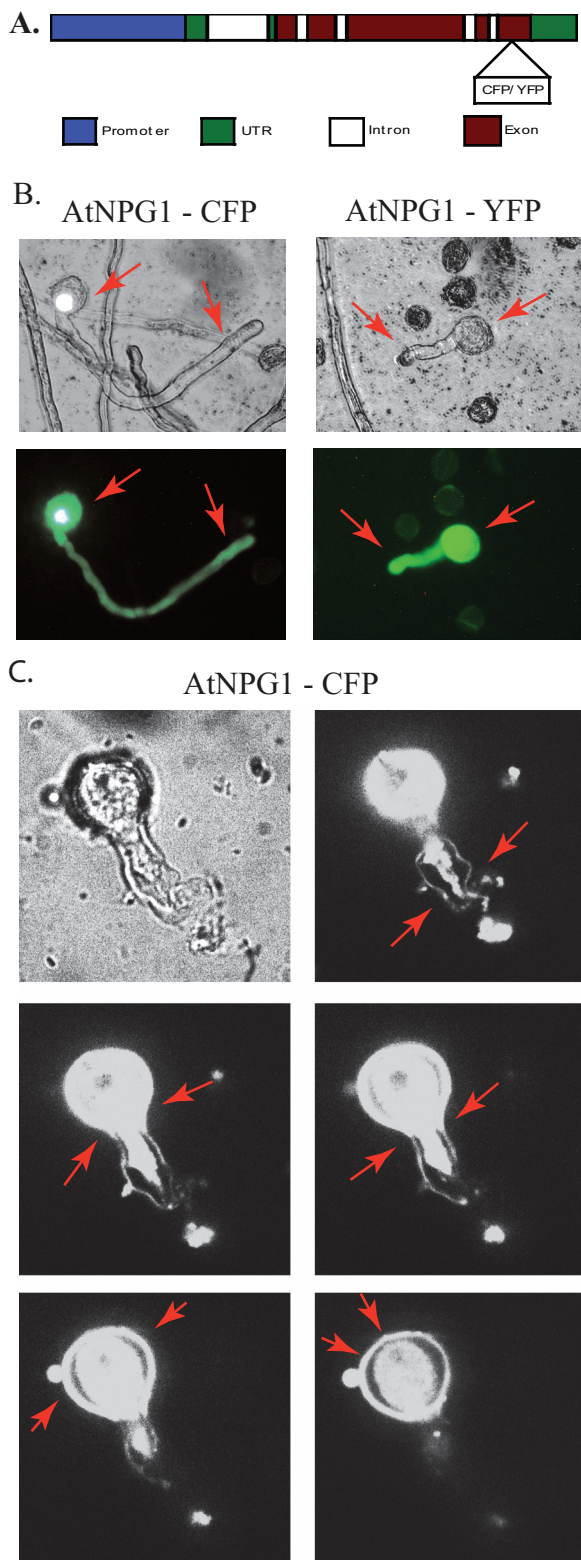


Figure 7 | Localization of *AtNPG1* in pollen and pollen tube. (A.) Schematic diagram of *AtNPG1*-YFP or -CFP (*Y/CFP*) fusion. The fusion construct includes *AtNPG1* promoter (blue), introns (white), exons (brown), *Y/CFP* reporter, 5' and 3' UTR (green). *Y/CFP* reporter was inserted into last exon. (B.) *AtNPG1* YFP or CFP fusions were expressed in tobacco pollen and pollen tube. Arrows indicate germinated pollen expressing CFP (left) or YFP (right). (C.) *AtNPG1*-CFP localizes to pollen tube cell wall. CFP signal was detected on pollen tube cell wall (arrows) after plasmolysis. Successive confocal sections (10 μ m thick) of a plasmolysed pollen grain were captured and five sections are shown.

Arrows indicate CFP in cell wall of pollen tube (first three panels) and pollen grain (last two panels). All confocal sections of this figure showing CFP localization are combined and presented in Supplemental movie 1.

AGC AGC ACC AGC AGG ATC-3', AttB1: 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG CTC GAT CCA CCT AGG CT-3', AttB2: 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GTA GCG AGA CCA CAG GA-3'. PCR products were first subcloned into the pDONR207 plasmid by BP clonase reaction (Invitrogen) and then transferred into the pMDC123 binary destination vector by LR clonase reaction. All recombinant clones were confirmed by *EcoRI* and *SmaI* restriction and final constructs were verified by sequencing and BLAST analysis (www.arabidopsis.org).

Arabidopsis transgenic plants were generated by *Agrobacterium*-mediated floral-dip transformation using GV3101 bacterial strain. Transformants were grown to maturity and CFP in pollen was observed using confocal microscopy. CFP fluorescence images were captured with Olympus IX81 confocal microscope and SlideBook4 software (Intelligent Imaging Innovations, Inc.).

Transient expression analysis in Tobacco pollen. *AtNPG1*-CFP and *AtNPG1*-YFP binary vector pMDC123 constructs were for transient expression in tobacco pollen. These constructs were introduced into pollen by helium-driven PDS-1000/He particle delivery system (Bio-Rad) following methodology described by³⁰.

Arabidopsis pollen germination analysis. Pollen germination on a glass-bottom 35 mm culture dish (Matte) was performed in a liquid medium essentially as described earlier⁵⁹. Arabidopsis pollen collected from open flowers was incubated on pollen germination medium in the dark at room temperature for 24 hours. Plasmolysis of pollen tubes was performed according to⁶⁰, followed by confocal microscopy and digital fluorescence image capturing. Olympus spinning disk confocal microscope was used to capture images of 10 μ m thick sections with SlideBook4 software.

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Author contributions

This study was conceived by A.S.N.R., M.G. and S.S. All authors were involved in experimental design. M.G. performed yeast two-hybrid screen and isolated PLLs. S.S. performed all other yeast two-hybrid assays, *in vitro* pull-down and localization studies. All authors contributed to writing of the manuscript.

Additional information

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