Supporting Online Material for

SRPP, a cell-wall protein, involved in development and protection of seeds and root hairs in *Arabidopsis thaliana*

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Materials and Methods

Peptide preparation and LC-MS analyses

Crude membrane fractions were prepared from roots of 14-d-old seedlings as previously reported (Fukao et al. 2011, Tanaka et al. 2014). The proteins in the crude membrane fractions were separated on a 12.5% SDS gel, which was stained using Flamingo Fluorescent Gel Stain (Bio-Rad). Proteins in each gel slice were isolated and treated with trypsin (Trypsin Gold, Mass Spec grade, Promega) in 50 mM ammonium bicarbonate. The digested peptides were filtered using Millipore Ultrafree-MC centrifugal filters (PVDF, 0.45 μm, Millipore) at 12,000× *g* for 15 min and used for LC-MS analysis. The peptides were loaded on a column (L-Column, CERI), and then eluted using a gradient of 5–45% (v/v) acetonitrile in 0.1% (v/v) formic acid. The eluted peptides were introduced into an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) with a flow rate of 500 nl min^{-1} and a spray voltage of 2.0 kV. The range of the MS scan was m/z 450–1,500. The three largest peaks were subjected to MS/MS analysis. MS/MS spectra were analyzed using the MASCOT server (version 2.4) in-house (http://www.matrixscience.com/) and compared with proteins registered in TAIR10. The following Mascot search parameters were used: threshold of the ion score cutoff, 0.05; peptide tolerance, 10 ppm; MS/MS tolerance, 0.5 D; and peptide charge, 2+ or 3+. The search was also set to allow one missed cleavage by trypsin, carbamidomethylation modification of cysteine residues, and variable oxidation modification of methionine residues.

RNA extraction and real-time RT-PCR

Total RNA fractions were prepared from roots of 18-day-old plants using a QIA shredder and RNeasy Mini kit (Qiagen). RNA (1 μg) was converted into cDNA using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). Real-time reverse transcription-PCR (RT-PCR) analysis was performed with a Thermal Cycler Dice™ Real Time System TP800 (Takara) using a THUNDERBIRD™ SYBR qPCR Mix (Toyobo). The primer sets used for the real-time RT-PCR were ATGGAGTCTTCAAGTCCCCAC (Fw) and CCGGAAGCATTCTTAGAATCTG (Rv) for *SRPP*,

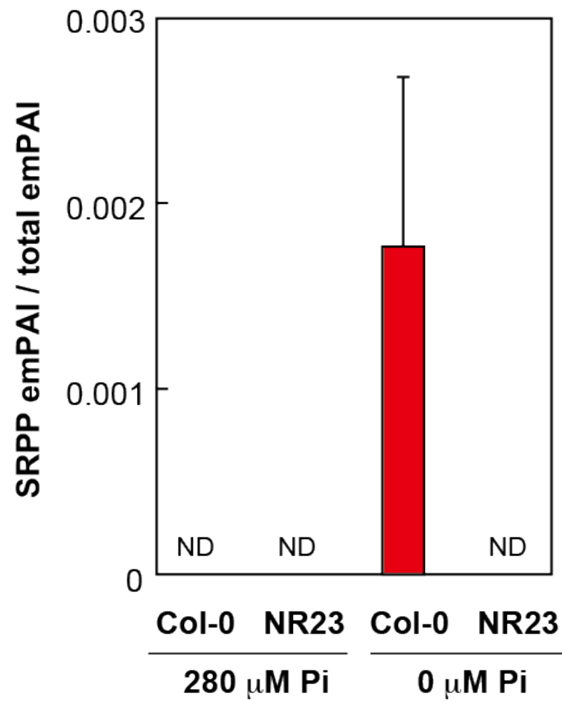
TAACGTGGCCAAAATGATGG (Fw) and GTTCTCCACAACCGCTTGGT (Rv) for *PP2A* (*PROTEIN PHOSPHATASE 2A*) (Nieto *et al*., 2015). Relative mRNA contents were normalized to the *PP2A* transcript.

