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Synergistic interaction of the two paralogous *Arabidopsis* genes *LRX1* and *LRX2* in cell wall formation during root hair development

N. Baumberger[1,†], M. Steiner[1], U. Ryser[2], B. Keller[1] and C. Ringli[1,*]

[1]*Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland, and*

[2]*Institute of Plant Biology, University of Fribourg, Ch. du Muse 10, CH-1700 Fribourg, Switzerland*

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[*]For correspondence (fax +41 1 6348204; e-mail chringli@botinst.unizh.ch).

[†]Current address: Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

Summary

LRR-extensins (LRX) form a family of structural cell wall proteins containing a receptor-like domain. The functional analysis of *Arabidopsis LRX1* has shown that it is involved in cell morphogenesis of root hairs. In this work, we have studied *LRX2*, a paralog of *LRX1*. *LRX2* expression is mainly found in roots and is responsive to factors promoting or repressing root hair formation. The function of *LRX1* and *LRX2* was tested by the expression of a truncated LRX2 and different LRX1/LRX2 chimaeric proteins. Using complementation of the *lrx1* phenotype as the parameter for protein function, our experiments indicate that LRX1 and LRX2 are functionally similar but show differences in their activity. Genetic analysis revealed that single *lrx2* mutants do not show any defect in root hair morphogenesis, but synergistically interact with the *lrx1* mutation. *lrx1/lrx2* double mutants have a significantly enhanced *lrx1* phenotype, resulting in frequent rupture of the root hairs soon after their initiation. Analysis of the root hair cell wall ultrastructure by transmission electron microscopy (TEM) revealed the formation of osmophilic aggregates within the wall, as well as local disintegration of the wall structure in the double mutant, but not in wild-type plants. Our results indicate that LRX1 and LRX2 have overlapping functions in root hair formation, and that they likely regulate cell morphogenesis by promoting proper development of the cell wall.

Keywords: LRR-extensins, cell morphogenesis, cell wall.

Introduction

The plant cell wall is a primary determinant of cell shape and ultimately of the overall plant morphology. While the cell turgor is the driving force of cell expansion, the cell wall regulates both the extent and the direction of this growth process (Pritchard, 1994). The primary cell wall is composed of a network of cellulose microfibrils and hemicelluloses, which are embedded in a matrix of pectins (Carpita and Gibeaut, 1993). The synthesis of the polysaccharides and the mechanisms that regulate their assembly in the extracellular matrix are still not well understood. Only few of the putative cell wall synthesis genes in the *Arabidopsis* genome have been functionally characterised (Carpita *et al.*, 2001). Mutations in two different cellulose synthase subunit genes, *RADIAL SWELLING1* and *PROCUSTE1,* provoke a reduction in crystalline cellulose in the primary walls and result in plants whose cells expand abnormally (Arioli *et al.*, 1998; Fagard *et al.*, 2000). Genes involved in the biosynthesis of the matrix polysaccharides, which, in contrast to the cellulose microfibrils, are produced in the endomembrane system, have also been identified (Edwards *et al.*, 1999; Favery *et al.*, 2001; Perrin *et al.*, 1999). Arabinogalactan proteins (AGPs) might regulate cell wall development, as precipitation of AGPs by Yariv agent disturbs cell wall assembly and the de-regulated growth of root epidermal cells in the *Arabidopsis reb1* mutant correlates with a strong decrease in AGP content (Ding and Zhu, 1997). Genetic evidence suggests that COBRA, a GPI-anchored extracellular protein, is involved in oriented cell expansion and thus cell wall assembly (Schindelman *et al.*, 2001).

Root hairs are particularly suitable to study polarised cell expansion and cell morphogenesis because they grow as long thin extensions of root epidermal trichoblast cells (Gilroy and Jones, 2000), free of any constraint imposed

by the surrounding cells. Root hair expansion takes place only at the apex of the hair by a mechanism known as tip growth. In plants, tip growth specifically occurs in root hairs and pollen tubes while the other cell types expand by diffuse growth (Yang, 1998). Three stages of root hair development have been defined. In the first step, formation of a bulge at the distal end of the trichoblast cell takes place. Subsequently, the establishment of a root hair structure on the bulge is initiated by slow tip growth. In the third phase, the root hair proper is formed by rapid tip growth (Dolan *et al.*, 1994).

A large number of *Arabidopsis* root hair mutants have been isolated, which display a wide array of different mutant phenotypes (for review, see Schiefelbein, 2000). For some of the mutants, the mutated genes have been identified. They encode a GTP-binding protein (*rhd3*; Wang *et al.*, 1997), a K^+ transporter (*trh1*; Rigas *et al.*, 2001), a cellulose synthase-like protein (*kojak*; Favery *et al.*, 2001; Wang *et al.*, 2001) or an actin isoform (*der1*; Ringli *et al.*, 2002), and thus are part of different cellular machineries required for proper root hair development.

We have recently characterised the new cell wall protein LRR-extensin1 (LRX1), which contains a leucine-rich repeat (LRR) and an extensin-like domain (Baumberger *et al.*, 2001). LRRs are usually involved in protein–protein or ligand–protein interactions and constitute the extracellular receptor domain of many plant-signalling proteins (Jones and Jones, 1997). However, LRRs can also be regulators of enzyme activity as shown for polygalacturonase-inhibiting protein (PGIP)s, which inhibit fungal polygalacturonases

Figure 1. Comparison of the duplicated *LRX1* and *LRX2*.
(a) Protein organisation of LRX2. The different domains are indicated. If differing, residues of LRX1 are indicated in italic letters and missing residues are symbolised by gaps. The consensus sequence for extracellular LRRs is shown in bold letters at the beginning of the domain. The solvent-exposed residues of the LRR domain are boxed and non-consensus positions that differ between LRX1 and LRX2 are bold. The extensin domains of LRX2 and LRX1 are shown on the left and on the right, respectively. Corresponding subdomains are indicated.
(b) Dotter alignment of the genomic regions harboring *LRX1* (BAC F21F1) and *LRX2* (BAC F24O1). The position of these BACs on chromosome 1 are shown on the left side. Genes collinear between the two BACs are indicated along the dot-blot axes. The smaller window is an enlargement of the *LRX1*- and *LRX2*-coding regions. Arrowhead: The homology between the repetitive extensin domains is reflected by the large number of lines.

