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RESEARCH PAPER

Proline-rich protein-like PRPL1 controls elongation of root hairs in *Arabidopsis thaliana*

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Abstract

The synthesis and composition of cell walls is dynamically adapted in response to many developmental and environmental signals. In this respect, cell wall proteins involved in controlling cell elongation are critical for cell development. Transcriptome analysis identified a gene in *Arabidopsis thaliana*, which was named proline-rich protein-like, *AtPRPL1*, based on sequence similarities from a phylogenetic analysis. The most resemblance was found to *AtPRP1* and *AtPRP3* from *Arabidopsis*, which are known to be involved in root hair growth and development. In *A. thaliana* four proline-rich cell wall protein genes, playing a role in building up the cross-connections between cell wall components, can be distinguished. *AtPRPL1* is a small gene that in promoter::GUS (β-glucuronidase) analysis has high expression in trichoblast cells and in the collet. Chemical or mutational interference with root hair formation inhibited this expression. Altered expression levels in knock-out or overexpression lines interfered with normal root hair growth and etiolated hypocotyl development, but Fourier transform-infrared (FT-IR) analysis did not identify consistent changes in cell wall composition of root hairs and hypocotyl. Co-localization analysis of the AtPRPL1–green fluorescent protein (GFP) fusion protein and different red fluorescent protein (RFP)-labelled markers confirmed the presence of AtPRPL1–GFP in small vesicles moving over the endoplasmic reticulum. Together, these data indicate that the AtPRPL1 protein is involved in the cell's elongation process. How exactly this is achieved remains unclear at present.

Key words: *Arabidopsis thaliana*, cell expansion, hypocotyl, pollen Ole allergen, root hairs.

Introduction

The plant cell wall is a rigid extracellular matrix that is highly dynamic and involved in many developmental processes, such as cell shape, anisotropic growth, defence against pathogenic attacks, and other processes (Cosgrove, 1999, 2000; Huckelhoven, 2007). In dicotyledons, the cell wall is composed of cellulose microfibrils that are tethered by hemicelluloses. Xyloglucan, the major hemicellulose in plants such as *Arabidopsis*, is associated with cellulose by hydrogen bonds (Valent and Albersheim, 1974; Hayashi, 1989; Acebes *et al.*, 1993; Hayashi *et al.*, 1994) and this cellulose–hemicellulose network is embedded in a highly hydrated pectin matrix (Carpita and Gibeaut, 1993). The cell wall also contains structural proteins that comprise up to 10% of its dry weight (Varner and Lin, 1989). According to the amino acid composition and the glycosylation pattern, these wall proteins can be divided into different groups based on the high abundance of certain amino acids (Johnson *et al.*, 2003).

The two major groups are the hydroxyproline-rich glycoproteins (HRGPs) and the glycine-rich proteins (GRPs) (Sachetto-Martins *et al.*, 2000). The former include extensins (Showalter, 1993; Cassab, 1998), arabinogalactan-proteins (AGPs) (Fincher *et al.*, 1983; Nothnagel, 1997), and the proline-rich proteins (PRPs) (Showalter, 1993; Cassab, 1998).

These structural cell wall proteins are secreted as monomers into the wall where they can be cross-linked. How these cross-links are made still remains largely unknown. There is, however, evidence for a peroxidase-mediated reaction that forms intermolecular isodityrosine links (Bradley *et al.*, 1992; Brisson *et al.*, 1994; Schnabelrauch *et al.*, 1996), which seems necessary to slow down or completely stop cell elongation in 1-aminocyclopropane-1-carboxylic acid (ACC)-treated *Arabidopsis* roots (De Cnodder *et al.*, 2005). Recently Cannon *et al.* (2008) showed that in the root-, shoot-, hypocotyl-defective (*rsh*) mutant a mutation is found in the gene encoding an extensin (AtEXT3) which is located in the cell plate, the cross wall, and in mature cell walls. This extensin is a HRGP and contains a hydrophobic isodityrosine cross-link motif (YVY). *In vitro*, a peroxidase catalyses the formation of insoluble RSH gels with concomitant cross-linking and it is suggested that *in planta* this positively charged extensin scaffold binds with negatively charged pectins to create an extensin pectate coacervate (Valentin *et al.*, 2010). MacDougall *et al.* (2001) indeed described that extensins have the potential to act as non-covalent cross-linking agents in pectin networks, forming elastic gels. Furthermore, extensin network formation can already occur without oxidative cross-linking, as shown by atomic force microscopy (Cannon *et al.*, 2008).

PRPs are a group of structural cell wall proteins that were first identified as wound-induced gene products in carrot storage roots (Chen and Varner, 1985; Tierney *et al.*, 1988), they are expressed in many plant species and their expression is spatially and temporally regulated during plant development. It has been shown that PRP genes are expressed during soybean leaf, root, stem, and seed coat development (Hong *et al.*, 1990; Kleis-San Fransisco and Tierney, 1990; Lindstrom and Vodkin, 1991; Wyatt *et al.*, 1992). PRP genes are also expressed in different stages of legume root nodule formation (Scheres *et al.*, 1990; Van de Wiel *et al.*, 1990; Wilson and Cooper, 1994), in the growth of bean seedlings (Sheng *et al.*, 1991), in immature maize embryos (Jose-Estanyol *et al.*, 1992), and in other tissues (Bernhardt and Tierney, 2000; reviewed in Johnson *et al.*, 2003). Four PRP genes were characterized in *Arabidopsis* (*AtPRP* genes) and, based on their DNA sequence identity, repetitive motifs, and domain organization, divided into two different classes (Fowler *et al.*, 1999). *AtPRP1* and *AtPRP3* are both exclusively expressed in roots, while *AtPRP2* and *AtPRP4* are expressed in leaves, stems, flowers, and siliques. Promoter analysis with β-glucuronidase (GUS) fusions showed that *AtPRP3* is expressed in root hairs. Experiments with molecules that induce or inhibit root hair initiation coupled to gene expression analysis in mutant backgrounds proved that developmental pathways involved in root hair formation regulate AtPRP3 and that AtPRP3 is contributing to the cell wall structure in *Arabidopsis* root hairs (Bernhardt and Tierney, 2000; Hu and Tierney, 2001).

In this study, the analysis of At5g05500, a putative structural protein whose expression is mainly found in the trichoblasts of the *Arabidopsis* root, is described.