References

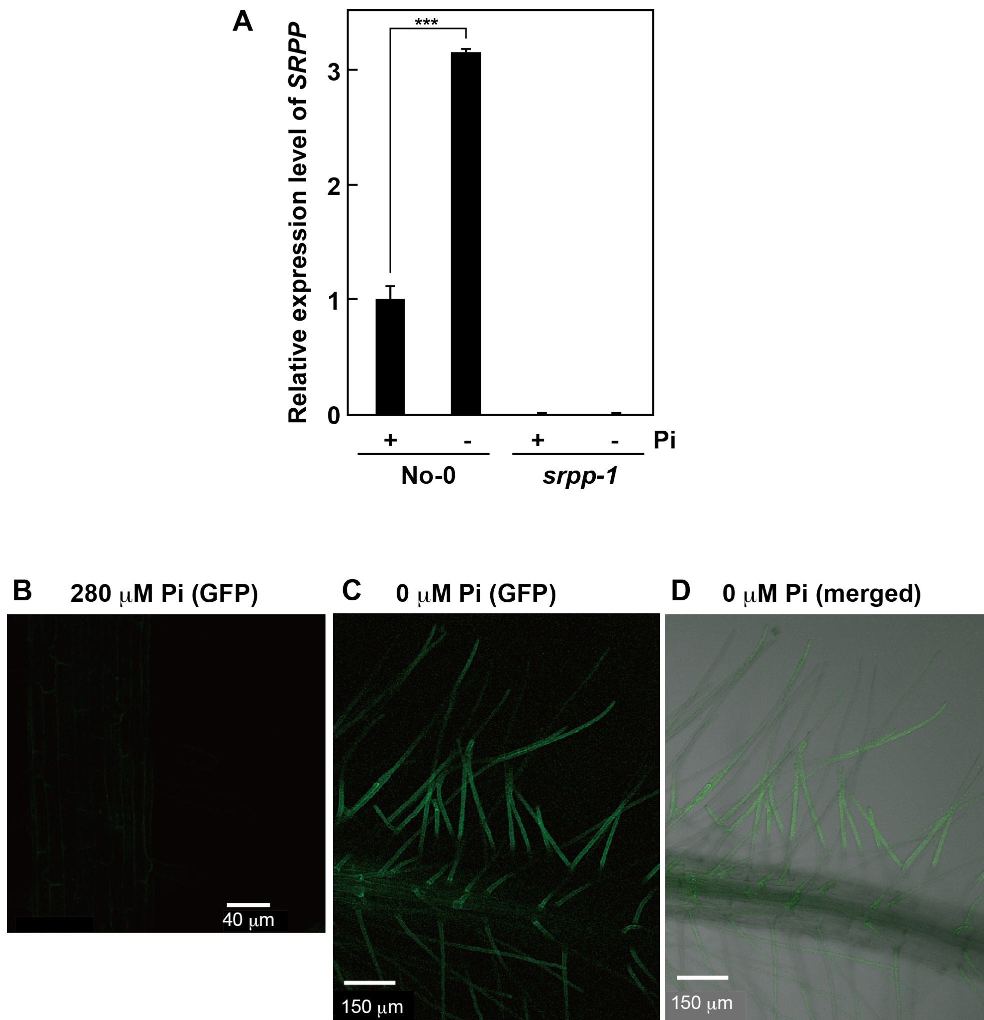
Fukao Y, Ferjani, A., Tomioka, R., Nagasaki, N., Kurata, R., Nishimori, Y. et al. (2011) The iTRAQ analysis reveals mechanisms of growth defects due to excess zinc in *Arabidopsis thaliana. Plant Physiol* 155:1893–1907.

Tanaka, N., Kato, M., Tomioka, R., Kurata, R., Fukao, Y., Aoyama, T. et al. (2014) Characteristics of a root hair-less line of *Arabidopsis thaliana* under physiological stresses. *J. Exp. Bot.* 65: 1497–1512.

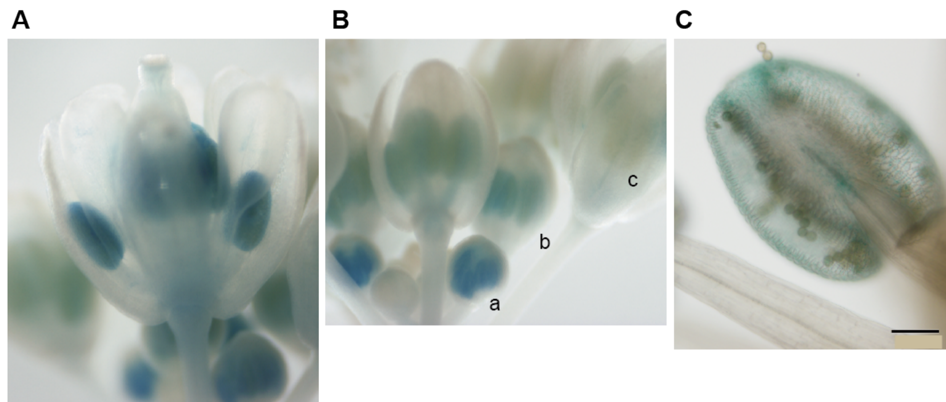
Nieto, C., Lóapez-Salmerón, V., Daviére, J.-M. and Prat, S. (2015) ELF2-PIF4 interaction regulates plant growth independently of the evening complex. Current Biol. 25: 187–193.