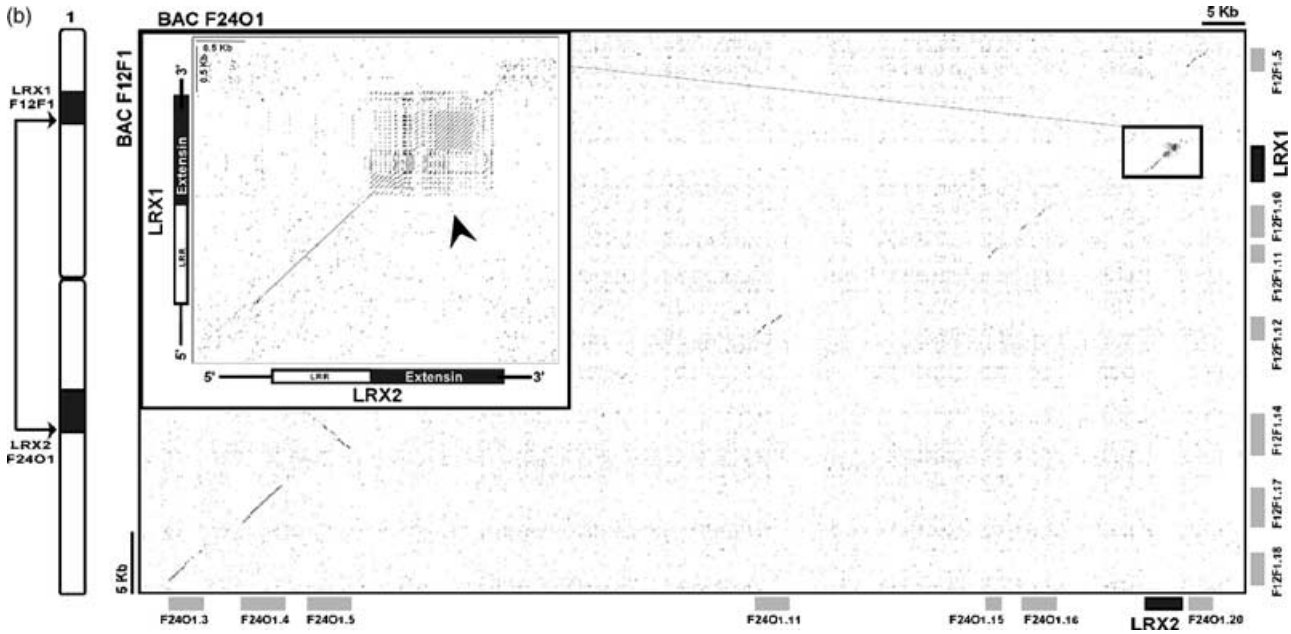

Figure 1. continuted

(De Lorenzo and Cervone, 1997). The second structural domain of LRX1 contains [Ser-Hyp_4] repeats characteristic of extensins, a subfamily of hydroxyproline-rich glycoproteins (HRGPs) (Cassab, 1998). Extensins can be insolubilised in the extracellular matrix to strengthen the cell wall or to lock the cell in shape after cessation of growth (Showalter, 1993). Recently, the role of an extensin in the correct positioning of the cell plate during cytokinesis in the *Arabidopsis* embryo has been genetically demonstrated (Hall and Cannon, 2002). *LRX1* is specifically expressed in root hairs, and *lrx1* mutants develop aberrant root hair structures resulting in frequent growth arrest. The modular structure of LRX1, together with the root hair phenotype in *lrx1* mutant plants, suggests that LRX1 is involved in the regulation of cell wall formation and assembly (Baumberger *et al*., 2001). *LRX1* is a member of a family of 11 *LRX* genes identified in the *Arabidopsis* genome. *LRX* genes are found in plant species of diverse origin, suggesting a fundamental role of LRX proteins during plant development. The *LRX* gene family can be divided into two classes, which encode proteins expressed in vegetative and reproductive tissue, respectively (Baumberger *et al*., 2003).

Here, we present the characterisation of the *Arabidopsis* gene *LRX2*, a paralog of *LRX1*. *LRX2* is expressed in root hairs, and genetic analysis of *lrx2* single and *lrx1/lrx2* double mutants indicates that LRX2 synergistically interacts with LRX1 during root hair development. Although *LRX1* and *LRX2* are highly homologous, there are differences in the physiological function of the proteins. The microscopic analysis of the extracellular matrix of *lrx1/lrx2* double mutants revealed irregular cell wall structures, demonstrating that LRX1 and LRX2 are involved in the correct development of cell wall architecture.

Results

The LRX1 *and* LRX2 *sequences are highly similar*

A BLAST search of the complete *Arabidopsis* genome sequence, performed with the LRR domain of LRX1 (accession number At1g12040), identified 10 additional genes encoding putative LRX proteins (Baumberger *et al*., 2003). The sequence showing the highest similarity to *LRX1* was named *LRX2 (*accession number At1g62440). *LRX2* is located on chromosome 1, BAC clone F24O1, and consists of an intronless open-reading frame of 2358 bp, encoding a protein of 786 amino acids. LRX2 shows the characteristic organisation of LRXs: a signal peptide, a LRR domain made of 10 repeats, a cystein-rich hinge region and a C-terminal extensin domain (Baumberger *et al*., 2003). With 87.6% of identity with LRX1, the 235-amino-acid-long LRR domain is the most conserved part of the protein. The 29 non-conserved amino acids are distributed over the 10 LRRs and occur at a similar frequency in the predicted solvent-exposed regions (xxLxLxx; see Figure 1a) and in the rest of the domain (90 and 87% identity, respectively). The extensin domain is organised in subdomains of repeated sequences, which are similar but not identical to the repeats found in the LRX1 extensin moiety (Figure 1a). The first extensin subdomain of LRX2, made of three P_4S_2KMSPSFRAT motifs followed by two P_4S_2kMSPSVrAY repeats, corresponds to

the three repeats of SP_5S_2KMSPSVRAY present in LRX1 (capital case characters represent residues present in more than 50% of the repeats, whereas lower case characters represent residues present in up to 50% of the repeats). Similarly, the second LRX2 extensin subdomain, made of two similar repeats based on the motif SP_4SP_4YIYS, has its equivalent in LRX1 in the form of six SP_4YVYS repeats. The third subdomain is characterised by 10 repeats containing a tyrosine pair and a terminal glutamine.

LRX1 and *LRX2* are located on the two copies of a large duplicated genomic fragment, as defined by Vision *et al*. (2000). The gene identity and order is conserved in the two chromosomal regions harbouring *LRX1* and *LRX2*, indicating that they are paralogous genes, which originated from one of the biggest genome duplication events in the evolutionary history of *Arabidopsis* (Figure 1b; Vision *et al*., 2000).