Materials and methods

Plant material and growth conditions

Plants were grown in a greenhouse with 16 h light, 8 h dark cycles at a temperature of 22 °C/18 °C during light and dark conditions, respectively, under a constant humidity of 70%. Dark-grown plants were grown on nutrient agar solidified medium (Estelle and Sommerville, 1987; Refrégier *et al.*, 2004); light-grown plants were grown on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.7% agar (Duchefa, The Netherlands).

Mutant analysis

Atprpl1-1 was obtained from FagDB (Samson *et al.*, 2002) in the Wassilevskija (Ws) background. Homozygous knock-out lines of *Atprpl1-1* were selected by PCR screening. RNA was extracted from 7-day-old seedlings using the RNeasy RNA extraction kit (Qiagen) and used to generate cDNA (Superscript III reverse transcriptase H; Invitrogen). Semi-quantitative reverse transcription–PCR (RT–PCR) was performed to analyse the expression of *AtPRPL1* in the T-DNA insertion lines with the primers OLERTfor 5′ ATGGA CTCA AGA GCCTTACCC 3′ (forward) and OLERTrev 5′ GTAAGTGGGTGGT GCAGTCG 3′ (reverse). ACT IN1 (At2g37620) was amplified as an internal control using the following primer set: RTactinfor 5′ GGCGATGAAGCTCAATCCAAA 3′ and reverse primer RTactinrev 5′ GGTC ACGACCAGCAAGATCAAG 3′.

Generation of transgenic plants

DNA was isolated from wild-type (Col-0) *Arabidopsis* leaves with a DNA extraction kit (Plant DNA Mini kit, Omega Biotek, Inc.) following the manufacturer's protocol. The promoter region, 2000 bp upstream of the ATG start codon, was selected to study the expression pattern of *PRPL1*. This region was amplified using the forward primer pPRPLfor2kb 5′ GGGGAC AAGTTTGTACA AAAAAGCAG GCTGCCTCAGCT TTACCAGCTTT 3′ for the 2 kb promoter and pPRPLfor446bp 5′ GGGACAAG TTTG TAC AAAAAAGCAGGCTCAAATTCCTCTGTTTGGTCCTT 3′ for the 446 bp promoter, and reverse primer pPRPLrev 5′GGGGAC CACTTTGT ACAAGAAA GCT GGGT GTTACTTGTTATA CACTCTGCTTCTTAAAC 3′ for both constructs, including Gateway-compatible recombination sites. Amplified fragments were first cloned into pDONR207 and sequenced before cloning into the final binary vectors pGWB3 (GUS; 446 bp promoter) and pGWB4 [green fluorescent protein (GFP) 2 kb promoter]. This resulted in the final vectors pAtPRPL1::GUS and pAtPRPL1::GFP.

Overexpression of *AtPRPL1* was achieved by amplifying the open reading frame (ORF) with primers PRP L1OEfor 5′ GGGG ACAAGTTT GTACAAA AAAGCAGG CTTC ATGGACTCAAGAGCCTTACCC 3′ (forward) and PR PL1OErev5′GGGGACCACTTTGTACAAGAAAGCTGGGTTT CACTAAGTG GGTGGTGC AGTCG 3′ (reverse). After sequencing, the amplified ORF was cloned in the binary pH2GW7,0 (Karimi *et al.*, 2002) that results in expression of the ORF under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter.

To determine the subcellular localization of AtPRPL1, a C-terminal fusion gene with GFP under control of a 35S promoter was constructed. The ORF was amplified with the primers PR PL1OEfor 5′ GGGGAC AAGTTTGTA CAAAAAAGCAGGCT TC AT GG ACTC AAGAGCC TTACCC 3′ (forward) and PR PL1OEGFPr 5′ GGGGAC CACTTT GTA CA GAAAGC TGGGTTGTAAGT GGGTGGTGCAGTCG 3′ (reverse) and cloned into pGWB5

generating 35S::PRPL1-GFP. As a second construct, the fusion gene was placed under control of the AtPRPL1-own promoter. As the ORF does not contain any introns, the promoter region (446 bp) and the ORF were amplified as one fragment with the primers PRPL1for446bp and PRP L1OEGFPr, and cloned into pDONR207. After sequencing, this fragment was recombined into pGWB4 generating pPRPL1::PRPL1-GFP.

All final vectors were transformed in *Agrobacterium tumefaciens* strain C58 that contained the pMP90 helper plasmid before *Arabidopsis thaliana* Col-0 was transformed by flower dip (Clough and Bent, 1998). Transgenic plants were selected on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose, 0.7% agar (Duchefa, The Netherlands), and the appropriate antibiotic. At least 30 independent transgenic lines were selected, and homozygous lines were identified after segregation analysis for further analysis.

Phylogenetic analysis

To elucidate the relationship of AtPRPL1 to other proteins, a phylogenetic tree was created from parsimony analysis. The protein sequence of AtPRPL1 was used to retrieve related sequences from the BLAST program (Altschul *et al.*, 1990). These retrieved sequences were aligned in ClustalW 1.87 and a phylogenetic tree was made from Neighbor–Joining analysis using Mega 5.05 software (Tamura *et al.*, 2011). The bootstrap values are shown in the tree, and 1000 replicates were used.

GUS staining

Seedlings were taken at different stages during plant development and fixed with 90% acetone for 20 min. After rinsing with distilled water, whole seedlings were stained for 16 h based on Jefferson *et al.* (1987) in GUS staining buffer [2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucoronide, 200 mM sodium phosphate (pH 7.0), 10 mM Na_2EDTA, 1 mM $K_4Fe(CN)_6$, and 1 mM $K_3Fe(CN)_6$] at 37°C. After incubation, seedlings were fixed in ethanol/acetic acid (3/1) and cleared with 8 M NaOH for 1 h. Images were taken with a Zeiss Axioskop equipped with a Nikon DXM 1200 or Nikon Ds-Fi1 digital camera.

GFP visualization

GFP was localized in pAtPRPL1::GFP and protein–GFP fusion lines using a Nikon C1 confocal microscope or a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (Ultra*VIEW* VoX; PerkinElmer). The seedlings were briefly dipped in 0.1 mg ml^{-1} propidium iodide to counterstain the cell walls and to aid cell-specific localization of the GFP.