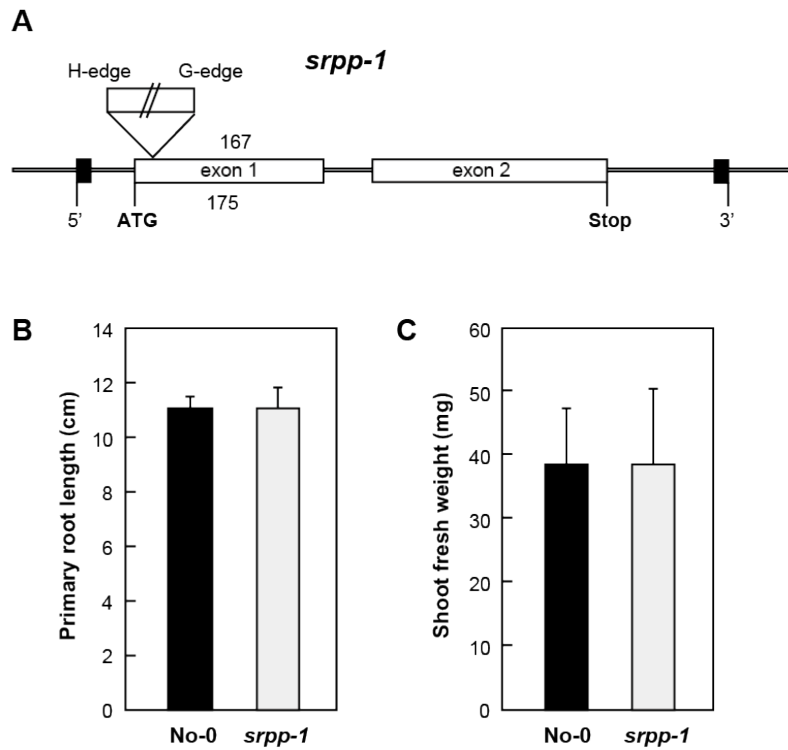
Supplementary Fig. S1. A protein identified specifically in root hairs of wild-type plants. Col-0 and NR23 plants were grown under normal and Pi-deficient conditions for 2 weeks. Crude membrane fractions were prepared and subjected to proteomics MS analysis. SRPP was identified only in the crude membrane fractions from wild-type plants under Pi-deficient conditions.



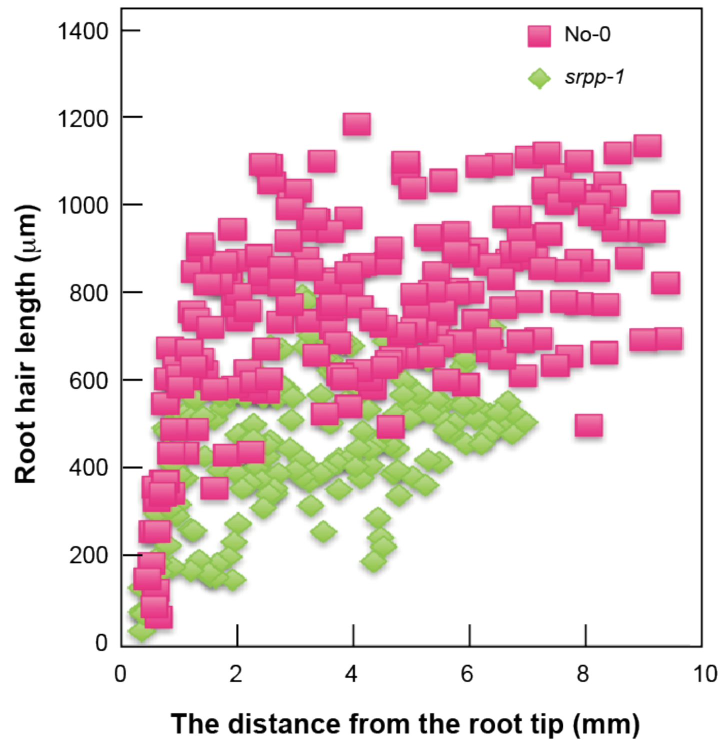
Supplementary Fig. S2. (A) Expression levels of *SRPP* in No-0 and *srpp-1* under Pi-deficient conditions. Total RNA fractions were prepared from roots of 18-d-old plants of No-0 and *srpp-1* and then subjected to real-time RT-PCR analyses of mRNA levels of *SRPP*. Three replicates with 50 plants were averaged and the SD is shown. Seedlings were grown in modified Hoagland medium with (+) or without (-) phosphorus. Values are expressed as mean ± SD; n = 3. Significant differences are indicated by asterisks (***P < 0.005). (B, C, D) SRPP is expressed in root hairs under Pi-deficient conditions. SRPP-GFP lines were germinated and selected on MS medium supplemented with kanamycin for 11 d. Seedlings were then transplanted to Hoagland medium containing 280 (A) or 0 μM (B, C) phosphorus in vertical plates and cultivated for 6 d. Roots were observed using a confocal laser scanning microscope. (C) Merged images of GFP (green) and differential interference contrast images.



