Plant Biotechnology 29, 373–381 (2012)
DOI: 10.5511/plantbiotechnology.12.0612a

Original Paper

Ectopic expression of the leucine-rich repeat-domain of cell wall extensin gene *NpLRX1* disturbs morphogenesis of leaf cells by its association with cell membranes in tobacco

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Received April 9, 2012; accepted June 12, 2012 (Edited by K. Kato)

Abstract *NpLRX1* (*Nicotiana plumbaginifolia Leucine-Rich Repeat-Extensin 1*) encodes a chimeric leucine-rich repeat (LRR)/extensin protein and is thought to regulate cell morphogenesis that is essential for plant tissue formation. We have reported that abnormal cell morphogenesis was observed in *NpLRX1*-RNAi-introduced tobacco adventitious buds. A similar phenotype was observed when truncated *NpLRX1* (*N/LRR*), a short N-terminal domain with LRR-domain, was expressed. Large gaps were observed between the cells with abnormal cell shapes in the leaves of the transgenic adventitious buds. The epidermal cells and the cuticle layer were defective in these leaves. The cells frequently swelled and the direction of cell division was sometimes out of order with an abnormal arrangement of microtubules in *N/LRR*-introduced tobacco BY-2 cultured cells. NpLRX1-GFP fusion proteins were detected on the cell membrane and in cell membrane-derived endocytic vesicles with partial degradation. These results suggest that ectopic expression of *N/LRR* causes defects in cell morphogenesis of tobacco tissues by the association of the LRR-domain with cell membrane.

Key words: Extensin, leucine-rich repeat domain, cell morphogenesis, cell membrane, tobacco

Plant cells are surrounded and attached to neighboring cells via the cell wall. The cell wall is composed of a network of cellulose microfibrils and hemi-cellulose, which are interconnected with xyloglucan chains and embedded in the hydrated pectin matrix (Carpita and Gibeaut 1993). In the cell wall, there are many kinds of proteins including proline-rich proteins (PRPs), glycine-rich proteins (GRPs), and hydroxyproline-rich proteins (HRGPs) (Showalter 1993). HRGPs are abundant cell wall structural proteins that have the highly glycosylated $Ser(Hyp)_4$ repeats and are thought to regulate the strength of the cell wall and cell shapes by cross-linking to each other and/or other cell wall components.

To correctly form organs and tissues, it is essential that each cell has contact with neighboring cells and adopts the proper shape. Because cells are surrounded by cell walls, the interchange of information among neighboring cells is performed through the cell wall. Thus, intercellular attachment mediated via the cell wall is very important for plant development and morphogenesis.

To identify the genes required for intercellular attachment, we have generated and analyzed *non-organogenic callus* with *loosely attached cells* (*nolac*) mutants, which were produced by the T-DNA tagging method using leaf disk cultures of haploid *Nicotiana plumbaginifolia* (Iwai et al. 2001, 2002). Because *nolac* mutants showed decreased intercellular attachment, these mutants cannot form adventitious shoots and generate paste-like callus or clusters of piecemeal small callus. One of the advantages of this system is that sexual reproduction is not required to generate and maintain the mutants. This enables us to analyze seriously weak mutants having defects in essential factors for life, such as enzymes required for normal cell wall synthesis. Even if the mutants have an embryo-lethal phenotype, we can maintain them as a non-organogenic callus in culture.

Recently, we reported the "*Nicotiana plumbaginifolia leucine-rich repeat-extensin 1*" (*NpLRX1*) gene, identified from the *nolac-K4* mutant (Chida et al. 2007). *NpLRX1* encodes a protein containing a short N-terminal domain,

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; LRR, leucine-rich repeat; LRX, leucine-rich repeat-extensin; *nolac*, non-organogenic callus with loosely attached cells; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; TB, Toluidine blue; DEX, Dexamethasone.

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This article can be found at http://www.jspcmb.jp/
Published online September 9, 2012

a leucine-rich repeat (LRR) domain, a cysteine-rich motif, and a C-terminal extensin domain. LRR domains have frequently been implicated in protein-protein or ligand-protein interactions, and in plants a large subclass of receptor-like kinases has extracellular LRRs in their receptor domain (Diévart and Clark, 2004; Jones and Jones, 1997). Extensins form an abundant group of cell wall structural proteins belonging to the family of HRGPs. Proposed functions of extensins include strengthening the cell wall and locking the cell shape after cessation of growth by insolubilization of the cell wall, with oxidative cross-linking to each other and/or other extracellular components (Bradley et al. 1992; Showalter, 1993).

