NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION (NPU), Is Essential for Primexine Deposition and Plasma Membrane Undulation during Microsporogenesis in Arabidopsis thaliana


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Primexine deposition and plasma membrane undulation are the initial steps of pollen wall formation. However, little is known about the genes involved in this important biological process. Here, we report a novel gene, \textit{NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION (NPU)}, which functions in the early stage of pollen wall development in \textit{Arabidopsis thaliana}. Loss of NPU function causes male sterility due to a defect in callose synthesis and sporopollenin deposition resulting in disrupted pollen in \textit{npu} mutants. Transmission electronic microscopy observation demonstrated that primexine deposition and plasma membrane undulation are completely absent in the \textit{npu} mutants. \textit{NPU} encodes a membrane protein with two transmembrane domains and one intracellular domain. In situ hybridization analysis revealed that \textit{NPU} is strongly expressed in microspores and the tapetum during the tetrad stage. All these results together indicate that NPU plays a vital role in primexine deposition and plasma membrane undulation during early pollen wall development.
INTRODUCTION

The pollen wall plays a pivotal role in pollen-stigma recognition, pollen hydration and pollen protection on the stigma and pollen tube elongation (Zinkl et al., 1999; Edlund et al., 2004; Scott et al., 2004). Pollen wall is organized in a highly complex manner, with three major layers: exine, intine and tryphine (Heslop-Harrison, 1971; Piffanelli et al., 1998). The exine is the outer layer of the pollen wall and includes the sexine (tectum and bacula) and nexine (foot layer). Located between the exine layer and plasma membrane, the intine is mainly comprised of cellulose, pectin and proteins (Brett and Waldron, 1990). Tryphine is secreted from the tapetal cells and deposited onto the exine layer during the later stages of pollen development.

Pollen wall development is a complicated process in which a set of elaborate and coordinated mechanisms are carried out by both the microspores and tapetum (Blackmore et al., 2007). It is initiated along with the termination of meiosis (Paxson-Sowders et al., 1997; Piffanelli et al., 1998). Close to the end of meiosis, the microspores are enclosed by the callose wall, which serves as a mold for primexine patterning and also protects the meiocytes and tetrads (Waterkeyn, 1970; Worrall D et al., 1992; Dong X et al., 2005). At the tetrad stage, the primexine is deposited between the plasma membrane and the inner callose wall (Fitzgerald and Knox, 1995; Paxson-Sowders et al., 1997), and the plasma membrane starts to invaginate and form the undulations which are common to various species (Takahashi 1989; Dickinson and Sheldon 1986; Fitzgerald and Knox, 1995; Dahl 1986). On the peaks of the undulations, probaculae are deposited in a well regulated fashion and eventually forms the mature pollen exine layer (Paxson-Sowders et al., 1997). Therefore, the primexine deposition and timely undulation of the plasma membrane both play important roles in early pollen wall formation (Rowley and Skvarla 1975; Takahashi M et al., 1991; Fitzgerald and Knox, 1995).
At present, a number of mutants have been identified that are involved in primexine formation and microspore membrane undulation in *Arabidopsis*. In the *nefl* mutant, the primexine is coarsely developed and some parts of the plasma membrane of the microspore are disrupted at the later stages. The NEF1 protein is predicted to be a plastid integral membrane protein (Ariizumi et al., 2004). In the mutant *dex1*, the primexine deposition is delayed and reduced in thickness, and the undulation of the microspore plasma membrane is scant (Paxson-Sowders et al., 1997, 2001). As a result, the exine pattern is not formed properly and the pollen wall does not form. The DEX1 protein is predicted to be a membrane associated protein that has at least two calcium-binding ligands (Paxson-Sowders et al., 2001). In the *rpg1* mutant, the primexine is irregularly deposited, and as a result only a spotted, irregular exine layer forms. The microspores are ruptured, with cytoplasmic leakage. The RPG1 protein is a membrane protein of the MtN3/saliva family that functions as a sugar transporter (Guan et al., 2008; Chen et al., 2010). These reported proteins are helpful in understanding the importance of primexine deposition and plasma membrane undulation on a molecular basis.