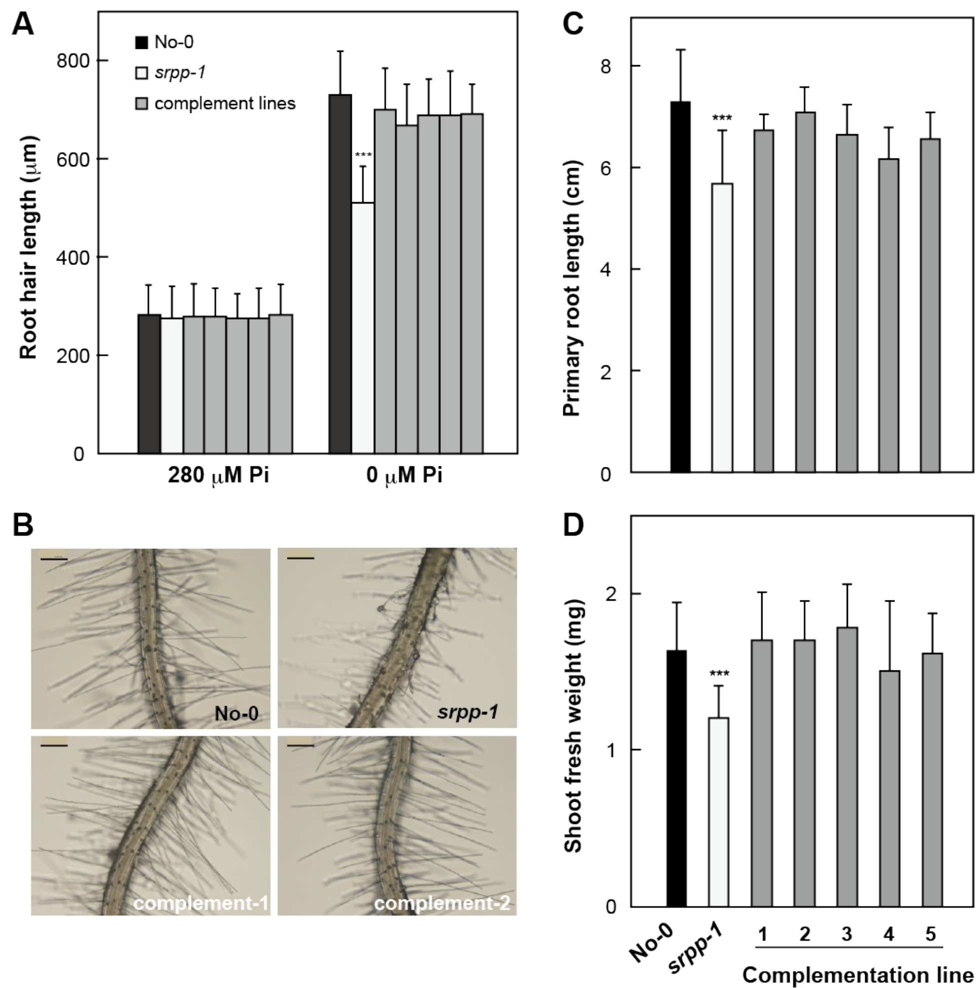
Supplementary Fig. S3. *SRPP* is expressed in anthers. *SRPP*p::GUS lines were germinated on MS medium for 3 weeks. Seedlings were then transplanted to vermiculite pots and cultivated for one month. (A) Flowers were GUS stained at an early stage and observed using a stereoscope. (B) Flowers with mixed stages from young (a), early developing (b), and developed stages (c) were observed. (C) An anther was observed using an optical microscope. Eight independent T_3 homozygous lines were observed. The epidermis of anthers exhibited GUS staining, especially in young developing anthers. Scale bar, 100 μm.



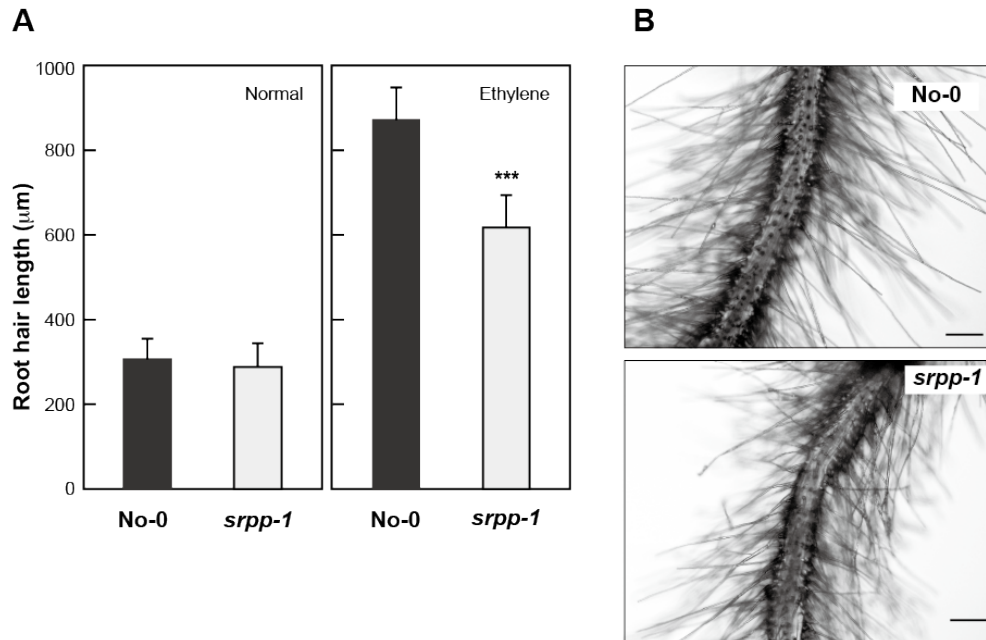
Supplementary Fig. S4. Transposon integration site in *srpp-1* and growth of *srpp-1*. (A) The transposon integration site in the *SRPP* gene is shown. Filled boxes show the 5'- and 3'-UTRs and open boxes show exons. The insertion site and the orientation of the transposon are indicated. (B, C) No-0 and *srpp-1* were grown on Hoagland medium in vertical plates for 24 d. Primary root length (B) and shoot fresh weight (C) were measured. Error bars show SDs; n = 10.