Transient expression to co-localize protein–GFP and mCherry-labelled markers for different organelles

A transient expression of mCherry-labelled markers for different organelles was performed in 35S::PRPL1-GFP and pPRPL1::PRPL1-GFP using the protocol described in Van Loock *et al.* (2010). The markers used were for the endoplasmic reticulum (ER), plasma membrane, peroxisomes, and the Golgi apparatus, and they are described in Nelson *et al.* (2007).

Transcript analysis

To quantify the expression levels of At5g05500 in the knock-out and the different generated transgenic lines, a quantitative RT–PCR (qRT–PCR) analysis was performed. RNA isolation was performed using TRIzol Reagent (Life Technologies) according to the guidelines of the manufacturer and its quality was checked with a nanodrop (ND1000). SuperScript™ II Reverse Transcriptase (Life Technologies) was used to perform cDNA synthesis according to the manufacturer's instructions. All samples were diluted 1:8 with RNase-free water prior to the qPCR. Gene expression analysis was performed using TaqMan Universal Master Mix II, with uracil-*N* glycoslyase (UNG) and ROX (passive reference dye) as a technical control. The TaqMan probe At02181637_s1 was used to quantify the expression of At5g05500. To normalize the samples, the expression level of actin 8 (At1g49240) was monitored with the probe At02270958_gH. All probes span intron-separated exons and were ordered from Life Technologies. Results were analysed with the StepOnePlus Real-Time PCR System (Life Technologies) software at a confidence level of 95%.

FT-IR analysis of etiolated hypocotyls and root hairs

Fourier transform-infrared (FT-IR) analysis was performed as described by Mouille *et al.* (2003).

For hypocotyls, 4-day-old dark-grown seedlings were squashed between two BaF_2 windows and rinsed thoroughly with distilled water before they were air-dried at 37 °C for at least 20 min. Four biological repeats were grown and five spectra for each repeat were used to analyse changes in cell wall composition.

To study root hairs, 1-week-old light-grown seedlings were collected in absolute ethanol, and then samples were rehydrated for a few hours in distilled water and air-dried at 37 °C for at least 20 min. Two biological repeats were grown, and spectra of 10 root hairs per seedling for each repeat were used to analyse changes in cell wall composition.

For both the hypocotyl and root hair study, the spectra were baseline corrected and area normalized before further analysis. A dendogram construction was performed as described before (Mouille *et al.*, 2003; Robin *et al.*, 2003). To analyse the spectral data with principal component analysis (PCA), WINDAS was used to normalize the data and to construct the data matrices. One-sample *t*-tests were executed to detect significant changes in the absorption at the different wavenumbers, which is indicative of compositional changes in the cell walls.

Results

AtPRPL1 is a proline-rich protein family member with an extensin-like domain

Several microarray data identified a large group of cell wall-related genes that are involved in *A. thaliana* root hair development (Ma and Bohnert, 2007; Velasquez *et al.*, 2011; Bruex *et al.*, 2012). One of these genes, At5g05500, is a cell wall-related gene that contains a pollen Ole allergen domain (PF01190, Pollen_*Ole_e*_I; Marchler-Bauer *et al.*, 2007) and an extensin (IPR006041) domain. This gene contains no introns and has an ORF of 552 bp that encodes a protein of 183 amino acids (Fig. 1a). At5g05500 has a putative signal peptide of 23 amino acids (Signal-3L; http://www.csbio.sjtu.edu.cn/cgi-bin/Signal3L.cgi) that with high probability targets the protein to the endomembrane system of the cell (*Arabidopsis* Cell eFP browser; based on Heazlewood *et al.*, 2007). Using a BLASTP similarity search (Altschul *et al.*, 1990, 1997) and the At5g05500 protein as a query sequence, phylogenetic analysis showed that the most closely related proteins are two unknown proteins of *Vitis vinifera* that share almost 70% identical amino acids with the At5g05500 protein. No closely related homologues were identified in *Arabidopsis*. A phylogenetic tree containing only *Arabidopsis* proteins was constructed with the Neighbor–Joining method using MEGA5.05 (Tamura *et al.*, 2011). Figure 1b shows a