In this paper, we report a new gene, *NPU*, which encodes a transmembrane protein in *Arabidopsis thaliana*. The callose synthesis is severely disrupted, and the absence of primexine deposition and plasma membrane undulation leads to the defects of microsporogenesis and male sterility in the *npu* mutants. Functional analysis of NPU indicates that it plays an important role in primexine deposition and plasma membrane undulation during pollen wall formation in *Arabidopsis*. 
RESULTS

The *npu-1* Mutant Exhibits a Male Sterility Phenotype

The *npu-1* mutant was isolated from a pool of T-DNA-tagged lines (Li et al., 2005). The mutant plants exhibited reduced fertility with normal vegetative growth (Fig. 1A). The average seed yield of the mutant plants was approximately 0.43% of the wild type. Backcrossing with wild-type pollen grains resulted in F1 plants with normal fertility. This result indicated that male fertility was hampered and female fertility was unaffected in the *npu-1* mutant. The progeny of the self-pollinated heterozygous plants segregated wild type and mutant phenotypes at a 3:1 (215:74) ratio, which indicates that the phenotype of *npu-1* is controlled by a single recessive locus.

Molecular Cloning of the *NPU* Gene

To identify the corresponding gene of the *npu-1* mutant, we performed thermal asymmetric interlaced PCR (Liu et al., 1995). A genomic DNA fragment that flanked the left border of T-DNA was obtained. Sequencing of the TAIL-PCR products indicated that the T-DNA was inserted into the seventh exon of a predicted open reading frame of At3g51610 (Fig. 2A). PCR analysis using T-DNA and genome-specific primers showed that all of the mutant plants analyzed (n> 80) were homozygous for the insertion (data not shown). Therefore, the phenotype of the *npu-1* mutants is evidently linked with the T-DNA insertion. To ensure that *NPU* is in fact At3g51610, a complementation experiment was conducted. A 4.7-kb DNA fragment containing the genomic sequence of *NPU*, a sequence 1.5-kb upstream from the translation initiation codon and 800bp downstream from the stop codon, was cloned from wild-type *Arabidopsis* and introduced into the homozygous mutant plants. A total of 40 transgenic plants were obtained and all of them exhibited normal fertility (Fig. 1A). This demonstrated that At3g51610 is the *NPU* gene and the 4.7 kb genomic DNA fragment contains sufficient information for normal *NPU* function.

We also obtained two additional *npu* mutant alleles. The T-DNA-tagged line SALK-062174 (*npu-2*) was obtained from the SIGnAL collection at the Arabidopsis...
Biological Resource Center (Fig. 1A). PCR analysis confirmed the T-DNA insertion into the second exon of *NPU* (Fig. 2A). The *npu-3* mutant was screened from an EMS-induced population in our laboratory. Sequence analysis revealed a point mutation from G to A, in 246th base after the start codon of the *NPU* genomic sequence in the mutant. This transition causes a premature termination at the second exon of At3g51610 (Fig. 2A). The phenotype of both *npu-2* and *npu-3* were similar to *npu-1* (Fig. 1A), except that they are completely male sterile.

**Microsporogenesis of the *npu* Mutant is Disrupted After Meiosis**

To analyze the male fertility defects of the *npu* mutant, Alexander staining (Alexander, 1969) was performed. As shown in Fig. 1B, wild-type pollen grains were stained purple. In the locules of the *npu-1* mutant, there were a few purple stained pollen grains. However, no normal pollen was observed in the locules of either the *npu-2* or *npu-3* mutant. These observations are in agreement with their fertility patterns, in that *npu-1* is partially sterile, and *npu-2* and *npu-3* are completely sterile.

We then performed anther cross sections to compare anther development in the wild-type and *npu-3* plants (Fig. 3, A to L). According to the 14 well-ordered anther development stages in *Arabidopsis* reported by Sanders (1999), the anther development in *npu-3* was similar to that of the wild-type from stage 1 to stage 6. However, at stage 7, the tetrads of *npu-3* seemed to be slightly different from that of wild type. The microspores were more closely wrapped inside the tetrad of *npu-3* mutant than that of wild type (Fig. 3, A and B). At stage 8, the wild type microspores were angular in shape, while the mutant microspores were round in appearance (Fig. 3, C and D). At stage 9, the wild type microspores became vacuolated and developed the basis of a regular exine wall (Fig. 3E), while the *npu-3* microspores began to degenerate (Fig. 3F). During stage 10 and 11, most microspores in *npu-3* degenerated (Fig. 3, H and J), while the wild type locules were filled with well-shaped microspores (Fig. 3, G and I). At stage 12, the microspores of the wild-type were released from the locules following anther dehiscence (Fig. 3K). However, the
microspores in *npu*-3 were completely aborted (Fig. 3L). These results showed that the microspore development started to be abnormal from stage 7. To further examine the defect of microspore development at stage 7, aniline blue was used to stain callose. The result showed that the callose wall between microspores in the tetrads of *npu*-3 was much thinner than that of wild type (Fig. 3M). It was reported that *CalS5* was related with callose synthesis and *A6* was proposed to be related with callose dissolution in *Arabidopsis* (Dong et al., 2005; Hird et al., 1993). The expression of *CalS5* was largely down-regulated in *npu*-3, while the expression level of *A6* was similar to that of wild type (Fig. 3N and O). All these demonstrated that *NPU* affected the callose synthesis in anther development.