In *N. tabacum,* leaf disks in which *NpLRX1*-RNAi is expressed formed pale green adventitious buds with thick and transparent leaves or white adventitious buds with a single leaf without a shoot meristem (Chida et al. 2007). In these leaves, the shapes of epidermal and mesophyll cells are aberrant and large spaces are present between cells. A similar abnormal cell morphogenesis was also observed in BY-2 (*Nicotiana tabacum* Bright Yellow-2) cells in which *NpLRX1*-RNAi was expressed. The cells were swollen and the direction of cell division was sometimes abnormal.

Genes encoding chimeric extracellular proteins composed of LRR and extensin domains have also been reported in other plant species, such as *Arabidopsis*, rice, maize and tomato, but their functions are not currently known in detail. In *Arabidopsis*, 11 *LRX* genes have been identified (Baumberger et al. 2003b) and four of them (*PEX1-4*) were specifically expressed in pollen and classified as reproductive LRXs, whereas the other seven genes (*LRX1-7*) were expressed in various tissues and classified as vegetative LRXs. Functional analysis of *Arabidopsis* LRXs has only been reported for *LRX1* and *LRX2*. *LRX1* and *LRX2* have overlapping functions in root hair formation and regulate cell morphogenesis by promoting proper development of the cell wall (Baumberger et al. 2001, 2003a; Ringli, 2005). In the *Arabidopsis lrx1* mutant, it was thought that defective cell expansion, resulting from spatially deregulated exocytosis or altered deposition of new cell wall materials, caused morphological defects in root hair. *LRX1* might have a function in fixing cell shape and in controlling the direction of cell expansion. However, the functions of the LRXs in the morphogenesis of the other organs remain unknown.

NpLRX1 is ubiquitously expressed in various organs, including the leaf, and was classified as a vegetative LRX, according to sequence similarity. The phenotypes of *NpLRX1*-RNAi introduced tobacco adventitious buds and the structure of NpLRX1 protein indicated that *NpLRX1* might have basic roles in proper cell morphogenesis that are essential for normal tissue development (Chida et al. 2007). To understand the function of *NpLRX1* for cell morphogenesis and cell adhesion, it is necessary to analyze the subcellular localization of *NpLRX1* and to identify the factors interact with *NpLRX1*.

In this study, the ectopic expression of truncated *NpLRX1* (*N*/*LRR*), a short N-terminal domain including LRR-domain, induced similar phenotypes to those observed in *NpLRX1*-RNAi transformants. We clarified that the NpLRX1 protein was localized on the cell membrane, with patch- or string-like arrangements, and also in cell membrane-derived endocytic vesicles. These results suggest that ectopic expression of *N*/*LRR* causes defects in cell morphogenesis of tobacco leaf tissues and BY-2 cells by the association of the LRR-domain with cell membrane.

Materials and methods

Plant materials and transformation

Nicotiana tabacum plants were grown on Murashige and Skoog (MS) medium prepared at 30 g l^{-1} sucrose and 0.7% agar. The transformation and regeneration were performed using the leaf disk co-cultivation method (Horsch et al. 1985). The transformed buds were selected using 100 mg l^{-1} kanamycin or 40 mg l^{-1} hygromycin. Suspension-cultured cells of tobacco BY-2 and BY-GT-16 cells were grown in 95 ml of modified Linsmaier and Skoog (LS) medium (Linsmaier and Skoog 1965) once per week at 25°C in darkness with agitation on a rotary shaker at 120 rpm. The cells were freshly sub-cultured every week.