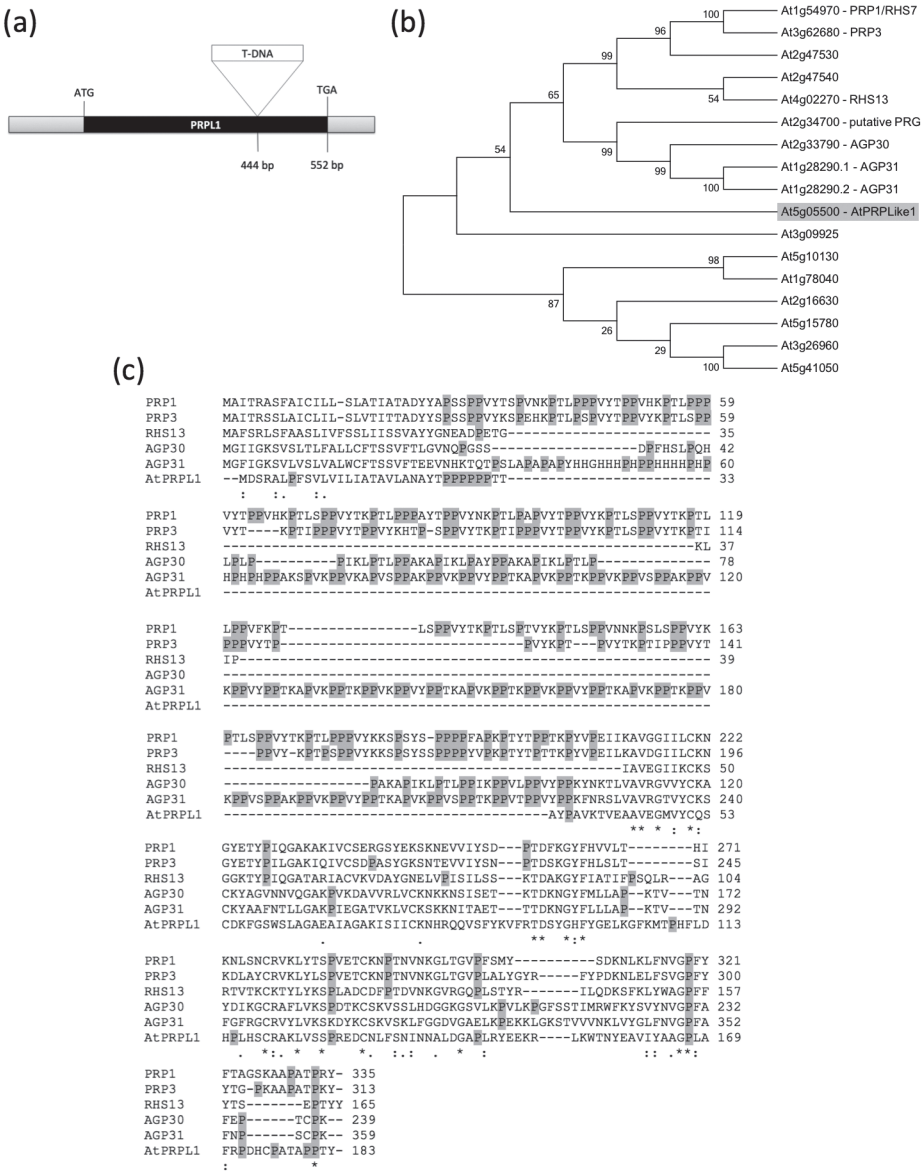

Fig. 1. *In silico* analysis of At5g05500. (a) Schematic representation of At5g05500 gene structure, including the start and stop codon, one exon, and the T-DNA insertion position in the mutant. (b) Phylogenetic tree made by Neighbor–Joining analysis using Mega 5.05 software of the 15 most related proteins in *Arabidopsis* identified by BlastP. The bootstrap values are shown in the tree, and 1000 replicates were used. (c) Alignment of the DNA sequence of At5g05500 and the five most related genes. Prolines are shaded in grey.

part of the tree with only the 16 most related *Arabidopsis* proteins. The alignment of At5g05500 with five characterized proteins of the upper cluster is presented in Fig. 1c. All proteins more or less share a common Pollen_*Ole_e*_I and extensin family domain. This pollen Ole domain is named based on the high similarity with the major olive pollen allergen Ole 1 to which >70% olive allergic patients are sensitive (Lauzurica *et al.*, 1988; Wheeler *et al.*, 1990; Villalba *et al.*, 1993). The most related of the characterized proteins in *Arabidopsis* are AtPRP1 and AtPRP3 (Fowler *et al.*, 1999; Bernhardt and Tierney, 2000), two proteins that are 32% and 30%, respectively, identical to the At5g05500 protein, and both are 43% similar. The At5g05500 protein has 17 proline residues (or 9%; see grey shaded Ps in Fig. 1c) and a length of 183 amino acids, whereas AtPRP1 and AtPRP3 have 23% and 22% proline residues and a length of 313 and 310 amino acids, respectively. The percentage of proline residues in the whole protein is clearly lower than in the two AtPRPs; however, the C-terminal parts of the proteins share the greatest sequence identity (Fig. 1c). This suggests that At5g05500 could belong to the PRP class of genes (Showalter, 1993; Fowler *et al.*, 1999). Given its close relationship to AtPRP1 and AtPRP3 and the lower percentage of proline, At5g05500 was named AtPRP-like1 (AtPRPL1). The hydrophobic isodityrosine cross-link motif (YVY), which is present in AtEXT3, a HRGP, is absent in AtPRPL1.

Expression pattern of AtPRPL1 *during different developmental stages*

In silico promoter analysis revealed cytokinin- and ABA-responsive elements and pollen and root hair *cis*-elements

(Supplementary Fig. S1 available at *JXB* online). The expression pattern of *AtPRPL1* was investigated using a 446 bp promoter fragment driving the GUS gene in homozygous T_3 lines. In dark-grown hypocotyls, *AtPRPL1* has a weak expression at the onset of the fast elongation, seen as faint blue staining after GUS assay (Fig. 2a). The expression level decreases once the fast elongation phase has started (Fig. 2b). No expression was detected after 4 d of growth, or after 8 d when the hypocotyl is fully grown (data not shown). These observations were confirmed in 2 kb promoter::GFP lines (data not shown).

AtPRPL1 is highly expressed in the root (Fig. 2a–d) and appears solely in the trichoblast cell file (overview of seedlings in Fig. 2c; close-ups in Fig. 2d). Also at the transition between hypocotyl and root (collet) *AtPRPL1* is expressed in root hair cells only (Fig. 2e). No expression was detected in a light-grown hypocotyl or in cotyledons or leaves at any developmental stage (Fig. 2c, e, and data not shown). A transient expression was detected in developing pollen (Fig. 2f), while it was absent in other flower parts. The expression pattern of *AtPRPL1* in roots was confirmed with GFP as reporter (Fig. 2g).

Since in the root, expression was found in trichoblasts only, the effect of ACC, which promotes root hair formation, and 1-α-(2-aminoethoxyvinyl)glycine (AVG), an inhibitor of ethylene biosynthesis, was studied on the expression pattern of *AtPRPL1* (Masucci and Schiefelbein, 1996). Whereas staining was absent in the atrichoblasts of an untreated plant (Fig. 3a), after ACC treatment the cells showing ectopic root hairs also expressed *AtPRPL1* (Fig. 3b). AVG treatment resulted in a strongly decreased or completely absent expression in correlation with decreased or absent root hair formation (Fig. 3c). These changes in *AtPRPL1* expression were confirmed in both an *eto2* and *ein2-1* background that mimick the changes in ethylene levels after ACC and AVG treatment (Fig. 3d, e).

Altered expression levels of AtPRPL1 *interfere with cell elongation*

Atprpl1-1, a T-DNA insertion line (*A. thaliana*, ecotype Ws) with an insertion in the exon (Fig. 1a), was obtained from the FlagDB collection (Samson *et al.*, 2002). A semi-quantitative RT–PCR analysis of *AtPRPL-1* expression in the wild type