LRX2 *is predominantly expressed in root hairs*

The expression pattern of *LRX2* was determined by Northern hybridisation and analysis of transgenic plants harbouring the *uidA* (*GUS*) gene under the control of the *LRX2* promoter (*pLRX2::GUS*). Northern blot hybridisation revealed that *LRX2* is mainly expressed in roots, while a very weak expression is also found in stem (Figure 2a). An identical pattern was obtained with RT-PCR using *LRX2*-specific primers (data not shown). The size of the hybridising transcript on Northern blots (2.6 kb) is in agreement with gene prediction. The predominant expression of *LRX2* in roots was confirmed in *pLRX2::GUS*-trangenic plants by the presence of GUS activity in root hair cells along the differentiation zone, as well as in the collet region and in the root meristematic region (Figure 2b). In some cases, a faint GUS staining was also observed in the inner cell layers of the root, particularly in the cortex of the hypocotyl and in the mesophyll cells of the leaves (data not shown). Agents promoting or repressing root hair formation, such as the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or ethylene biosynthesis inhibitor L-α-(2-aminoethoxyvinyl)-glycine (AVG), have a strong impact on *LRX2* expression. Plants treated with ACC formed ectopic root hairs and showed an elevated level of *LRX2* expression. In contrast, plants treated with AVG stopped root hair formation and showed a reduced level of *LRX2* expression. *LRX2* expression was also strongly reduced in the *rhd6* mutant (Masucci and Schiefelbein, 1994), which only sporadically forms root hairs (Figure 2c). These data suggest that *LRX2* expression is predominantly associated with the formation of root hairs. Pollen tube is the other cell type that expands by tip growth. However, our analysis by Northern blots and staining for GUS activity in the *pLRX1::GUS*- and *pLRX2::GUS*-trangenic plants did not reveal expression of the two genes in pollen (data not shown).

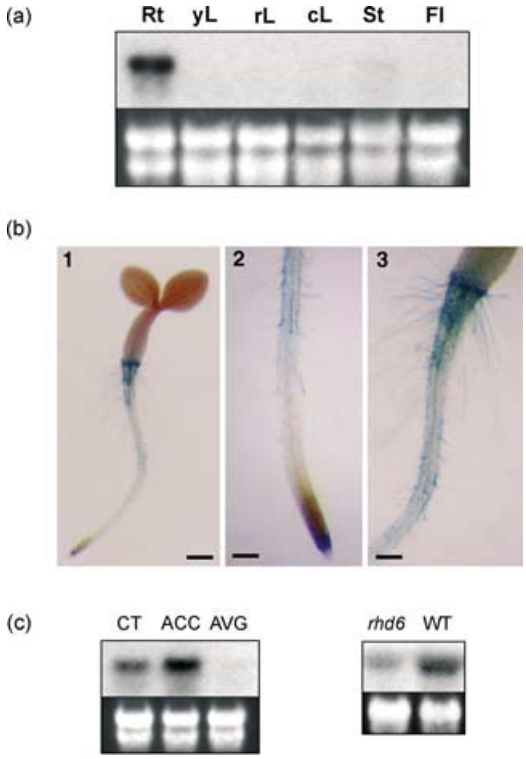

Figure 2. *LRX2* is predominantly expressed in root hairs.
(a) Organ-specific expression of *LRX2*. Total RNA was extracted from roots of vertically grown seedlings (Rt), developing leaves of 2-week-old seedlings (yL), mature rosette leaves (rL) and cauline leaves (cL), stem (St) and flower buds (Fl) of 6-week-old plants.
(b) Transgenic seedlings containing 1.5 kb of *LRX2* promoter sequence fused to the *uidA* (*GUS*) gene were histochemically stained to reveal the tissue-specific *LRX2* expression. GUS activity (blue staining) was detected in trichoblast cells along the root differentiation zone (1, 2) and in the collet region (1, 3). GUS activity was also detected in the meristematic region at the root tip (2). Bars: 200 μm (1); 100 μm (2, 3).
(c) *LRX2* expression in AVG- and ACC-treated seedlings and in the *rhd6* mutant. For Northern analysis, 5 μg of RNA per lane was blotted and hybridised with a *LRX2*–specific probe (upper panels). Ethidium bromide-stained rRNAs were used as a loading control (lower panels).

LRX1 *and* LRX2 *are functionally similar but not identical*

Overexpression of the N-terminal moiety of LRX1 (from the ATG start codon to the end of the LRR domain) in wild-type plants results in a dominant *lrx1*-like phenotype, indicating that this truncated protein has a high affinity towards, and thus titrates out, a putative interacting partner of the endogenous LRX1 (Baumberger *et al*., 2001). The physiological action of the corresponding fragment of LRX2 (Figure 3a) was tested by overexpression in wild-type plants. The truncated LRX2 protein accumulated, as shown by Western blot experiments (Figure 3g), but did not result in an aberrant root hair phenotype (Figure 3h). Thus, the N-terminal moieties of LRX1 and LRX2 have different properties in terms of binding specificity and/or affinity towards their interaction partner. To analyse this difference in more detail

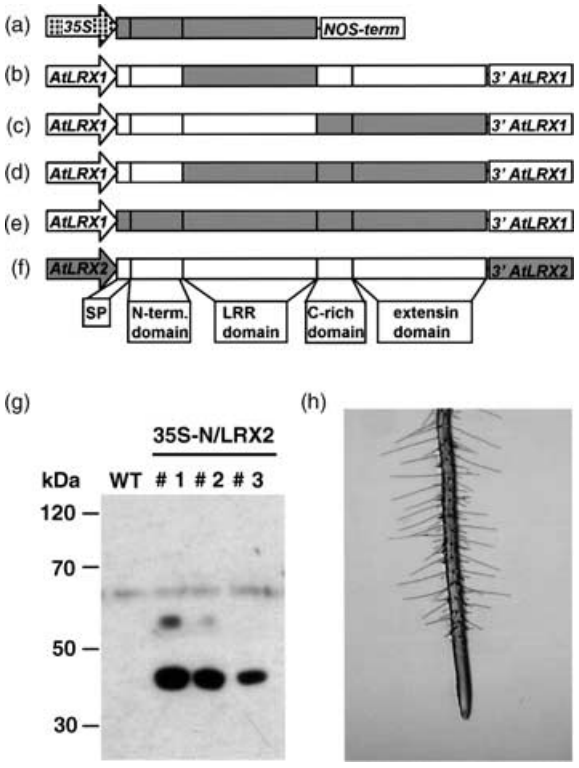

Figure 3. Truncated and chimaeric constructs used for complementation of the *lrx1* mutation.
(a) Overexpression construct of the LRX2 N-terminal moiety (N/LRX2, grey) with the 35S CaMV promoter (dotted arrow).
(b–f) In the chimaeric constructs, the domains of LRX1 (white) were replaced by their equivalents of LRX2 (grey). The open-reading frames were under the regulation of 1.5 kb of 5′- and 0.8 kb of 3′-*LRX1* sequence.
(g) Protein of a wild-type Columbia plant and of three independent lines transformed with a 35S CaMV promoter::*N/LRX2* construct was extracted, separated by denaturing SDS–PAGE and immunolabelled with a polyclonal anti-LRX2 antiserum.
(h) Seedlings of N/LRX2-overexpressing lines were vertically grown on MS medium and were microscopically examined after 3 days.
The identity of the different domains is indicated in (f). SP: signal peptide.