Plasmid construction

To construct *35S promoter::NpLRX1*, the coding region of *NpLRX1* was amplified using L445-Dnega U (5′-CGGGATCCCAA AAT AGT AGC AAT GCG GCC -3′) and NpLRX1-SacAS (5′-CGAGCTCTCA ATA GTA TGG AAT GAC TG-3′) and cloned into the *Bam*HI and *Sac*I sites of the pBI121 vector. Underlining indicates the restriction enzyme recognition site in the primers for the introduction of the amplimer into vectors. To construct *35S promoter::NpLRX1*-RNAi, the RNAi construct cloned into the pDONR/Zeo vector (Invitrogen) used in a previous report (Chida and Yazawa et al. 2007) was introduced into the RNAi vector pK7GWIWG2(I) and pH7GWIWG2(I) vector (Karimi et al. 2005) using LR clonase. To construct the *35S promoter::N/LRR-Myc*, the NpLRX1 coding region without the extensin domain (amino acids 1-385) was amplified using LRR attB1 forward (5′-aaaaagcaggctTCA AGA AAA TAG TAG CAA TGC GGC C-3′) and LRR attB2 reverse (5′-agaaagctgggtTCA CGA CAG CAG ATA TAG CAG AGC GA-3′) primers. Small letters indicate the portion of the attB1 and attB2 sites, respectively, which were included in the primers for use in Gateway cloning. The amplified fragment was subsequently cloned into the pDONR/Zeo vector (Invitrogen) using the Gateway

method. The resulting construct was named pDONR-N/LRR. The fragment was subsequently introduced into the pGWB17 Gateway vector (provided by Tsuyoshi Nakagawa, Shimane University). To construct a glucocorticoid dexamethasone (DEX)-inducible *N/LRR-Myc* expression vector, the fragment was amplified from the pGWB17-*N/LRR-Myc* vector using primers LRX1-XhoS (5′-CTCGAGATA GTA GCA ATG CGG CCT CCT -3′) and LRR-SpeAS (5′-ACTAGTGCT CTA AGC GCT ACC GTT CAA G-3′) and cloned into the *Xho*I and *Spe*I sites of the pTA7002 vector (Aoyama et al. 1995). To construct *35S promoter::N/LRR of AtLRX1*, the fragment was amplified with the primers AtLRX1-BamS (5′-GGATCCATG TTG TTC CCT CCT CTT CGT TC-3′) and AtLRR-SacAS (5′-GAGCTCTAT AGG AGG ACG ACC GGA GAA GT-3′) and cloned into the *Bam*HI and *Sac*I sites of the pBI121 vector. To construct *35S promoter::NpLRX1-GFP*, the full-length *NpLRX1* was amplified using the primers L445-Dnega U (5′-CGGGATCCCAA AAT AGT AGC AAT GCG GCC -3′) and (5′-GGATCCATA GTA TGG AAT GAC TGG TGG TGG T-3′) and cloned into the corresponding site of a modified pBI121 vector, pBI121 *35S promoter::GFP*, in which the *GUS* gene was replaced with the *GFP* gene. To construct *35S promoter::N/LRR-GFP*, the expression fragment of pDONR-*N/LRR* was transferred into the pGWB5 Gateway vector (provided by Tsuyoshi Nakagawa, Shimane University). These constructs were introduced into the *Agrobacterium tumefaciens* strain LBA4404 C58 Rif (pGV2260) by the freeze-thaw method.

Transformation of BY-2 and BY-GT-16 cells

The BY-2 and BY-GT-16 cells at 4 days after inoculation onto a new medium were collected and spread on sterilized filter paper (Whatman). The plasmid construct described above (2 μg) was used to coat gold particles and cells were bombarded with a gene gun using the plasmid-coated gold particles (PDS-1000/He, Bio-Rad). The bombarded cells on the filter paper were cultured for 24 h on solid LS medium with 0.8% agar and subsequently transferred onto new solid LS medium containing 100 mg l^{-1} kanamycin or 40 mg l^{-1} hygromycin (40 μg/ml) to select transformed cells. After 2 weeks, the colonies derived from the transformed cells formed on filter paper were suspended and cultured in 5 ml of liquid LS medium containing appropriate antibiotics for 1 week at 25°C in darkness with agitation on a rotary shaker at 120 rpm. The increased cells were maintained in 95 ml volume, as described above.

Transient expression of NpLRX1 and N/LRR in onion epidermal cells

Plasmid construct (2 μg) were used to coat gold particles. The onion inner-layer of the epidermis was bombarded with a gene gun using the plasmid-coated gold particles (PDS-1000/He, Bio-Rad). The bombarded onion epidermis was incubated at 25°C on MS medium (pH 5.8).