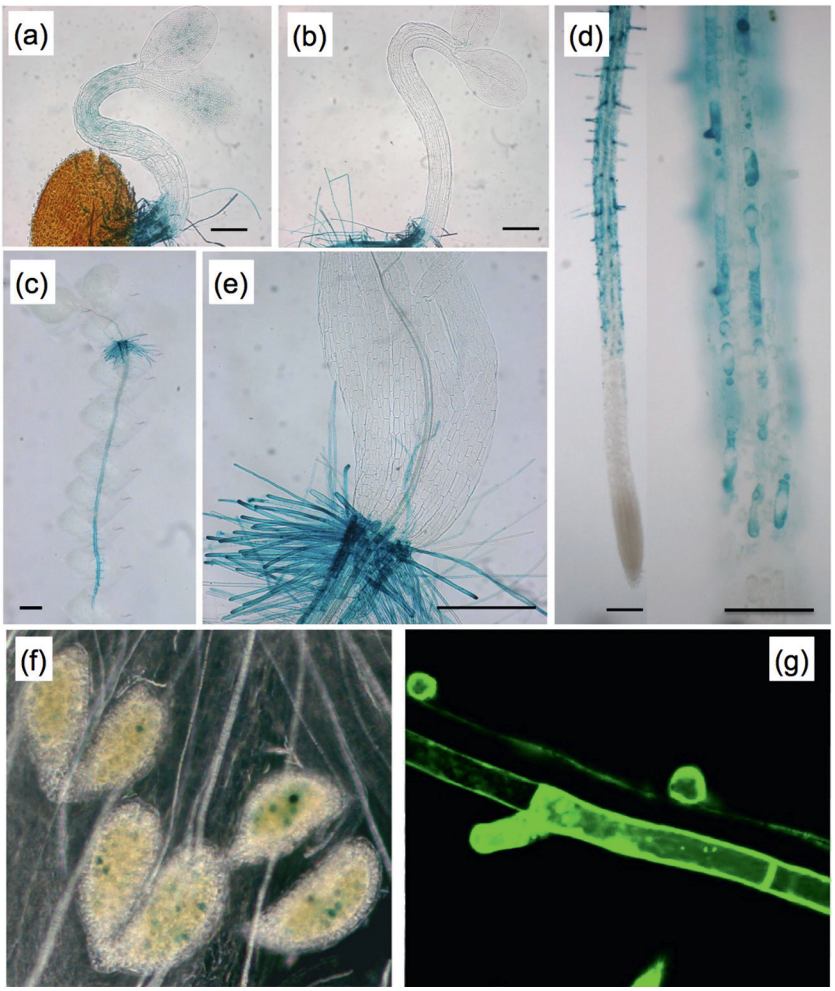

Fig. 2. Expression analysis of At5g05500 using promoter–reporter lines. Bright-field images showing (a) faint GUS activity in a young etiolated hypocotyl, (b) absent GUS activity in a young etiolated hypocotyl where fast cell expansion has started, (c) enrichment in the root of a whole seedling, (d) trichoblast-specific expression in a root and in its close-up, (e) expression in root hairs at the collet, (f) expression in pollen. (g) represents a confocal image of promoter-driven GFP in the trichoblast cell files only. Scale bars are 200 μm in (a, b, and e), 100 μm in (d), and 1 mm in (c).

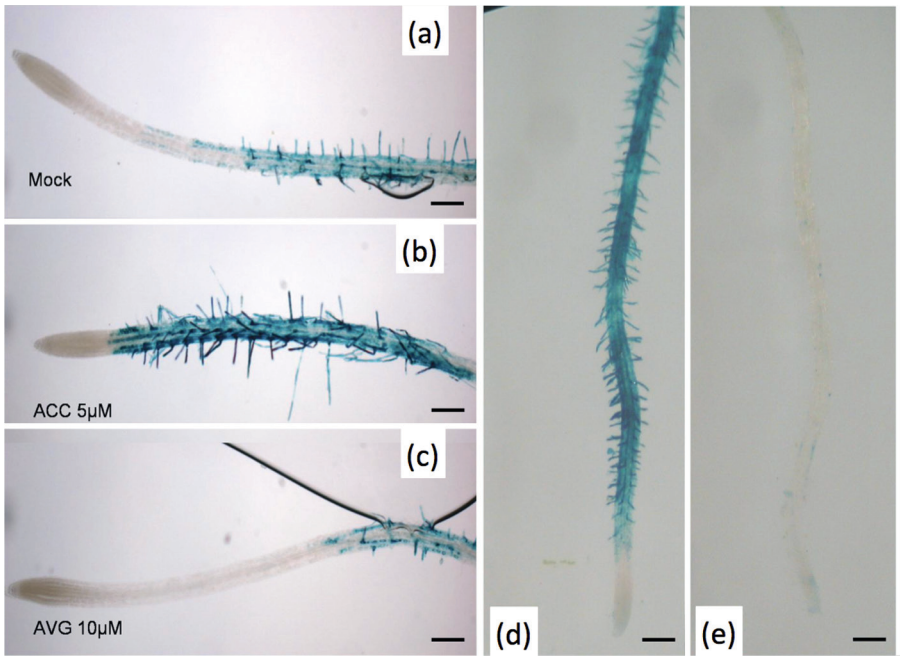

Fig. 3. Effect of altered ethylene presence and mutated background on *AtPRPL1* expression. (a) GUS activity is seen in the trichoblast cell files of a mock-treated root. (b) Treatment with the ethylene precursor ACC induces ectopic root hair formation and concomitant extra AtPRPL1 expression. (c) AVG-inhibited ethylene production interferes with root hair initiation/formation and reduces *AtPRPL1* expression. (d, e) Both ethylene effects are mimicked in the *eto2* and *ein2-1* backgrounds. Scale bars are 100 μm.

and *Atprpl1-1* with actin and elongation factor as an internal control proved that it was a null mutant. Phenotypic analysis of root and etiolated hypocotyl length shows no significant difference between the knock-out line and the wild type (Fig. 4a, b). However, a clear reduction in root hair length was observed in the knock-out line (Fig. 4c). Wild-type root hairs were 536 ± 12 μm long, whereas in the knock-out they were shorter: 393 ± 12 μm. Supplementary Fig. S16 in Velasquez *et al.* (2011) mentions two other insertion lines in the same gene (in an exon and in the promoter), but at other positions, and these show a root hair length reduction as well, confirming that the absence of this gene causes the phenotype described here.

To analyse further the role of *AtPRPL1* in cell elongation, transgenic lines overexpressing the ORF of *AtPRPL1* under control of the constitutive CaMV 35S promoter (35S::AtPRPL1) were generated. Homozygous plants were identified after segregation analysis on hygromycin, and a semi-quantitative RT–PCR was performed to identify lines with a clear overexpression. Relative quantification with qPCR analysis revealed an *AtPRPL1* expression increase of 19 and 16.5 times in 35S::AtPRPL1-10 and 35S::AtPRPL1-28 respectively.