**Neither Primexine Deposition Nor Microspore Plasma Membrane Undulation Was Observed in *npu*-3**

To further elucidate the cause of pollen degeneration in *npu* plants, we compared the ultrastructure of the microspore development in the wild-type and *npu*-3 plants by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). As shown in Fig. 1C, the wild-type anther was filled with mature pollen grains after dehiscence, and the pollen grains were plump in shape. By contrast, broken pollen and pollen remnants were observed in the *npu*-3 anther (Fig. 1D). The mature wild type pollen grains were covered with orderly reticulate pollen exine (Fig. 1E). Nevertheless, the reticulate exine pattern was seldom observed on the surface of the *npu*-3 pollen grains (Fig. 1F). These results indicate that the exine pattern formation and the sporopollenin deposition were defective in *npu*-3 pollen.

To further clarify the details of abnormal exine development of *npu*-3 pollen, we performed TEM observation. At the early tetrad stage in the wild type, both the primexine deposition and regular plasma membrane undulation were observed (n=25), and the probaculae was observed growing on the membrane undulation peaks (Fig. 4, A and C). By contrast, neither primexine deposition nor regular undulation of the microspore membrane occurred around the microspores of *npu*-3 (n=28), and the
globular sporopollenin was observed on the surface of plasma membrane (Fig. 4, B and D). After the microspores released from tetrads, the primexine was still visible, and the exine layer formed a regular pattern in wild type (Fig. 4, E). In contrast, the exine pattern was not observed in the npu-3 mutants, and the globular sporopollenin was randomly deposited on the plasma membrane (Fig. 4, F). Later, the primexine disappeared and the typical exine pattern successfully formed, including the tectum, and foot layer in wild type (Fig. 4, G). However, much sporopollenin was accumulated irregularly on the pollen surface of the npu-3 mutant plants instead of the typical exine pattern (Fig. 4H). These TEM observations showed that primexine was not deposited on the plasma membrane and the undulation did not form in npu-3 during microsporogenesis, which resulted in the altered exine pattern formation in the npu-3 mutants.

**NPU Encodes an Unknown Protein Localized to the Plasma Membrane**

The *NPU* gene encodes a functionally unknown protein of 230 amino acids that is predicted to localize to the plasma membrane. Residues 35-177 were predicted to be on the cytoplasmic side of the membrane with residues 1-14 and 201-230 being extracellular. To confirm the subcellular localization of the NPU protein, we fused this gene with GFP driven by the cauliflower mosaic virus 35S promoter. The construct was transformed to the protoplast of the wild-type. As shown in Fig. 2C, the GFP signal could only be detected on the plasma membrane. This result confirmed that NPU is a plasma membrane protein.

Pfam database (http://www.sanger.ac.uk/Software/Pfam/) analysis showed that there was only one copy of the *NPU* gene in *Arabidopsis*. Furthermore, homologues of the NPU protein were identified in various plant species by BLASTp and tBLASTn searches in the NCBI database (Fig. 5A) and The Institute for Genomic Research Functional Genome Database (Fig. 5B). No significant sequence similarity was found outside the plant kingdom (Fig. 5, A and B). The homologs of NPU showed strong conservation in plants, including a rice (*Oryza sativa*) protein with 70% identity, a
grape (*Vitis vinifera*) protein with 81% identity, a poplar (*Populus trichocarpa*) protein with 77% identity, a maize (*Zea mays*) protein with 69% identity, a castor bean (*Ricinus communis*) protein with 75% identity, a sorghum protein (*Sorghum bicolor*) with 70% identity, a moss (*Physcomitrella patens subsp.*) protein with 38% identity, and a green algae (*Chlamydomonas reinhardtii*) with 38% identity to NPU protein, respectively. However, the function of these proteins is presently unknown.