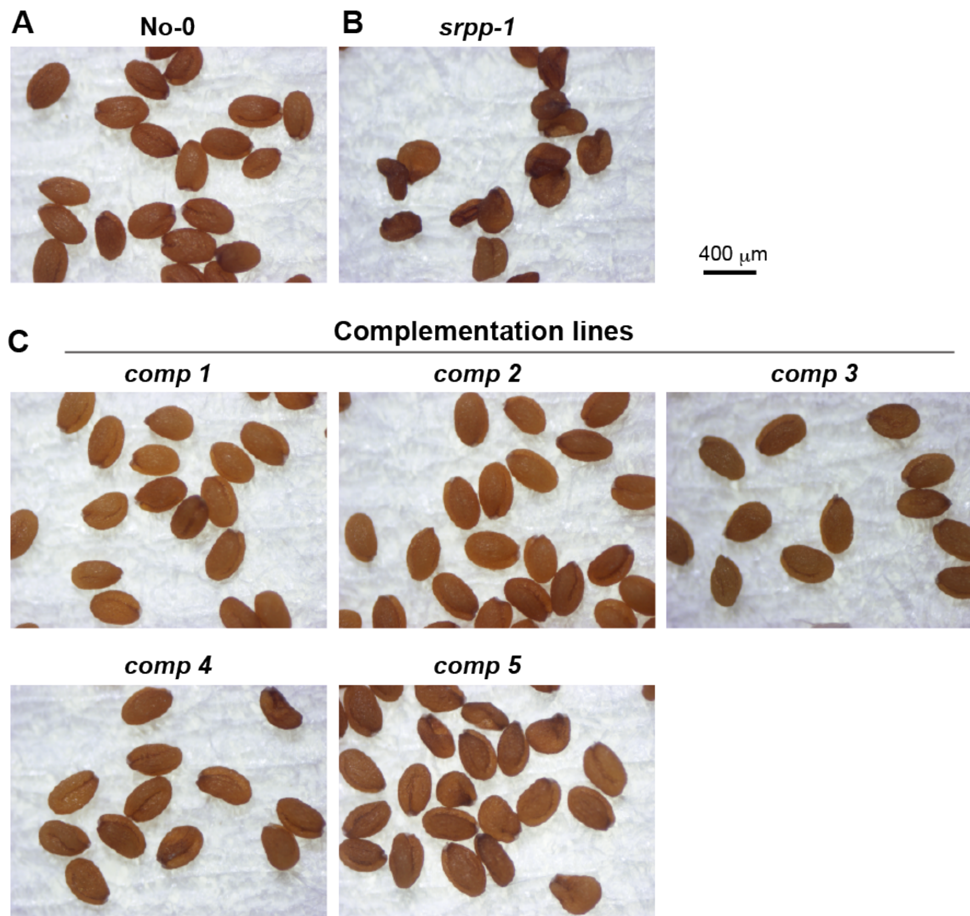
Supplementary Fig. S5. The length of each root hair and its distance from the root tip on No-0 and *srpp-1* roots under Pi-deficient conditions. No-0 and *srpp-1* were grown on Hoagland medium without Pi in vertical plates. Root hairs on 14-d-old roots of each of five seedlings were observed and the length of each root hair (on the *y*-axis) is plotted against the distance from the root tip (on the *x*-axis).