Hypocotyls grown for 4 d in the dark were analysed and this revealed that the overexpression of *AtPRPL1* reduced the hypocotyl length by 40% and 20% (Fig. 4b). Wild-type hypocotyl length was 13.16 ± 0.21 mm; 35S::AtPRPL1-10 and 35S::AtPRPL1-28 had hypocotyls of 9.79 ± 0.42 mm and 10.34 ± 0.36 mm, respectively. The overexpression also caused a significant increase in the root hair length when compared with the control plants. Root hair lengths of overexpression lines (489 ± 9 μm for 35S::AtPRPL1-10 and 507 ± 6 μm for 35S::AtPRPL1-28) were clearly longer, namely 24.74% and 27.80%, than those of the wild type (392 ± 6 μm) (Fig. 4c).

FT-IR analysis of root hair cell walls reveals subtle compositional changes

As the PRPL1 protein is located in the root hairs, the composition of the cell wall of root hairs in wild-type plants, *Atprpl1-1*, and 35S::AtPRPL1-10 was investigated by FT-IR. Figure 5a shows the comparison of the average spectra where Ws serves as the wild type for *Atprpl1-1* and Col-0 for 35S::AtPRPL1. These spectral data were analysed with one-sample *t*-test to reveal significant changes. The region between wave number 1384 cm^{-1} and 1307 cm^{-1} and between 1446 cm^{-1} and 1415 cm^{-1} showed that the null mutant *Atprpl1-1* contains cell walls with significantly altered composition. However, those changes cannot be accurately assigned to specific bonds (Fig. 5b).

The comparison of the spectra of the overexpressing line and wild type showed a significantly changed absorption in the region between 1550 cm^{-1} and 1500 cm^{-1}. This region contains wave numbers that correspond to amide I (1546 cm^{-1} and 1511 cm^{-1}) (Sene *et al.*, 1994). Another region with a significantly changed absorption in the overexpression lines, from 1141 cm^{-1} to 1070 cm^{-1}, contains mainly wave numbers that correspond to cellulose (1091 cm^{-1}) and xyloglucan (1120 cm^{-1}) (Kacuráková *et al.*, 2000; Wilson *et al.*, 2000; Carpita *et al.*, 2001). Therefore, the data of AtPRPL1 overexpression strongly suggest that the presence of AtPRPL1 in root hairs is positively correlated with the presence of polysaccharides such as cellulose and xyloglucan.

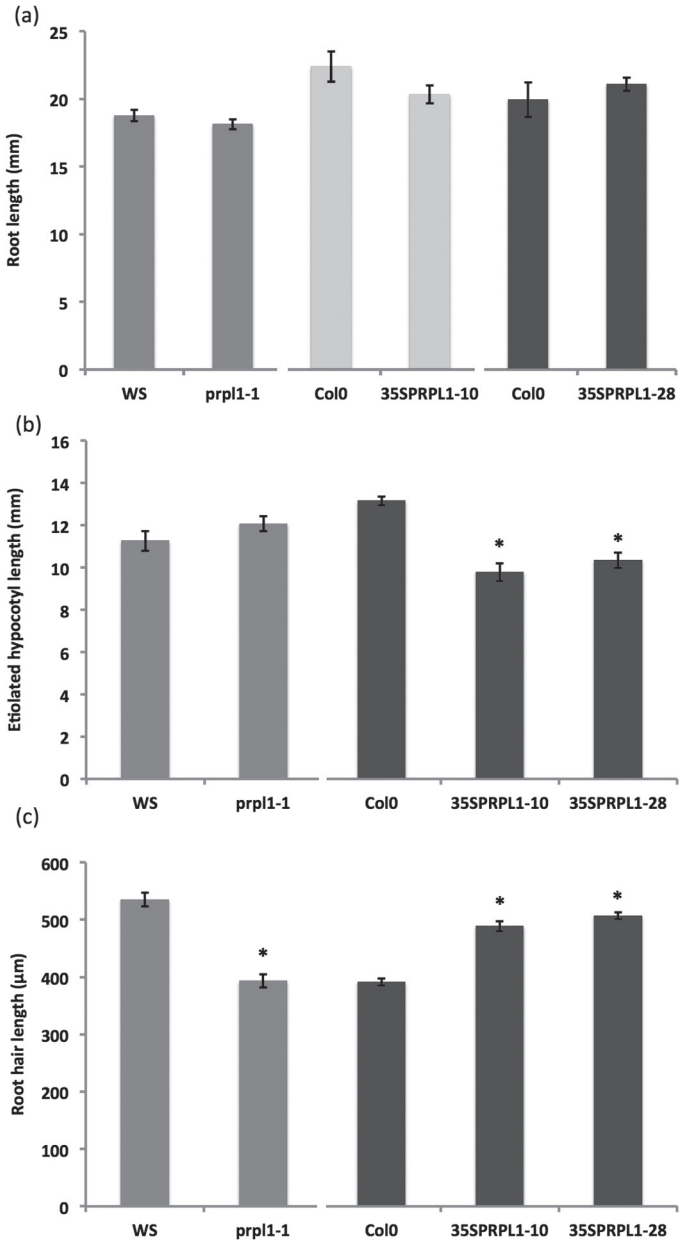

Fig. 4. Root, etiolated hypocotyl, and root hair phenotyping in the wild type, knock-out, and overexpression lines of AtPRPL1. (a) Root length, (b) etiolated hypocotyl length, and (c) root hair length of wild type, knock-out, and overexpression plants. Asterisks refer to significant changes compared with the corresponding wild types (the wild type and lines that are statistically compared with it are presented in the same greyscale). Data shown are the average of three representative biological replicates having at least 15 seedlings; error bars represent the SE. Student's *t*-test, $P<0.05$.

FT-IR analysis of etiolated hypocotyl cell walls reveals no compositional changes and suggests that AtPRPL1 is not a non-conventional AGP

As shown in the phylogenetic analysis, *AtPRPL1* is closely related to *AtPRP* genes and to *AGP30* and *AGP31*. To test whether AtPRPL1 is an AGP or not and to identify potential cell wall compositional changes that could explain the hypocotyl phenotype in the overexpression lines, the composition of the cell wall of 4-day-old dark-grown hypocotyls in wild-type and overexpression plants was investigated by FT-IR microspectroscopy (Mouille *et al.*, 2003; Pelletier *et al.*, 2010). Spectra were taken next to the central cylinder in the middle of the hypocotyl. Student's *t*-tests on the comparison of the average spectra sampled did not identify consistent significant changes in the cell wall composition of both overexpression lines when compared with the wild type (Supplementary Fig. S2 at *JXB* online), indicating that AtPRPL1 is not an AGP and that altering levels of *AtPRPL1* affect wall composition in a complex fashion.