**NPU is Highly Expressed in Microspores and Tapetal Cells**

We performed semi-quantitative RT-PCR to analyze the expression levels in roots, stems, leaves, inflorescences and 10-day-old seedlings. The results showed that *NPU* was expressed in all of these organs, with the strongest expression in buds (Fig. 6A). To obtain the precise expression pattern of *NPU* during anther development, RNA in situ hybridization experiments were performed. The hybridization signal was first detected in the microsporocytes and tapetal cells at stage 5 (Fig. 6B), and became stronger at stage 6 (Fig. 6C). The signal was the strongest in tetrads and tapetal cells at stage 7 (Fig. 6D), and decreased significantly during stages 8 and 9 in developing microspores and tapetal cells (Fig. 6, E and F). These results were in accord with the TEM observations of *npu-3* and together suggested that the -NPU functions mainly at the tetrad stage.
Discussion

*npu* Is a Novel Male Sterile Mutant With a Defect in Sporopollenin Deposition

In *Arabidopsis*, many male sterile mutants have been reported. In this study, we have identified a new male sterile mutant of *npu*. Of the three alleles, the weak allele *npu-1* exhibits reduced male fertility. However, both *npu-2* and *npu-3* are completely male sterile. This shows that *NPU* gene is essential for male fertility. In *npu-1*, T-DNA was inserted in the 3' terminal of this gene, while the T-DNA insertion in *npu-2* and the point mutation in *npu-3* were located in the 5' terminal (Fig. 2A). The position of the T-DNA insertion in this gene was consistent with their fertility phenotype. Cytological analysis revealed that male sterility is caused by the defect in pollen wall formation (Fig. 3). Callose synthesis, callose dissolution, sporopollenin synthesis and deposition play important roles in pollen wall formation during anther development (Worrall et al. 1992; Dong et al. 2005; Azevedo et al. 2009). Our researches showed that callose synthesis and sporopollenin deposition were abnormal in *npu* mutants. The high expression of *NPU* at stage 7 (Fig. 6D), the defective tetrads in the *npu* mutants (Fig. 4, G and I) and the largely down-regulated expression of *CalS5* in *npu* buds indicate that NPU functions at the early stages of pollen wall formation. Besides, primexine deposition and plasma membrane undulation were defective in *npu* mutants, which led to the defect in sporopollenin deposition (Fig. 4). This further confirms that *NPU* is responsible for the early stages of pollen wall formation.

Primexine and Plasma Membrane Undulation

Fitzgerald & Knox (1995) have reported the events in early pollen wall development using rapid freeze-substitution technology. The microspores in the tetrads were initially surrounded by a callose wall after meiosis. After the primexine matrix had assembled between the microspore plasma membrane and callose wall, the plasma membrane gradually undulated and formed crypts. Several mutants were reported to affect the early events of pollen wall development. In the *dex1* mutant, the primexine matrix assembly is both reduced and delayed, and the plasma membrane undulation of
*dex1* fails to form (Paxson-Sowders et al., 1997, 2001). In the *nef1* mutant, the primexine matrix assembly appears to be coarser than that of the wild type, and the undulation of the *nef1* microspore plasma membrane appears to be abnormal (Ariizumi et al., 2004). NPU is a critical protein in primexine matrix assembly, as the primexine matrix is completely absent in *npu* mutants (Fig. 4).

Southworth & Jernstedt (1995) proposed a “Tensegrity Model” to explain the processes of exine patterning. After the primexine matrix is secreted from microspores, the hydrated primexine matrix exerts osmotic pressure on the microspore cell surface resulting in a change in the cytoskeletal tension. Subsequently, the plasma membrane begins to undulate and the exine layer is assembled. In *npu-3*, the primexine matrix is completely absent and plasma membrane is not undulated (Fig. 4). Based on this model, we believe that the plasma membrane undulation is dependent on the primexine matrix during primexine formation. Due to the lack of primexine matrix, the osmotic pressure is not exerted on the microspore surface, and the plasma membrane fails to undulate in the *npu* mutants.