Detection of GFP fluorescence in transgenic cells

Because many extensins and *Arabidopsis* LRX1 have been reported to be localized in the cell wall, we observed the GFP image under the conditions for the observation of the GFP image present in apoplasts (Tanaka et al. 2004). To visualize GFP, the samples were bathed in 20 mM PIPES-KOH (pH 7.0). In the plasmolysis experiment the samples were treated with 0.5 M mannitol and observed using a laser-scanning confocal microscope (Leica). GFP fluorescence was induced by excitation at 488 nm with an argon laser. BY-GT16 cells were observed using a fluorescence microscope (IX-70; Olympus) equipped with a CSU10 scanning head (Yokogawa, Tokyo, Japan) and a cooled CCD camera head system (CoolSNAP HQ; PhotoMetrics, Huntington Beach, Canada).

Fluorescence analysis with ER-tracker and FM4-64

FM4-64 and ER-tracker stains were performed as previously reported (Kutsuna and Hasezawa, 2002; Villarejo et al. 2005). BY-2 cells expressing NpLRX1-GFP were centrifuged and resuspended in medium containing 1 μM ER-Tracker Red dye (Molecular Probes) or 32 μM FM4-64 (Invitrogen). After incubation for an appropriate time at room temperature, cell suspensions were centrifuged and resuspended in fresh medium without dye. Cells were transferred to glass slides and observed using a fluorescence microscope (Leica).

Toluidine blue (TB) staining

TB staining was performed as described previously (Tanaka et al. 2004). The adventitious buds were submerged in 0.05% (w/v) TB (Sigma) solution, which had been filtered through a fiber media filter (pore diameter, 0.2 μm; Millipore). After 2 min, the TB solution was removed and buds were washed gently with water to remove excess TB from the plant.

Subcellular fractionation and detection of NpLRX1-GFP fusion protein by immunoblot

Suspension-cultured cells were collected by filtration on filter paper and a subcellular fractionation was performed as described previously (Satoh and Fujii 1984). Cells (1 g) were homogenized with a glass-Teflon homogenizer in 2 ml of extraction buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, and Protease inhibitor cocktail, Roche) on ice. The homogenate was filtered through four layers of MiraCloth to obtain a cell wall-rich fraction. The filtrate was centrifuged at 800×g at 4°C for 10 min to obtain a nucleus-rich fraction. The supernatant was centrifuged at 4,500×g to obtain a mitochondria-rich fraction, and subsequently centrifuged at 200,000×g for 30 min at 4°C to obtain soluble and microsome-rich fractions. The precipitates and 80% (v/v) acetone-precipitate of the soluble fraction were resuspended in sample buffer for SDS-PAGE and were boiled.

For immunoblot analysis, the proteins were resolved by 12% SDS-PAGE and electro-blotted onto polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore). Membranes were blocked in Tris-buffer saline (TBS; 10 mM Tris-HCl, pH 8.0 and 150 mM NaCl) containing 3% (w/v) bovine serum albumin (BSA) for 1 h at room temperature. Horseradish peroxidase-