Subcellular localization of PRPL1

To investigate the subcellular localization of *AtPRPL1*, the ORF was fused to a GFP-coding sequence and used to generate transgenic lines with stable expression of 35S::AtPRPL1-GFP. Using confocal and spinning disc microscopy AtPRPL1–GFP was located in small vesicles that were actively moving around in the cell (see Supplementary Video S1 at *JXB* online). In plants expressing AtPRPL1-GFP driven by its own promoter, GFP was visible in similar moving vesicles, but only in the trichoblast cell files, confirming the expression analysis (data not shown). In comparison with the 35S promoter, the promoter of *AtPRPL1* was, however, much weaker. To assess whether the movement was actin or microtubule based, 35S::AtPRPL1-GFP plants were grown for 2 d in the dark and treated with 5 µM latrunculin or 10 µM oryzalin to disrupt actin bundles and microtubules, respectively (see Supplementary Videos S2 and S3 at *JXB* online). The movies were created with the same settings of the confocal microscope, and bleaching of the GFP can be seen near the end of the movies. Only latrunculin (Supplementary Video S2 at *JXB* online) was capable of stopping the movement of the GFP-enriched vesicles, suggesting that they move on actin. To identify the nature of the AtPRPL1–GFP-enriched vesicles, different cell organelle markers (Nelson *et al.*, 2007) were transiently expressed in 35S::AtPRPL1-GFP roots (Fig. 6a–d) according to Van Loock *et al.* (2010). From these co-localization experiments with plasma membrane-, Golgi apparatus-, peroxisome-, and ER-specific red fluorescent protein (RFP), it seems that AtPRPL1–GFP is localized on the ER, or in vesicles that move along or on the ER. To confirm these results, the mCherry-fused ER marker ER-rb *CD3-960* (Nelson *et al.*, 2007) was transformed in the 35S::AtPRPL1-GFP background. As in the transient expression, AtPRPL1–GFP is localized in small vesicles that move 'on the ER' in roots (Fig. 6e–g). From the pictures and the movies that were generated, it remains unclear whether or not the AtPRPL1–GFP is transferred from the ER to the cell wall.

Discussion

In this study, a novel PRP-like protein encoded by At5g05500, which is specifically expressed in root hair cells, was identified. The protein contains a pollen Ole allergen domain (pfam01190, Pollen_Ole_I; Marchler-Bauer *et al.*, 2007) and an extensin (IPR006041) domain. Phylogenetic analysis showed that

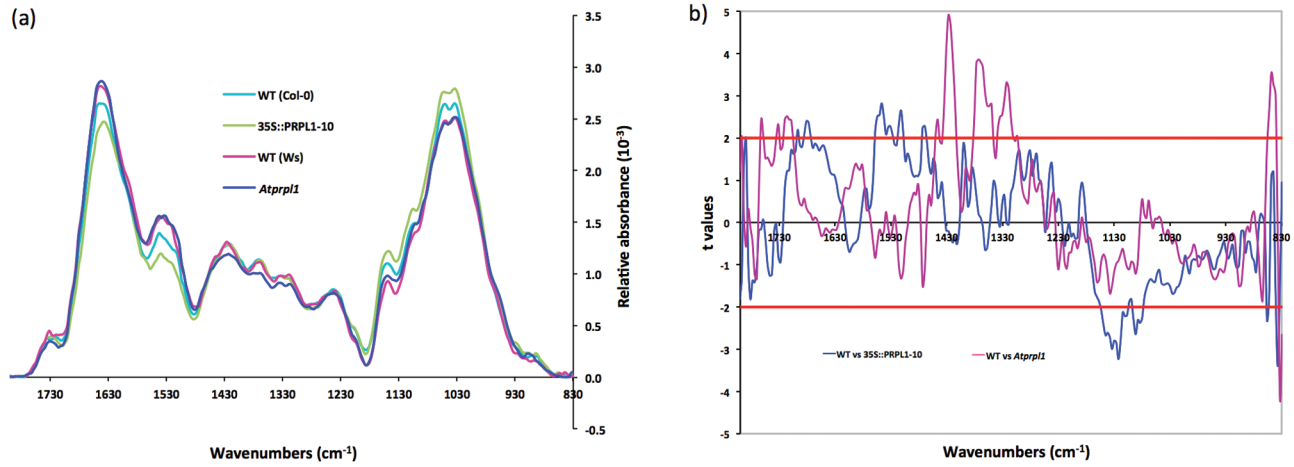

Fig. 5. Cell wall compositional changes between root hairs of the wild type, knock-out, and overexpression lines of AtPRPL1 revealed by FT-IR. (a) Comparison of the average spectra of *Atprpl1-1* (dark blue trace) and its corresponding WT (pink trace) and of 35S::AtPRPL1-10 (green trace) and its corresponding WT (light blue trace). (b) Student's *t*-test (P=0.05) of a comparison of FT-IR spectra sampled from root hairs. Comparison between WT and *Atprpl1-1* (violet trace) and WT and AtPRPL1-1 overexpression (blue trace). For *t*-values >2 the absorbance for the corresponding wavenumber is significantly higher in the wild type. Horizontal red lines mark the P=0.01 significance level.