**The Putative Role of NPU**

The NPU protein was predicted to have two extracellular regions, two transmembrane regions and one intracellular region (Fig. 2B). Its subcellular localization in the plasma membrane was confirmed in our research (Fig. 2C). NPU functions sporophytically in anther development because wild type and *npu-3* male sterile phenotype segregated in F2 population with 3:1. It is in agreement with its expression at transcription level in microsporocytes and tapetal cells in anther development (Fig. 6D). In the knockout mutant of NPU (*npu-3*), primexine could not form (Fig. 4). It was reported that the synthesis, secretion and siting of primexine were all controlled by individual microspores (Fitzgerald & Knox, 1995; Perez-Munoz et al., 1993 a, b). Therefore, the NPU protein is likely to be located on the membrane of microspore. It is believed that primexine is composed of polysaccharide material (Heslop-Harrison, 1968). Another
membrane integral protein RPG1, which is involved in the exine patterning in Arabidopsis, has recently been demonstrated to be a sugar transporter (Guan et al., 2008; Chen et al., 2010). Therefore, the NPU protein may also function as a sugar transporter on the microspore membrane, which is responsible for transporting the polysaccharide material essential for primexine matrix formation.

Callose is composed of polysaccharide (β-1,3 glucan), and CalS5 was reported to be responsible for the synthesis of callose deposited at the primary cell wall of meiocyte and tetrads (Dong, et al., 2005). In situ hybridization result also shows that NPU initially expresses at meiocytes with its highest expression at tetrad stage (Fig. 6). The expression pattern of NPU is similar to CalS5 in anther development. It was reported that plant cell maintain a relatively stable level of sugar concentration, and high or low level of sugar may inhibit or activate some other gene expressions (Koch, 1996). In npu mutant, the block of primexine matrix material might lead to a high level of sugar inside microspores/microsporocytes which inhibit the expression of CalS5 in the mutant. However, there might exist other mechanisms that the knockout of NPU affects CalS5 expression. The downregulated expression of CalS5 further affects the callose synthesis as shown in Figure 3. Therefore, the low callose accumulation in npu mutant might be a side effect of the block of primexine matrix formation.
MATERIALS AND METHODS

Plant Material
Arabidopsis plants used in this study are in the ecotype Columbia-0 background. The npu mutant was isolated from a population of T-DNA tagged transformants provided by Dr. Zuhua He (Li et al., 2005). Plants were grown under long-day conditions (16 h of light/8 h of dark) in an approximately 22°C growth room. The herbicide Basta was used to monitor the segregation of the T-DNA inserts.

Microscopy
Plants were photographed with a Nikon digital camera (Coolpix 4500). Flower images were taken using an Olympus dissection microscope with an Olympus digital camera (BX51). Alexander solution was used as described (Alexander, 1969). Plant material for the semi-thin sections was prepared and embedded in Spurr’s resin as previously described (Owen and Makaroff, 1995). For SEM examination, fresh stamens and pollen grains were coated with 8 nm of gold and observed under JSM-840 microscopy (JEOL). For TEM observation, Arabidopsis floral buds from the inflorescence were fixed and embedded as described (Zhang et al., 2007).

Protein Structure Prediction and Phylogenetic Analysis
The SOSUI, TMHMM and SMART programs were run respectively to predict the transmembrane, intracellular and extracellular regions of NPU. The NPU protein sequence was used to search for NPU homologues using the BLASTp and tBLASTn programs. Multiple sequence alignment of full-length protein sequences was performed using ClustalX 2.0, and were displayed using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed and tested by MEGA 4.0 based on the neighbor-joining method (http://www.megasoftware.net/).
Identification and Complementation of the NPU Gene