and to investigate possible functional redundancy between LRX1 and LRX2, the LRR or extensin domain of LRX1 was replaced by the equivalent of LRX2. These chimaeric constructs, under the regulation of 1.5 kb of 5′- and 0.8 kb of 3′-*LRX1* sequence (Figure 3b,c), were transformed into the *lrx1* mutant background, and complementation of the *lrx1* phenotype was used as a parameter for protein function. The analysis of the root hair phenotype of four independent T_2 lines for each construct revealed a wild-type-like phenotype, indicating that both chimaeric constructs are functional and complement the *lrx1* mutation. A third construct in which both the LRR and the extensin domain of LRX1 were replaced by those of LRX2 (Figure 3d) gave the same result (data not shown). This demonstrates that, under these experimental conditions, the LRX2 LRR and extensin domain driven by the *LRX1* promoter can functionally replace the corresponding domains of LRX1. To test whether the N-terminus preceding the LRR domain of LRX1, a part of the protein that had not been exchanged in the chimaeric constructs, determines the specificity of the protein, we generated additional transgenic *lrx1* plants expressing the full-length *LRX2* gene under the regulation of the *LRX1* promoter (Figure 3e). This construct also complemented the *lrx1* mutation, indicating that the N-terminal domains of LRX1 and LRX2 do not specify interaction with different ligands (data not shown). Finally, the full-length *LRX1* expressed under the regulation of *LRX2* promoter (1.3 kb and 0.4 kb of 5′- and 3′-non-transcribed sequence, respectively; Figure 3f) was also able to complement the *lrx1* mutation (data not shown). Thus, the *LRX1* and *LRX2* promoters are regulated in a similar way in root hairs.

lrx2 *null mutants are indistinguishable from the wild type but enhance the effect of the* lrx1 *mutation*

An *lrx2* loss-of-function mutant was identified in an *En-1* mutagenised *Arabidopsis* population (Wisman *et al.*, 1998). The mutant line carried a transposon insertion at the 3′ end of the LRR-coding domain. A stable *lrx2* mutant allele created by excision of the transposon at the *LRX2* locus was identified by PCR screening. The footprint left in the *lrx2* stable mutant allele caused a frame shift, which only allowed the translation of a truncated protein completely lacking the extensin domain (Figure 4a). Northern hybridisation indicated that the amount of *LRX2* transcript was strongly reduced in *lrx2* mutant plants compared to the wild type, suggesting that the *lrx2* mutation also reduced

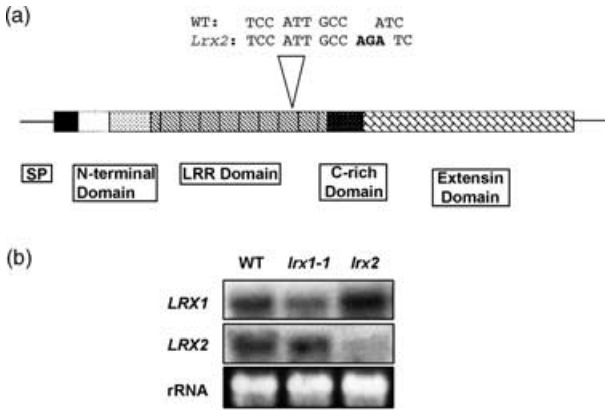

Figure 4. *En-1* transposon-insertion mutant of *lrx2*.
(a) Position and footprint sequence of the *lrx2* mutation. The two-nucleotide footprint left by the autonomous excision of the *En-1* transposon in the eighth LRR disrupts the *LRX2* coding sequence by introducing a frame shift mutation.
(b) Expression of *LRX1* and *LRX2* in *lrx1* and *lrx2* mutants. Total RNA was extracted from 4-day-old seedlings grown vertically on MS plates. Five micrograms of RNA was blotted and hybridised sequentially with an *LRX1*- and an *LRX2*-specific probe (upper panels). Ethidium bromide-stained rRNA was used as a loading control (bottom panel).

the stability of the *lrx2* transcript. The expression of the wild-type *LRX2* gene in the *lrx1* mutant was similar to its expression in wild-type plants. Interestingly, the level of *LRX1* transcript appeared slightly higher in *lrx2* mutants than in wild-type plants, suggesting that the plant compensated the absence of functional LRX2 by increasing the level of expression of LRX1 but not vice versa (Figure 4b).

The *lrx2* plants were morphologically identical to wild-type plants both in soil and *in vitro.* With regard to the predominant *LRX2* expression in roots and its correlation with root hair growth, we checked the root morphology and root hair formation with light microscopy and low-temperature scanning electron microscopy (LTSEM). The *lrx2* root development was indistinguishable from that in wild-type plants (Figure 5a,c,e,f).