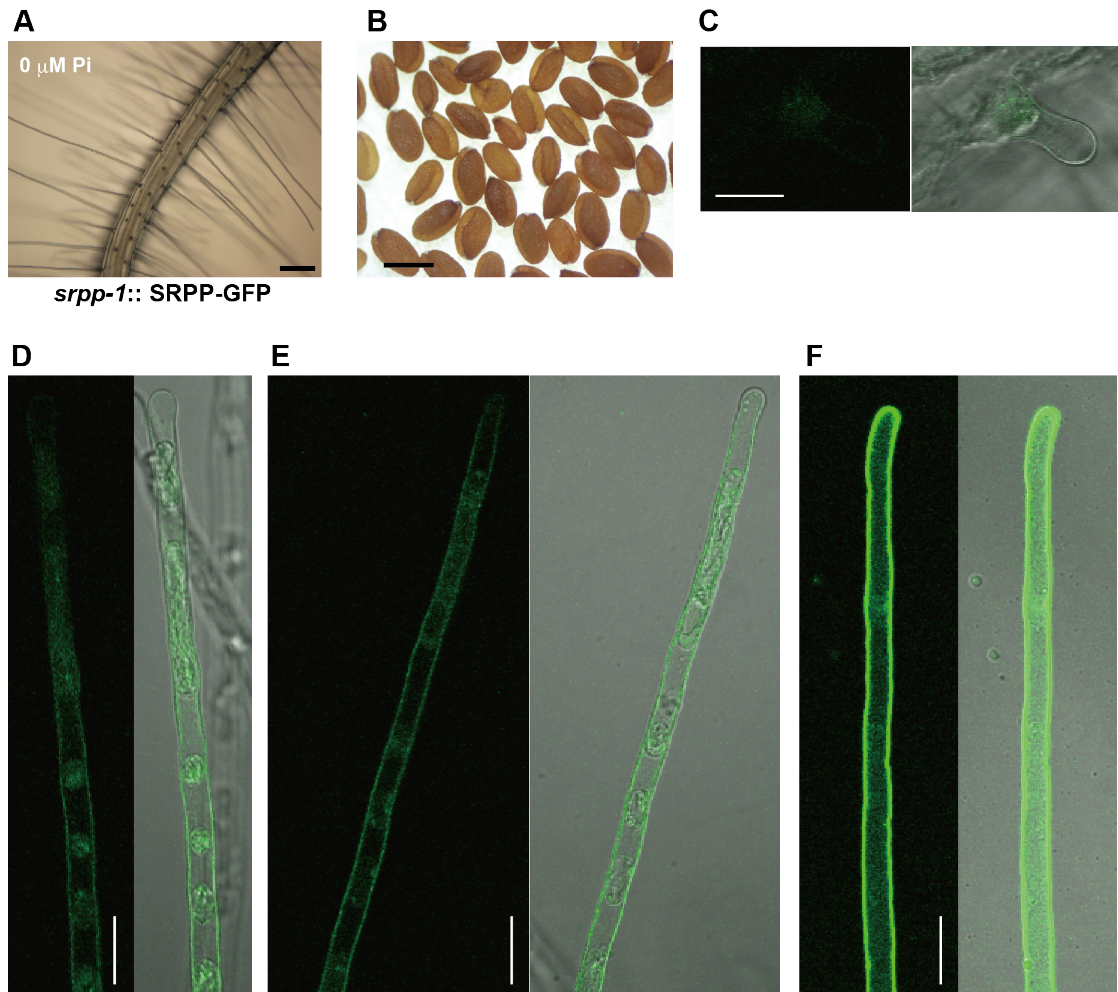
Supplementary Fig. S6. The root hair phenotype of *srpp-1* and the growth of primary roots and shoots under Pi-deficient conditions was restored in the complementation lines. No-0, *srpp-1*, and five complementation lines were grown on Hoagland plates containing 0 or 280 μM Pi for 13 d. (A) Each of the five seedlings were used to measure root hair length. Mature root hairs at the region around 30 mm (under normal conditions) or slightly less than 10 mm (under Pi-deficient conditions) from the root tip were measured. Error bars show the SDs; $n = 50$. Asterisks indicate statistical differences at $^{***}P < 0.005$ compared with No-0. (B) In seedlings grown on the 0 μM Pi Hoagland plates, typical root hairs at the region around 15 mm from the root tip were observed using an optical microscope. (C, D) No-0, *srpp-1*, and complementation lines were grown on Hoagland plates containing 0 or 280 μM Pi for 24 d. Primary root length (C) and shoot fresh weight (D) were measured. Error bars show the SDs; $n = 10$. Asterisks indicate significant differences at $^{***}P < 0.005$ compared with No-0.