The identification of the T-DNA insertion was performed using primers that specifically amplify the BAR gene of T-DNA, Bar-F (5'-GCACCATCGTCAACCACACTAC-3') and Bar-R (5'-TGCCAGAAACCCACGTCTAT-3'). For TAIL-PCR, T-DNA left border primers AtLB1 (5'-ATACGACGGATCGTAATTTGTC-3'), AtLB2 (5'-TAATAACGCTGCGGACATCTAC-3') and AtLB3 (5'-TTGACCATCATCATTGCTG-3'), and genomic DNA of the mutant plants were used. The TAIL-PCR procedure and arbitrary degenerate primers were as described (Liu et al., 1995). Close linkage of the T-DNA insertion site and mutant phenotype were analyzed with primer AtLB3 and plant-specific primers, LP (5'-GACTAGGTAATTGATATTGAACC-3') and RP (5'-GTTATGTATAGTGCTTGTG-3'). A DNA fragment of 4.7-kb, including 1.5-kb upstream and 800-bp downstream sequences, was amplified using KOD polymerase (Takara Biotechnology) with primers, CMP-F (5'GAATTCATTTAGAAACAACGACCACCAT3') and CMP-R (5'GTCGACGGGTAAGAGATCCTAACACGCG-3'). The fragment was verified by sequencing and was cloned into a pCAMBIA1300 binary vector (CAMBIA; www.cambia.org.au). The plasmids were transformed into Agrobacterium tumefaciens GV3101 and then introduced into the homozygous mutant plants. Seeds were selected using 20mg/L hygromycin for transformants that were fertile with a homozygous background. The primers used for the verification of the background of the transformants were AtLB3 and plant-specific primers CMPV-F (5'-ATGAAAAACCTTCAACGTCTAT-3') and CMPV-R (5'-TGTCTGTCTGTGAGATGCGATTACTA-3').

Expression Analysis

The full-length cDNA of the wild-type plants without the stop codon was cloned for the GFP-fusion with the primers GFP-F (5'-CCCGGGATGGCGGGCATGCGCTGCTAT-3') and GFP-R (5'-GGTACCCGATGCGGAGATGTGCT-3').
ATCCCCCTTGCCAAATTCTCC-3'). The cDNA was cloned into the pMON530 vector with eGFP. The primers used for the expression analysis of CalS5 and A6 genes were as follows: CalS5 primers: (F: 5'-ATTATTGCAGCTGCTAGAGATG-3' and R: 5'-CTTGTTCAGAGGTTCTGGCTT-3'); A6 primers: (F: 5'-TACCTAAACCGACGAACA-3' and R: 5'-ATGCCAATAAATGGGAGAC-3')

Semiquantitative RT-PCR with 30-cycles was used to analyze the expression level of the NPU gene. The primers were as follows RT-F (5'-CAGGATTACGACCGTGGAAC-3') and RT-R (5'-CATTCATGCTGCTGCTTCC -3'). The DIG (for digoxigenin) RNA Labeling Kit (Roche) and PCR DIG Probe Synthesis Kit (Roche) were used for the RNA in situ hybridization experiment. An NPU specific cDNA fragment of 304bp was amplified and cloned into the pSKvector. Antisense and sense digoxigenin-labeled probes were prepared as described (Zhu et al., 2008).

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Figure Legends

Figure 1. Characterization of the npu mutants.
A. The wild type (Col and Ler), npu allele mutant and the NPU complemented plants. Col: Columbia; Ler: Landsberg erecta. B, Alexander staining of the wild-type (Wt) and npu anthers. SEM examination of dehiscent anthers and pollen grains of the wild-type,(C, E) and npu-3 (D, F) plants. C and D, Dehiscent anthers. Wild-type anther contains numerous pollen grains, while the npu-3 anther is filled with degenerated microspores. Bars=100μm. E, Wild-type pollen grains with a regular reticulate exine pattern. Bar= 5μm. F, Ruptured npu-3 microspores of stages 9 and 10 with defective exine pattern formation. Bar=2μm.

Figure 2. Characterization of the NPU gene.
A. Gene structure of NPU and T-DNA insertion. Black boxes, exons; gray lines, introns; light gray lines, untranslated regions. npu-1, npu-2, and npu-3 are allele mutant lines. Left (LB) and right (RB) borders of T-DNA sequences are shown. DNF and DNR, the left border and right border of the DN fragment. B, Predicted protein structure of NPU. Gray box, plasma membrane; black bar, transmembrane regions. C. Subcellular localization of 35S:GFP inflorescence in transgenic wild-type protoplast. Green fluorescence indicates the localization of 35S:GFP protein. Bar = 20μm. D, Subcellular localization of NPU:GFP inflorescence in transgenic wild-type protoplast. Green fluorescence indicates the localization of NPU:GFP protein; red fluorescence is the autofluorescence of chloroplasts. Bar = 20μm.