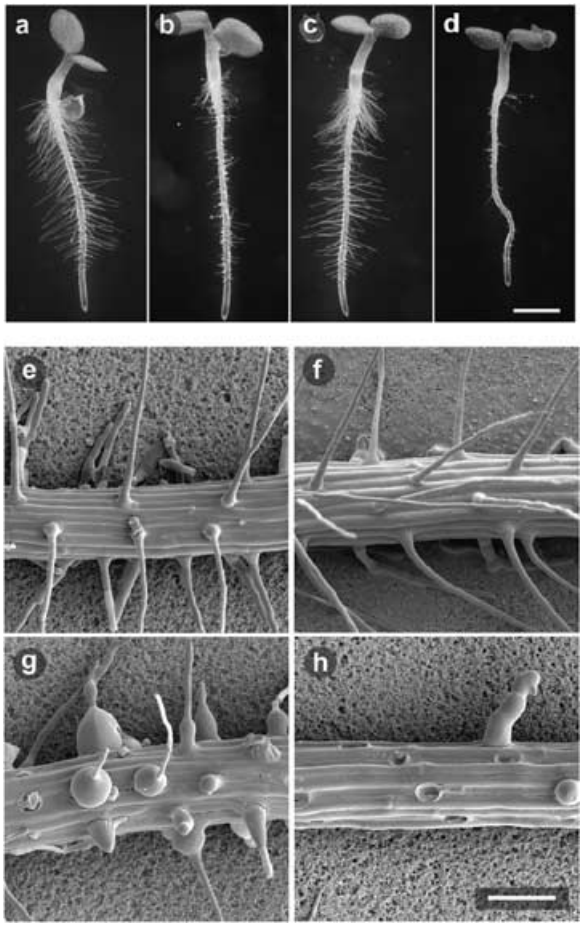

Figure 5. *lrx2* and *lrx1/lrx2* mutant phenotypes.
Wild-type, *lrx1*, *lrx2* and *lrx1/lrx2* seedlings were grown for 3–4 days on vertical MS plates, and were either observed under a stereomicroscope (a–d) or plunge-frozen in liquid propane and observed at low temperature using a scanning electron microscope (e–h). Roots of the wild type (a, e) and *lrx2* (c, f) were undistinguishable. Root hairs in the *lrx1* mutant frequently had a swollen basis, and were shorter, irregular in diameter, branched and sometimes collapsed (b, g). The majority of the root hairs in *lrx1/lrx2* collapsed or were very short (d, h).
Bars: 1 mm (a–d); 200 μm (e–h).

To investigate the possible genetic relationship between *LRX1* and *LRX2,* we generated a *lrx1/lrx2* double mutant. Homozygous *lrx1/lrx2* plantlets displayed a severely impaired root hair development and mostly appeared as morphologically hairless (Figure 5d). Thus, *lrx2* strongly enhances the *lrx1* phenotype, indicating a synergistic interaction between the two genes. When root hairs developed, they had aberrant shapes similar to the root hair defects observed in *lrx1*, i.e. they showed reduced length, bending, branching and swelling. However, as revealed by LTSEM (Figure 5h) and propidium iodide staining (data not shown), most of the trichoblast cells in *lrx1/lrx2* double mutants were dead. Traces of collapsed root hairs could be identified in these dead trichoblasts, indicating that hairs initiated but ruptured shortly afterwards. Other parameters of root hair development such as density and epidermal cell specification were not affected in the double mutant, and neither the shape and size of the epidermal part of the trichoblast cells nor the root anatomy appeared to be modified by the mutations (data not shown). No difference compared to the wild-type phenotype was observed in any other part of the plant. Transformation of the *lrx1/lrx2* double mutant with the genomic clone coding for *LRX2* (see Experimental procedures) resulted in plants exhibiting the *lrx1* single mutant phenotype (data not shown). Thus, the enhanced root hair phenotype of the *lrx1/lrx2* double mutant was indeed caused by the mutation in *LRX2*.

Aberrant cell wall architecture in the lrx1/lrx2 *double mutant*

The rupture observed in most root hairs in the *lrx1/lrx2* double mutant, as well as the cell wall localisation of LRX1 (Baumberger *et al.*, 2001), strongly suggests that LRX1 and LRX2 contribute to cell wall integrity in root hairs. Therefore, we investigated the ultrastructure of the root hair cell walls of *lrx1/lrx2* seedlings by transmission electron microscopy (TEM). Regions of the *lrx1/lrx2* root containing root hairs were first identified, selected on semi-thin sections and subsequently used for thin sectioning and TEM. Thus, there was a bias towards intact root hairs, i.e. less severe phenotypes, as most of the root hairs in *lrx1/lrx2* were collapsed. The cell wall of wild-type root hairs was made up of a regular pattern of fibrillar material, and had a constant thickness, regular density and smooth outer surface (Figure 6a). In contrast, the extracellular matrix of *lrx1/lrx2* double mutants was of irregular thickness and variable electron density, and had irregular inclusions of osmophilic material. Frequently, the mutants had a dispersed cytoplasm and lacked a distinct plasma membrane, suggesting that these root hair cells were already dead (Figure 6b–d). Cell death was most likely a consequence of the *lrx1/lrx2* mutations rather than an artefact during sample fixation

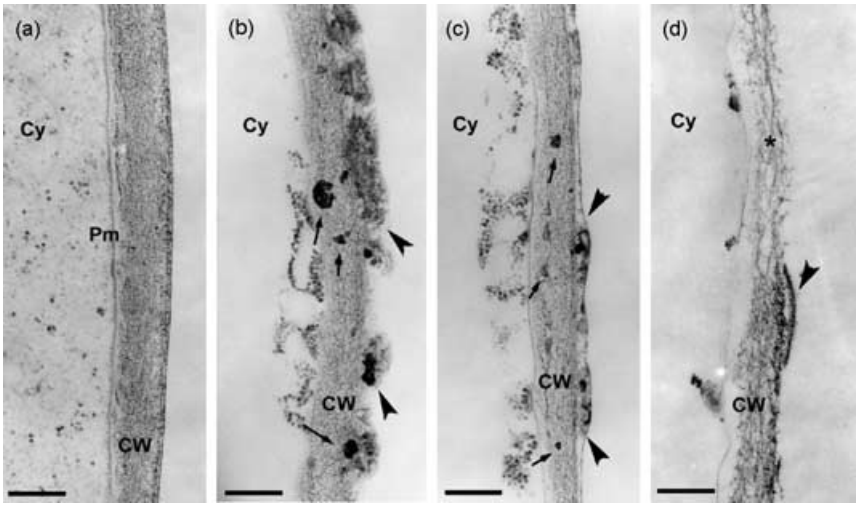

Figure 6. *lrx1/lrx2* double mutants show an aberrant cell wall architecture.
The ultrastructures of the extracellular matrix of wild-type (a) and *lrx1/lrx2* root hairs (b–d) were compared by transmission electron microscopy. Longitudinal sections (a, c, d) and a transversal section (b) of lateral cell walls of mature root hairs are shown. Wild-type hairs have a cell wall of regular density, constant thickness and smooth outer surface. The separation (in *lrx1/lrx2* cell walls) between an irregular outer, electron-dense layer and an inner layer mostly similar to the wild-type cell wall is indicated by arrowheads. The arrows point at inclusions of osmophilic material embedded in the surrounding fibrillar matrix. Occasional loosening of the secondary inner layer was also observed in cell walls of *lrx1/lrx2* plants (asterisk in (d)).
Cy: cytoplasm; CW: cell wall; Pm: plasma membrane. Bar: 0.25 μm.