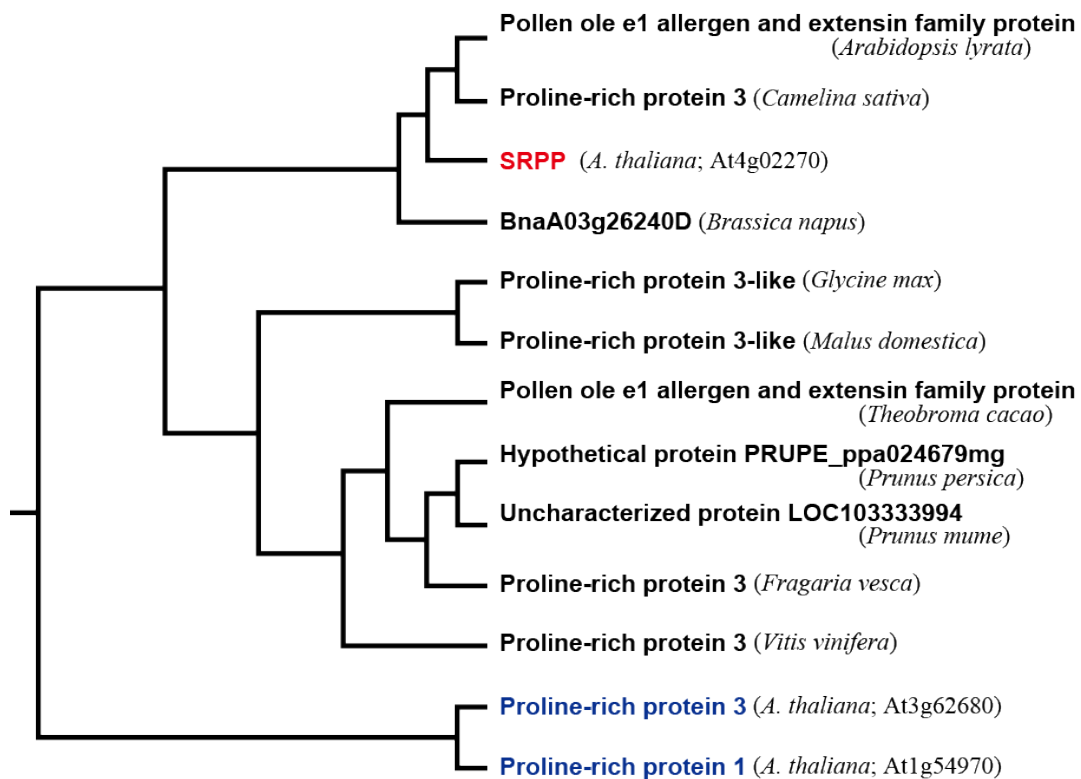
Supplementary Fig. S7. Root hairs of *srpp-1* were shorter than those of No-0 when treated with ethylene. No-0 and *srpp-1* were grown on MS vertical plates for 5 d, and then put into an airtight and transparent box to treat with 10 ppm ethylene for 1 week. The control plants were grown under the same conditions without ethylene. After incubation for 1 week, the ethylene concentration in the box was approximately 8 ppm. (A) Each of the five seedlings was used to measure the length of mature root hairs. Error bars show the SDs; $n \geq 80$. Asterisks indicate significant differences at $^{***}P < 0.005$ compared with No-0 (two-sided Student's *t*-test). (B) Typical root hairs of No-0 and *srpp-1* that were around 10–20 mm from the root tip were observed using an optical microscope. Scale bars, 200 μm.



Supplementary Fig. S8. The phenotype of *srpp-1* seeds was restored in the complementation lines. No-0 (A), *srpp-1* (B), and complementation lines (C, *comp1* to *comp5*) grown on MS plates for 3 weeks were transplanted to pots containing vermiculite and cultivated for approximately 2.5 months. Plants were watered with 2,000-fold diluted Hyponex solution. After plants had matured and dried, seeds were harvested and observed using a stereoscope.



Supplementary Fig. S9. The *SRPP*p::SRPP-GFP/pGWB604 was introduced into *srpp-1*. After selection with 10 mg ml^{-1} Basta, multiple T_2 heterozygous or T_3 homozygous lines were used for experiments. *srpp-1*::SRPP-GFP lines grown for 11 d were transplanted to Hoagland plates without Pi and cultivated for 6 d. (A) Root hairs of 17-d-old *srpp-1*::SRPP-GFP seedling. (B) Seeds harvested from *srpp-1*::SRPP-GFP. (Left panels, C to F) Various elongation stages (C, early; D and E, middle stage; F, fully elongated) of root hair cells of *srpp-1*::SRPP-GFP, which were treated with plasmolysis by 20% (w/v) sucrose for 10 min observed and then observed by CLSM. (Right panels, C to F) Merged images of GFP (green) and differential interference contrast images. Bars, 200 μm (A), 400 μm (B), and 20 μm (C to F).



Supplementary Fig. S10. Phylogenetic tree of SRPP. A rooted phylogenetic tree of 10 related orthologous proteins (in black) and the two highly related paralogous proteins (in navy) was made by CLUSTALW (http://www.genome.jp/tools/clustalw/) based on the amino acid sequences.

A. thaliana SRPP
Pollen ole e1 allergen and extensin family protein (*A. lyrata*)
BnaA03g26240D (*Brassica napus*)
Hypothetical protein PRUPE_ppa024679mg (*Prunus persica*)
Proline-rich protein 3 (*Fragaria vesca*)
Proline rich protein 3-like (*Glycine max*)
Proline-rich protein 3 (*Arabidopsis thaliana*, At3g62680)
Proline-rich protein 1 (*Arabidopsis thaliana*, At1g54970)

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MAFSRLS---FAASLIVFSSLIISSVAYYGNEAD----------------------------------------- 31
MAFSRLS---FAASLVVFSSLIISSVAYYGNEAD----------------------------------------- 31
MAFSRLS---FAASLVVFSSLIISSVANYGNEVN----------------------------------------- 31
MALTRFL---FATSMLLLTFLVIASASDYGSAPKP-KVDDH---------------------------------- 37
MALTRFL---FPTCLVVLSLLLIDVSADYGYASKPPQVEQPKPPQVGK--------------------------- 45
MKMAYFHSTCFLVFMLIASLLVPGRGGDYG--------------------------------------------- 30
MAITRSS----LAICLILSLVTITTADYYSPSSPPVYKSPEHKPTLPSPVYTPPVYKPTLSPPVYT----KPTIP 67
MAITRAS----FAICILLSLATIATADYYAPSSPPVYTSPVNKPTLPPPVYTPPVHKPTLPPPVYTPPVHKPTLS 71