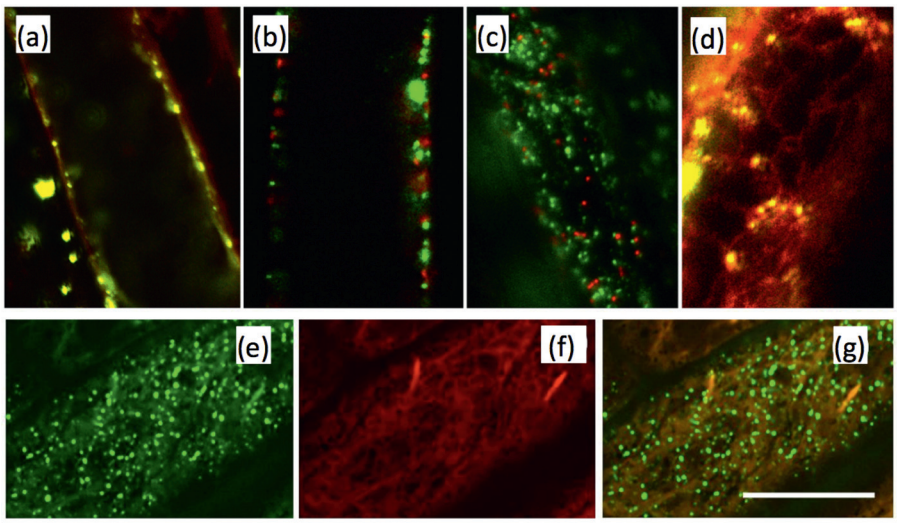

Fig. 6. Protein–GFP localization. Transient co-localization analysis of AtPRPL1–GFP and RFP-tagged markers for the (a) plasma membrane, (b) Golgi apparatus, (c) peroxisomes, and (d) ER. (e) AtPRPL–GFP, (f) mCherry-fused ER marker ER-rb *CD3-960*, and (g) overlay of both in 35S::AtPRPL1-GFP lines stably transformed with the ER marker. Scale bar=10 μm.

At5g05500 is closely related to *AtPRP3* and *AtPRP1*, identified by Fowler and co-workers (1999). At5g05500 is a small gene that contains no introns and codes for a small protein of only 183 amino acids. The deduced protein contains an N-terminal putative signal peptide of 20 amino acids that probably directs this protein to the ER and possibly to the cell wall. This was confirmed in transgenic plants bearing protein–GFP constructs and using co-localization experiments. The protein is most related to PRPs, which indicates that *AtPRPL1* could be a member of this PRP family (or subfamily) of genes. Also *AGP30*, reported to be more related to *PRP* genes than to other *AGP* genes (Showalter *et al.*, 2010), and *AGP31* were closely related to At5g05500. FT-IR results on transgenic lines overexpressing AtPRPL1 excluded that it is a NcAGP since an increase in a specific type II arabinogalactan side chain was not seen at the 1078 cm^{-1} wavenumber (Kacuráková *et al.*, 2000). Furthermore, AGP30 has been reported to be more related to PRPs than to other AGPs (Showalter *et al.*, 2010), strengthening the present findings. As the closest relatives of At5g05500 are PRPs and as the percentage of proline residues in the protein is slightly lower, At5g05500 was named a proline-rich protein-like gene, *AtPRPL1*.

Expression analysis of *AtPRPL1* using promoter::GUS and promoter::GFP lines indicated that its expression was positively correlated with the occurrence and outgrowth of root hairs. The expression after treatments (ACC and AVG) or in mutants (*eto2* and *ein2-1*) that induced or inhibited the initiation of root hairs confirmed this. The two closest relatives in *Arabidopsis*, *AtPRP1* and *AtPRP3*, were also shown to be involved in root hair growth (Bernhardt and Tierney, 2000; Hu and Tierney, 2001). *AtPRP3* is expressed during root hair initiation and outgrowth of the root hairs. A null mutant of *AtPRP3* shows an aberrant phenotype in root hair branching (Bernhardt and Tierney, 2000; Hu and Tierney,

2001). From the present expression data and the effect of the knock-out mutation and overexpression of AtPRPL1 on root hair length, it can be concluded that *AtPRPL1* is positively involved in root hair formation, similar to *AtPRP3*.

The plant cell wall is a complex and diverse structure and consists of the polysaccharides cellulose, cross-linking glycans, pectins, and structural proteins. All components are organized into a complex extracellular matrix (Carpita and Gibeaut, 1993). Interactions between structural cell wall proteins and other cellular matrix components are critical for the integrity of this matrix and correct self-assembly of the cell wall (Cannon *et al.*, 2008). PRPs belong to a superfamily of cell wall proteins called HRGPs. Besides the lightly glycosylated PRPs, moderately glycosylated extensins (EXTs) and highly glycosylated arabinogalactan-proteins (AGPs) also belong to the HRGPs (Showalter *et al.*, 2010). HRGPs contribute to form an independent structure-determining network (Bernhardt and Tierney, 2000). Recently it was reported that post-translational modifications, such as proline hydroxylation and *O*-glycosylation, of HRGPs are essential in root hair growth (Velasquez *et al.*, 2011, 2012*a*, *b*), whereby prolyl 4-hydroxylase (P4H) is the enzyme that converts proline to hydroxyproline. Velasquez *et al.* (2011) showed aberrancies in root hair elongation caused by inhibition of P4H activity. PRPs are thought to be secreted into the wall where, according to their ability, associate with and become covalently cross-linked to components within the cell wall (Bradley *et al.*, 1992; Showalter, 1993). It was shown here that AtPRPL1 is a member of PRPs and suggest that it somehow participates in determining the root hair cell wall structure during plant development, being a member of the HRGP network. From the clear root hair phenotypes and PRPL1's location in vesicles that move on/along the ER, it seems that AtPRPL1 is essential for creating a wall that grows normally. Differences in cell wall composition were seen in the FT-IR analysis of knock-outs and overexpression plants, but only in root hair walls, suggesting that *AtPRPL1* affects cell wall composition in a rather complex manner. How AtPRPL1 interferes with cellulose deposition and xyloglucan sequestering into the root hair cell wall remains unknown. It could, however, be postulated that it somehow functions as a kind of chaperone in moving vesicles, but this has to be elucidated. How exactly it manages to play its role remains unclear. Furthermore, co-expression analysis revealed that At4g02270 (a closely related pollen Ole e 1 allergen and extensin family protein) is co-expressed with *AtPRPL1* (www.genevestigator.ethz.ch; Zimmermann *et al.*, 2004), pointing to possible redundancy. However, as seen here, the knock-out of At5g05500 already showed a clear root hair phenotype, suggesting that multiple knock-outs in extensin(-like) genes which were identified by Velasquez *et al.* (2011) could have even much more severe effects.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. *In silico* analysis of the putative promoter sequence of At5g05500.

Figure S2. Cell wall compositional changes between etiolated hypocotyls of the wild type and two overexpression lines of AtPRPL1 revealed by FT-IR.

Video S1. Movie showing green AtPRPL1–GFP proteins in 35S::AtPRPL1-GFP lines grown in control conditions.

Video S2. Movie showing green AtPRPL1–GFP proteins in 35S::AtPRPL1-GFP lines after treatment with 5 µM latrunculin.

Video S3. Movie showing green AtPRPL1–GFP proteins in 35S::AtPRPL1-GFP lines after treatment with 10 µM oryzalin.

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