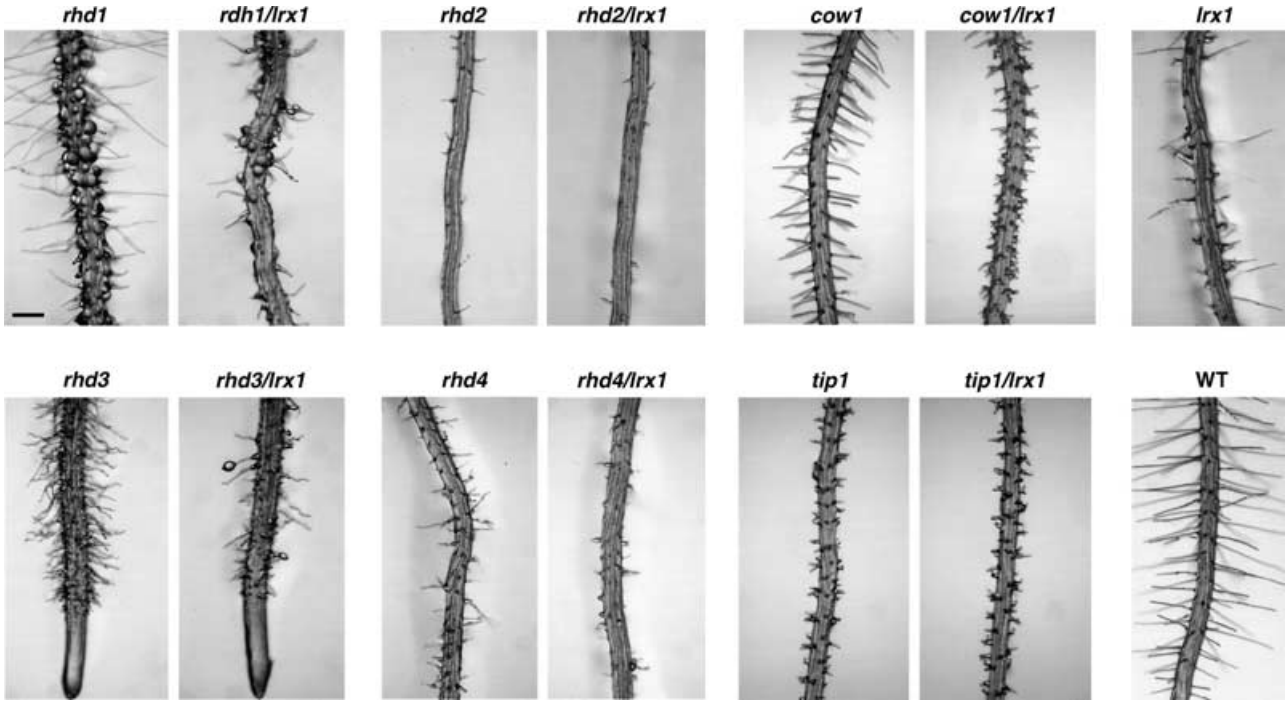

Figure 7. Genetic interaction of *lrx1* with other root hair mutants.
Seedlings of single and double mutants were grown for 3–4 days on vertical MS plates and observed under a stereomicroscope. *lrx1/rhd1*, *lrx1/rhd3* and *lrx1/rhd4* show additive phenotypes. *rhd2* and *tip1* are epistatic to *lrx1*. The root hairs of *lrx1/cow1* indicate a synergistic effect of *lrx1* and *cow1*.
Bar: 200 μm.

because it was also observable in light microscopy and LTSEM (Figure 5 and data not shown).

Genetic interaction of lrx1 *and* lrx2 *with other root hair mutants*

To further investigate the function of *LRX1* and *LRX2* during root hair development, we generated double mutants between *lrx1* or *lrx2* and other root hair mutants (*rhd1*, *rhd2*, *rhd3*, *rhd4*, *cow1* and *tip1*; Grierson *et al.*, 1997; Ryan *et al.*, 1998; Schiefelbein and Somerville, 1990). Seedlings of *lrx1/rhd1*, *rhd3* and *rhd4* double mutants show additive phenotypes with frequently ruptured root hairs, as observed in *lrx1* mutants, and bulges, wavy root hairs or almost naked roots, respectively (Figure 7). This indicates that *LRX1* acts in parallel with *RHD1*, *RHD3* and *RHD4*. In contrast, *rhd2* and *tip1* were clearly epistatic to *lrx1*, and the phenotype of the double mutants was undistinguishable from that of *rhd2* or *tip1* (Figure 7). Interestingly, *lrx1/cow1* displayed a novel phenotype: root hairs were shorter than those in the single *cow1* mutant, developed as multiple hairs (up to four shafts per bulge) and had a swollen basis. In parallel, the ruptured root hair phenotype characteristic of *lrx1* mutants was suppressed (Figure 7). In contrast to *lrx1,* the *lrx2* mutation had no effect on any other root hair mutation, and the double mutants systematically showed the phenotype of the other parent (data not shown). Therefore, the synergistic effect of *lrx2* on root hair development is specific for the *lrx1* mutation.

Discussion

LRX2 is a paralog of the *LRX1* gene that arose from a duplication of the genomic region encompassing *LRX1* about 100 million years ago (Baumberger *et al.*, 2003; Vision *et al.*, 2000). As *LRX1*, *LRX2* is also involved in root hair development. Northern blot experiments and the complementation of the *lrx1* mutation by an *LRX1* promoter::*LRX2* and an *LRX2* promoter::*LRX1* construct show that the expression profiles and the function of the two genes are similar. In fact, the LRR and extensin domains of LRX1 and LRX2 can be mutually replaced, revealing that these two proteins, and most likely LRX proteins in general, are multidomain proteins with functionally independent and exchangeable modules. However, LRX2 appears to have a lower affinity for the putative interaction partner of LRX1 than LRX1, as the dominant negative effect by the overexpressed LRX1 N-terminal moiety (Baumberger *et al.*, 2001) was not observed with the equivalent construct of LRX2. This finding provides a possible explanation as to why the endogenous *LRX2* cannot substitute for *LRX1*, resulting in an *lrx1* mutant phenotype. Despite this difference, *LRX2* is important for root hair development, and a mutation in *LRX2* has a synergistic effect on the *lrx1* mutant phenotype.