Figure 3. Anther development, callose wall and expression analyses of A6 and CalS5 in the wild-type and npu-3 mutant plants.
A and B, Anthers of tetrad stage. npu-3 tetrads (A) seemed to be closely compacted compared with those of the wild type (B). C and D, anthers at stage 8. npu-3 microspores (C) are round compared with wild-type microspores (D). E and F, Anthers at stage 9. npu-3 microspores began to be degenerated (F). G and H, Anthers
at stage 10. Cytoplasm of npu-3 microspores was disintegrated. (H). I and J, Anthers at stage 11. Most npu-3 microspores were degenerated (J). K and L, Anthers at stage 12. Remnants of microspores were observed in npu-3 anther locule (L). M, Callose wall of npu-3 mutant was thinner around the microspores compared with that of the wild type. N, Semiquantative RT-PCR analyses of A6 and CalS5 in wild-type and npu-3 floral buds. TUB, TUBULIN expression as a control. O, Real-time PCR analysis of CalS5 in wild-type and npu-3 floral buds. E, epidermis; En, endothecium; MSp, microspore; PG, pollen grain; RM, remnants of microspores; T, tapetum; Tds, tetrads. Bars =10µm.

**Figure 4.** Ultrastructure of the pollen wall development in wild-type (A, C, E and G) and npu-3 (B, D, F and H) plants. A and C, Tetrad stage. Primexine was deposited between the plasma membrane and the inner callose wall, and the probaculae was growing on the peaks of the undulation. Arrowheads showed the microspore membrane undulation. B and D, Tetrad stage. Arrow indicated the absence of primexine deposition and microspore membrane undulation. E and F, Released microspore stage. Baculae and tectum were formed in wild-type microspores; no baculae or tectum was formed on npu-3 microspores, only globular sporopollenin. G and H, Stage 9 microspore. Sporopollenin was randomly accumulated around the degenerated npu-3 microspores. Ba, baculae; CW, callose wall; DMsp, degenerated microspore; Msp, microspore; Pe, primexine; Pm, plasma membrane; Pb, probaculae; SP, spotted sporopollenin; Tc, tectum. Bars = 1µm.

**Figure 5.** Phylogenetic analysis of NPU and homologous proteins.
A, Multiple alignments of NPU and its homologues. Black bars, putative transmembrane regions; Black thin line, predicted intracellular region; Black curved sweeping lines, predicted extracellular domains. Protein sequence of NPU homologs in plants are as follows: Sh, Sorghum bicolor, SORBIDRAFT_09g005000; Zm, Zea mays, LOC100278212; Os, Oryza sativa, Os05g0168400; Rc, Ricinus communis, XP_002532862.1; Pp, Populus trichocarpa, XP_002323065.1; Vv, Vitis vinifera,
XP_002270471.1; Ph, *Physcomitrella patens*, XP_001784831.1; Ch, *Chlamydomonas reinhardtii*, XP_001690235.1. B, Unrooted phylogenetic tree of NPU and its homologous proteins. Protein sequences of NPU and its homologs were analyzed with the neighbor-joining method by MEGA 4.0 software. The numbers at the nodes represent percentage bootstrap values based on 1000 replications.

**Figure 6.** Expression analysis of *NPU*.

A, Semiquantitative RT-PCR of RNA isolated from various tissues with *NPU* and β-**TUBULIN** specific primer sets. Inf, Inflorescence; R, root; S, stem; L, leaf; B, buds; Sdl, seedling. B, In situ hybridization of the *NPU* transcript in a stage 5 anther with an antisense probe. C, In situ hybridization of the *NPU* transcript in a stage 6 anther with an antisense probe. D, In situ hybridization of the *NPU* transcript in a stage 7 anther with an antisense probe. F, In situ hybridization of the *NPU* transcript in a stage 8 anther with an antisense probe. G, In situ hybridization of the *NPU* transcript in a stage 9 anther with an antisense probe. H, In situ hybridization of the *NPU* transcript in a stage 7 anther with a sense probe.
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**Figure 1.** Characterization of the *npu* mutants.

A, The wild type (Col and Ler), *npu* allele mutant and the *NPu* complemented plants. Col: Columbia; Ler: Landsberg erecta. B, Alexander staining of the wild-type (Wt) and *npu* anthers. SEM examination of dehiscent anthers and pollen grains of the wild-type, (C, E) and *npu-3* (D, F) plants. C and D, Dehiscent anthers. Wild-type anther contains numerous pollen grains, while the *npu-3* anther is filled with degenerated microspores. Bars=100μm. E, Wild-type pollen grains with a regular reticulate exine pattern. Bar= 5μm. F, Ruptured *npu-3* microspores of stages 9 and 10 with defective exine pattern formation. Bar=2μm.
Figure 2. Characterization of the NPU gene.