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---------------------------------------------------------------------------
---------------------------------------------------------------------------
---------------------------------------------------------------------------
---------------------------------------------------------------------------
---------------------------------------------------------------------------
PPVYTPPVYKHTP-SPPVYTKPTIPPPVYTPPVYKPTLSPPVYTKPTIPPPVYTP--------------PVYKPT 127
PPVYTKPTLPPPAYTPPVYNKPTLPAPVYTPPVYKPTLSPPVYTKPTLLPPVFKPTLSPPVYTKPTLSPTVYKPT 146

----------------------------------------------PETGK----------LIP--IAVEGIIKC 48
----------------------------------------------PETGK----------LIP--IAVEGVIMC 48
----------------------------------------------PETGK----------FIP--VAVEGVIMC 48
-----------------------------------------DTPKLPKVEKPKPQEVYAKQLPH-IIGIQGLVLC 70
-----------------------------------PEAPGVEKPNPPQVEKPKLDGSYGELLPHNLFGIGGLVLC 85
----------------------------------------------PEEEK----------LLSKTIGIQGIVYC 49
---PVYTKPTIPPPVYTP----PVY-KPTPSPPVYKKSPSYSSPPPPYVPKPTYTPTTKPYVPEILKAVDGIILC 194
LSPPVNNKPSLSPPVYKPTLSPPVYTKPTLPPPVYKKSPSYS-PPPPFAPKPTYTPPTKPYVPEIIKAVGGIILC 215
                                                                    1st

KSGGKTYPIQGATARIACVKVDAYGNELVPISILSSKTDAKGYFIATIFPSQLRAG----RTVTKCKTYLYKSPL 119
KSGGKTYPIQGATARIACVKVDAYGKEIVPISIMSSKTDAKGYFFATIFPSQLRAG----RTVTKCKAYLYKSPI 119
KSGDKSYPIQGATARIACVKTDAYGKEIVPISIMSSKTDAKGYFFATLFPSQLRAG----RTVTKCKVFLYQSPI 119
KSGLKSFPIKGAVARITCLAEDEYGYETAPFSILSGATDAKGYFFATLSPSELQD----KWKLTECKAFLDYSPF 141
TSGLKAFPIQQAVARITCVGEDENGYETAPFSMLAGATDAKGYFFATLSPSKLEDSYNKKWKLTECKAFLDSSPL 160
KSASKLTPLEGALTRIACEAVDEYGFETTPFSFLSEATDSKGYFLATLSPQEVEG----KGVLKECRAFLDASPL 120
KNGYETYPILGAKIQIVCSDPASYGKSNTEVVIYSNPTDSKGYFHLSLTS---------IKDLAYCRVKLYLSPV 260
KNGYETYPIQGAKAKIVCSERGSYEKSKNEVVIYSDPTDFKGYFHVVLTH---------IKNLSNCRVKLYTSPV 286
                                                              2nd

ADCDFPTDVNKGVRGQPLSTYR--ILQDKSFKLYWAGPFFYTSE--PTYY       165
ADCDFPTDVNKGVRGQTLSKYR--VLEDKSFKLYWAGPFFYTSE--PTYY       165
ADCDFPTDVNKGVRGMSLNKYR--VLEDKSFKLYWAGPFFYTSE--PTYY       165
QYCQVPTDVNHGITGHLLASYR--IINTKKIKLYSVGPFFYTSETEPKSTPNNGY  194
ESCKVPTDANHGISGAPLASYR--TLNAKNMKLFSVGPFFYTSE--PKPAPN-GY  210
NNCSYPTDVNKGISGAVLRFHR--FLHDKKMKLYTVGPFQFTAS--PTPISD-GY  170
ETCKNPTNVNKGLTGVPLALYGYRFYPDKNLELFSVGPFYYTG-PKAAPATPKY   313
ETCKNPTNVNKGLTGVPFSMY------SDKNLKLFNVGPFYFTAGSKAAPATPRY   335
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Supplementary Fig. S11. Sequence alignment of SRPP and homologous proteins. The primary sequences of SRPP (blue) and seven homologous proteins, such as Pro-rich proteins, are shown. Predicted signal sequences are underlined. Highly conserved residues, which are identical or conservative (L/I, F/Y, S/T, and K/R) in seven of the eight proteins, are shown in red. AGI codes are shown for proteins of *A. thaliana*. Two highly conserved regions of 53 and 50 residues in SRPP are each marked by a double underline.

Table S1 Primer sets used for gene cloning.

A. *SRPP*p::SRPP

Fw: 5’-CACCATGTTAGAGGAGAGTACATG-3’

Rv: 5’-GTACGATCACCATTATGCATAGTC-3’

B. *SRPP*p::GUS

Fw: 5’-CACCATGTTAGAGGAGAGTACATG-3’

Rv: 5’-TGAGAGGCGTGAGAAAGCC-3’

C. *SRPP*p::SRPP-GFP

Fw: 5’-CACCATGTTAGAGGAGAGTACATG-3’

Rv: 5’-GTAGTAAGTAGGTTCAGACGTGTAGAAG-3’