The functional similarity of LRX1 and LRX2 LRR domains is obviously independent of a number of differences in their protein sequences. Crystallographic studies of LRR domains have shown that the xxLxLxx motifs form the surface of interaction with the ligand and are therefore essential for the recognition specificity (Kobe and Deisenhofer, 1995; Papageorgiou *et al.*, 1997). An analysis performed on a *PGIP* gene has confirmed that single mutations in those solvent-exposed regions can be sufficient to generate new specificities (Leckie *et al.*, 1999). Surprisingly, a total of five solvent-exposed amino acids differ between LRX1 and LRX2, obviously without affecting the function of the proteins profoundly. Therefore, the specificity for the putative ligand does not reside in the three LRRs (the 3rd, 8th and 9th), which contain these variable residues. By comparison with the LRR domains of other members of the *Arabidopsis* LRX family, two solvent-exposed amino acids in the 6th LRR (the first A and the D residue) are specific to LRX1 and LRX2 (Baumberger *et al.*, 2003) and might be important determinants of specificity.

LRX1 and PEX1, an LRX1-like protein of maize, are insolubilised in the cell wall matrix (Baumberger *et al.*, 2001; Rubinstein *et al.*, 1995). This suggests that the LRX extensin moiety targets the LRR domain to the proper position in the cell wall and subsequently becomes insolubilised. The fact that a truncated LRX1 protein lacking the extensin domain is unable to complement the *lrx1* mutation (Ringli and Keller, unpublished data) provides further evidence for the importance of the extensin domain. The domain swap experiments indicate that the putative anchoring/targeting function is equally well performed by the extensin domains of LRX1 and LRX2. The presence of three subdomains is the most striking similarity between the two largely conserved extensin domains. Therefore, the particular function of the extensin domain is probably determined by motifs belonging to these three subdomains. Alternatively, the structure of these subdomains, particularly their glycosylation pattern rather than their primary sequence, might be the major determinant of the conformation and hence the function. In future experiments, the functionally equivalent extensin domains of LRX1 and LRX2 will be compared and analysed in more detail to better understand the basis of extensin activity. With the identification of *lrx1* and *rsh*, an extensin mutant defective in the positioning of the cell plate during cytokinesis (Hall and Cannon, 2002), genetic tools have become available to investigate the functional importance of extensin motifs through complementation experiments.

The extracellular matrix of root hairs consists of a primary cell wall layer formed at the growing tip and a secondary layer, which is later deposited along the root hair shaft (Sassen *et al.*, 1985). *lrx1/lrx2* double mutants show an aberrant and irregular cell wall structure (Figure 6). The primary layer of the cell wall appears thicker and fragmented compared to wild-type plants. In some cases, similar

defects are observed in the secondary cell wall layer, which is normally formed by deposition of multiple layers of ordered cellulose microfibrils (Sassen *et al.*, 1985). In *rhd4* mutants, a similar increase in the thickness of the primary cell wall is observed and can be explained by the continuous deposition of new cell wall material during reduced tip growth characteristic of this mutant (Galway *et al.*, 1999). The structure of the extracellular matrix in *lrx1/lrx2* double mutants, however, appears less regular than in *rhd4* plants, suggesting that the observed enlargement in the double mutants is rather the result of an uncontrolled assembly of stochastically deposited cell wall material. The frequent burst of root hairs indicates that the observed irregular structure weakens the extracellular matrix that can rarely resist the turgor pressure of the protoplast. These results suggest that LRX1 and LRX2 are involved in the assembly of the primary cell wall layer at the tip as well as of the secondary layer along the lateral walls of the root hair, which is consistent with the previously determined localisation of LRX1 (Baumberger *et al.*, 2001).

In the double mutant analysis, *tip1* and *rhd2* are epistatic to *lrx1*, suggesting that TIP1 and RHD2 either function in the same process but at an earlier stage than LRX1, or are required for LRX1 activity. As TIP1 and RHD2 are postulated to be involved in early root hair development and subsequent tip growth, respectively (Schiefelbein *et al.*, 1993; Wymer *et al.*, 1997), LRX1 probably functions only after tip growth is initiated. The additive phenotypes of the *lrx1/rhd1*, *lrx1/rhd3* and *lrx1/rhd4* mutants indicate that RHD1, RHD3 and RHD4 act independently of *lrx1*. In contrast, these three *rhd* mutations suppress the phenotype of *kojak*, which encodes a cellulose synthase-like protein and exhibits fragile root hairs that frequently collapse (Favery *et al.*, 2001). The distinct phenotypes of *lrx1* and *kojak* in the double mutant backgrounds indicate that the cell wall defects are of different nature. The suppressed root hair rupture observed in *lrx1/cow1* plants indicates that COW1 might act in a pathway that counteracts LRX1, and thus mutations at both loci partially neutralise each other. The absence of a synergistic effect of *lrx2* on any of the root hair mutants indicates that LRX2 functions specifically in LRX1-dependent root hair development.

Duplicated genes can have different evolutionary fates leading to silencing, acquisition of new functions (neofunctionalisation) or deviation from the original function (subfunctionalisation) with a sufficient overlap to maintain the original function of the ancestral gene (Lynch and Conery, 2000; Ohno, 1973). A possible consequence of subfunctionalisation can be that single mutants of a gene family are indistinguishable from wild-type plants under standard growth conditions. In contrast, double mutants can exhibit a striking phenotype. The overlapping functions of *LRX1* and *LRX2* and the striking double mutant phenotype indicate that the two genes are the result of a subfunctionalisation process. Functional redundancy has also been investigated for the *ACTIN* gene family of *Arabidopsis* (Meagher *et al.*, 1999). While the *act7* mutant is not distinguishable from the wild type and the *act2* mutation causes aberrant root hair development, the *act2/act7* double mutant is affected in almost every aspect of development (Gilliland *et al.*, 2002; Ringli *et al.*, 2002). These examples show that the functional contribution of the members of a gene family might appear subtle but can, in fact, be substantial and thus reveal the importance of functionally redundant multigene families for the fitness of an organism.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia was used for all experiments. The *lrx1* line is a footprint mutant caused by the excision of *En-1*, with a 6-bp deletion in the sixth LRR. The *rhd1, rhd2, rhd3* and *rhd4* mutants were obtained from the *Arabidopsis* Biological Resource Center. The *rhd6* and *tip1* were a gift from J. Schiefelbein, and *cow1-2* was provided by C. Grierson. The *lrx2* mutant was isolated from an *En-1* mutagenised *Arabidopsis* population (Wisman *et al.*, 1998) by PCR screening, as described by Baumberger *et al.* (2001), using the *LRX2* gene-specific primers lrx2MUT1f, lrx2MUT1r, lrx2MUT2f and lrx2MUT2r, and a probe derived from the *LRX2* ORF bp114–1181, spanning the LRR domain (referred to as *LRX2* probe). The precise position of the *En-1* insertion was determined by cloning and sequencing the PCR products spanning the right and left border of the *En-1* element. Mutant plants were backcrossed at least four times with wild-type plants to remove additional insertions. Plants carrying an *lrx2* allele with a footprint after excision of *En-1* were identified by PCR and confirmed by sequencing. All experiments described in this paper were performed with plants carrying a footprint allele of *lrx2*. The plants were grown as described by Baumberger *et al.* (2001).