A, Gene structure of NPU and T-DNA insertion. Black boxes, exons; gray lines, introns; light gray lines, untranslated regions. npu-1, npu-2, and npu-3 are allele mutant lines. Left (LB) and right (RB) borders of T-DNA sequences are shown. DNF and DNR, the left border and right border of the DN fragment. B, Predicted protein structure of NPU. Gray box, plasma membrane; black box, transmembrane region. C, Subcellular localization of 35S:GFP inflorescence in transgenic wild-type protoplast. Green fluorescence indicates the localization of 35S:GFP protein. Bar = 20 μm. D, Subcellular localization of NPU:GFP inflorescence in transgenic wild-type protoplast. Green fluorescence indicates the localization of NPU:GFP protein; red fluorescence is the autofluorescence of chloroplasts. Bar = 20 μm.
Figure 3. Anther development, callose wall and expression analyses of A6 and CalS5 in the wild-type and npu-3 mutant plants.

A and B, Anthers of tetrad stage. npu-3 tetrads (A) seemed to be closely compacted compared with those of the wild type (B). C and D, anthers at stage 8. npu-3 microspores (C) are round compared with wild-type microspores (D). E and F, Anthers at stage 9. npu-3 microspores began to be degenerated (F). G and H, Anthers at stage 10. Cytoplasm of npu-3 microspores was disintegrated (H). I and J, Anthers at stage 11. Most npu-3 microspores were degenerated (J). K and L, Anthers at stage 12. Remnants of microspores were observed in npu-3 anther locule (L). M, Callose wall of npu-3 mutant was thinner around the microspores compared with that of the wild type. N, Semiquantitative RT-PCR analyses of A6 and CalS5 in wild-type and npu-3 floral buds. TUB, TUBULIN expression as a control. O, RT-PCR analyses of CalS5 in wild-type and npu-3 floral buds. E, epidermis; Ep, endothecium; MSP, microspore; PG, pollen grain; RM, remnants of microspores; T, tapetum; Tds, tetrads. Bars =10μm.
**Figure 4.** Ultrastructure of the pollen wall development in wild-type (A, C, E and G) and *npu-3* (B, D, F and H) plants. A and C, Tetrad stage. Primexine was deposited between the plasma membrane and the inner callose wall, and the probaculæ were growing on the peaks of the undulation. Arrowheads showed the microspore membrane undulation. B and D, Tetrad stage. Arrow indicated the absence of primexine deposition and microspore membrane undulation. E and F, Released microspore stage. Baculæ and tectum were formed in wild-type microspores; no baculæ or tectum was formed on *npu-3* microspores, only globular sporopollenin. G and H, Stage 9 microspore. Sporopollenin was randomly accumulated around the degenerated *npu-3* microspores. Ba, baculæ; CW, callose wall; DMsp, degenerated microspore; Msp, microspore; Pe, primexine; Pm, plasma membrane; Pb, probaculæ; SP, spotted sporopollenin; Tc, tectum. Bars = 1 μm.
Figure 5. Phylogenetic analysis of NPU and homologous proteins.

A, Multiple alignments of NPU and its homologs. Black bars, putative transmembrane regions; Black thin line, predicted intracellular region; Black curved sweeping lines, predicted extracellular domains. Protein sequence of NPU homologs in plants are as follows: Sh, *Sorghum bicolor* SORDIDRAFT_09g005000; Zm, *Zea mays*, LOC100278212; Os, *Oryza sativa*, Os05g0168400; Rc, *Ricinus communis*, XP_002532862.1; Pp, *Populus trichocarpa*, XP_002323065.1; Vv, *Vitis vinifera*, XP_002270471.1; Ph, *Physcomitrella patens*, XP_001794831.1; Ch, *Chlamydomonas reinhardtii*, XP_001690235.1. B, Unrooted phylogenetic tree of NPU and its homologous proteins. Protein sequences of NPU and its homologs were analyzed with the neighbor-joining method by MEGA 4.0 software. The numbers at the nodes represent percentage bootstrap values based on 1000 replications.
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