DNA primers

The following DNA primers were used for plasmid constructs and screening of the *En-1* mutagenised population. The numbers in parentheses refer to the position of the 5′ end of the primer relative to the transcription start of *LRX2*.

lrx2MUT1f: 5′-GTTGTTTCCTTCTACTTCTTTACGGTCTC-3′ (−5)
lrx2MUT1r: 5′-GGAGTTATACCAAGCAGCATTTGTCAG-3′ (1450)
lrx2MUT2f: 5′-ATGCCCTAACGGAAGGTGACATTTCG-3′ (907)
lrx2MUT2r: 5′-GATAGGCGGAAGAGGTGTGTCTTCG-3′ (2301)
pLRX2GUSf: 5′-AAAAGCTTTAGTTGGAGGTTAATTTACGC-3′ (−1510)
pLRX2GUSr: 5′-AATCTAGAAGAGACGTAAAGAAGTAGAAG-3′ (31)

LRX2 *genomic clone isolation*

A λZapII *A. thaliana* genomic library (Stratagene, La Jolla, CA, USA) was screened with the ^{32}P-labelled *LRX2* probe. A genomic fragment corresponding to bp 77782–84352 of the BAC clone F24O1 (accession: AC003113), containing the *LRX2* gene (accession: At1g62440) with 1.55 kb of 5′- and 2.6 kb of 3′-untranslated sequence, was isolated, cloned and sequenced (referred to as pλLRX2).

Constructs and plant transformation

For the *LRX2* promoter*::GUS* fusion construct (*pLRX2::GUS*), 1.5 kb of the promoter region was amplified by PCR from pλLRX2 with the primers pLRX2GUSf and pLRX2GUSr, digested with *Xba*I and *Hin*dIII, and cloned into pGPTV-KAN (Becker *et al.*, 1992).

The 35S-N/LRX2 construct was obtained by PCR amplification of the *LRX2*-coding region from the ATG to the C-rich hinge domain with oligonucleotides containing an *Eco*RI and an *Xba*I site, respectively. After control sequencing, the fragment was cloned into pART7 (Gleave, 1992) cut with the same enzymes to obtain the 35S-N/LRX2 fusion, and subsequently digested with *Not*I and cloned into the binary plant transformation vector pART27 (Gleave, 1992) cut with *Not*I. For the domain swap constructs, the *LRX1* construct including the c-myc tag (Baumberger *et al.*, 2001) was used, and an *Xho*I and a *Pst*I site was introduced by silent mutagenesis at the 3′ end of the c-myc tag and in the hinge region, respectively. In pλ*LRX2*, the *Xho*I site was already present at the equivalent position and the *Pst*I site was introduced by silent mutagenesis at the same position as in *LRX1*. In addition, an *Spe*I site was introduced at the stop codon as it is present in *LRX1*. The *Xho*I/*Pst*I and *Pst*I/*Spe*I fragments encompassing the LRR- and the extensin-coding region, respectively, were exchanged by digestion with the corresponding enzymes. For the *LRX1* promoter::*LRX2* and the *LRX2* promoter*::LRX1* constructs, a *Pst*I site was introduced in pλ*LRX1* and pλ*LRX2* (containing the *Spe*I site at the stop codon) in a signal peptide-coding region conserved between both genes. The *Pst*I/*Spe*I fragments of *LRX2* and *LRX1* were mutually exchanged. These constructs were directly cloned into pART27 for plant transformation.

Plant transformation and selection of transgenic plants was performed as described by Baumberger *et al.* (2001).

GUS histochemical analysis, ACC and AVG treatments

Histochemical staining for GUS activity was performed as described by Baumberger *et al.* (2001). For AVG and ACC treatments, plants were grown on the surface of normal MS medium for 3 days, transferred onto MS plates supplemented with 20 μM of AVG and 10 μM ACC (Sigma, Buchs, Switzerland), respectively, and grown for five additional days before RNA isolation. As control, plants were transferred onto MS plates without additions and subsequently grown for 5 days.

Phenotype observations

Light microscopic observations were carried out with a Leica stereomicroscope LZ M125. For scanning electron microscopy, seedlings grown on the surface of MS medium were transferred onto humid nitrocellulose membranes on metal stabs and plunge-frozen in liquid propane at −190°C. Frozen samples were stored in liquid N_2 until partial freeze-drying was performed in high vacuum ($<2 \times 10^{-4}$ Pa) at −90°C for 30 min. Sputter-coating with platinum was performed in a preparation chamber SCU 020 (BAL-TEC, Balzers, Liechtenstein) before observation at −120°C in an SEM 515 scanning electron microscope (Philips, FEI Co., the Netherlands).

Transmission electron microscopy

The first 5-mm of 3–4-day-old seedlings grown at the surface of vertical MS plates were excised and placed on small pieces of nitrocellulose membrane held at the tip of a thin metal wire and were mechanically plunge-frozen in liquid propane at −190°C. Samples were then placed in 1 ml of anhydrous acetone, 0.25% glutaraldehyde and 0.5% OsO_4 (Wild *et al.*, 2001), and were freeze-substituted for 48 h at −88°C. The temperature was then gradually increased over 12 h to 20°C, and the samples were kept for 1 h in ice-cooled water. The substitution solution was then replaced by ice-cold acetone three times, and samples were gradually infiltrated in Spurr resin. Thin blocks of 0.25 mm (made of two layers of Aclar® sheet (Plano, Wetzlar, Germany) separated by spacers of the same material) were polymerised at 70°C for 3 days. Embedded samples were checked under a binocular, and roots containing several root hairs were selected for sectioning. Thin blocks were mounted, and ultrathin sections of silver to gold interference were sectioned on an ultramicrotome (Reichert Ultracut E, Leica Microsystems, Switzerland), and were stained with 2% uranyl acetate in distilled water and alkaline lead citrate for 15 min each. Sections were observed with a transmission electron microscope (Philips CM 100 BIOTWIN, FEI Company, the Netherlands) at 80 kV using a 30-μm objective diaphragm and a LaB_6 cathode.

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