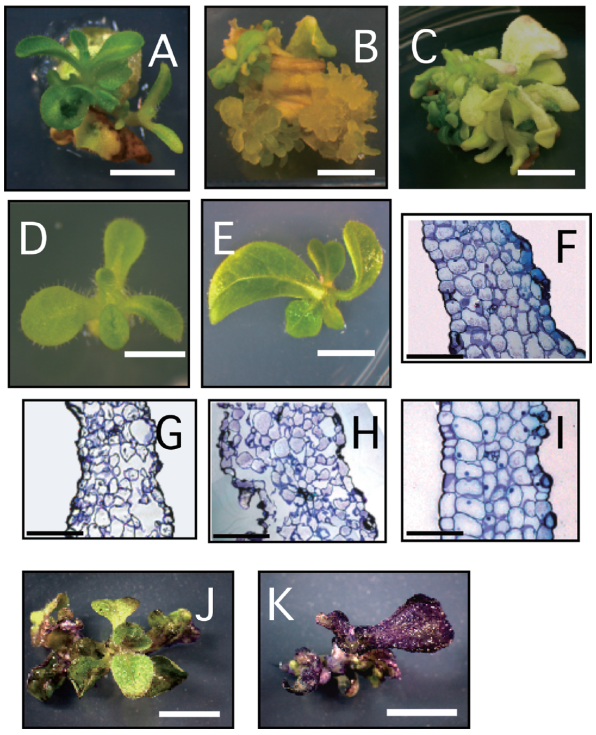

Figure 1. Phenotypes of transgenic adventitious buds generated from *N. tabacum* leaf disks. (A) *35S promoter::GFP*, (B) *35S promoter::NpLRX1*-RNAi, (C) *35S promoter::N/LRR-Myc*, (D) *35S promoter::NpLRX1* (full length), (E) *35S promoter::N/AtLRR*. (F)–(I) Transverse sections of leaves of the adventitious buds in a–d, respectively. (J)–(K) The toluidine blue (TB) test detects defects in the cuticle. The transgenic adventitious buds harboring DEX-inducible *N/LRR-Myc* constructs were stained with TB: (J) grown on medium containing ethanol as a control, (K) containing 10 μM DEX. A deficient cuticle allows TB to permeate the epidermal surface. Bars: 10 mm (A–E, J, K) and 1 mm (F–I).

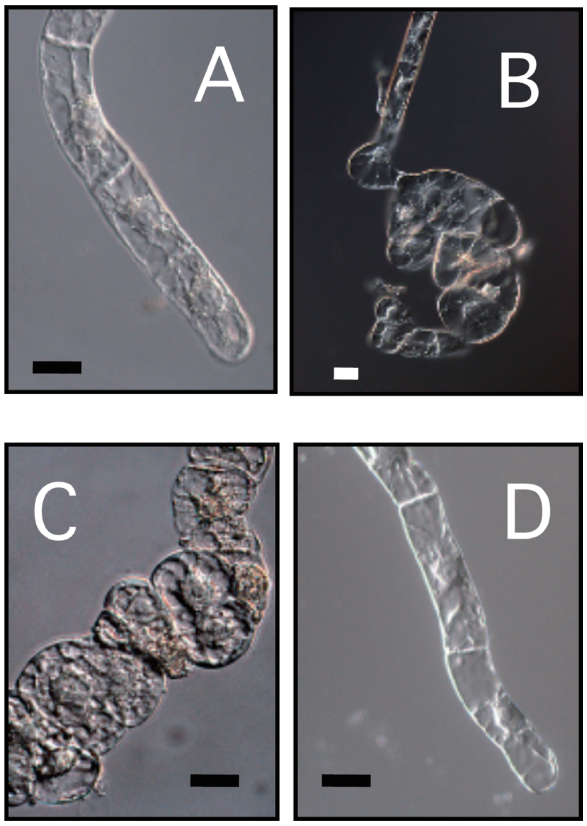

Figure 2. Phenotypes of transgenic BY-2 cells. BY-2 cells were cultured for 7 days and observed. (A) Control BY-2 cells. BY-2 cells expressing (B) *N/LRR-Myc*, (C) *NpLRX1*-RNAi and (D) *NpLRX1* (full length). The expression of each gene was controlled by the 35S promoter. The cells expressing *N/LRR-Myc* and *NpLRX1*-RNAi were frequently swollen and irregular in shape. Bars: 10 μm.

conjugated anti-GFP antibodies were used for detection, diluted 1 : 5000 in PBS-T. Incubations were done at room temperature and were followed by three washes of 10 min each in PBS-T. Chemiluminescence detection was performed with SuperSignal West Dura Extended Duration Substrate (Pierce) and LAS1000 (Fuji photo film).

Results

Morphological features of transgenic adventitious buds

The *NpLRX1*-RNAi construct driven by the 35S promoter was introduced into *N. tabacum* leaf discs. The NpLRX1-specific region between the LRR and the extensin domains was used as an RNAi trigger region because this region had 97% similarity to that of NtLRX1 and did not have significant homology to that of other NpLRXs. In the generated adventitious buds, almost no *NtLRX1* transcripts were detected by semi-quantitative RT-PCR (data not shown). These buds were showed abnormal morphology, compared to the control buds in which the *GFP* gene was expressed (Figure 1A, 1B). Most of the buds had transparent, pale green, and thick leaves. A white single leaf without shoot meristem was occasionally formed on the callus. The buds in which *N/LRR-Myc* (1–384 amino acid region of *NpLRX1* fused with a c-Myc tag) was expressed under the control of the 35S promoter and showed similar morphology to those transformed with *NpLRX1*-RNAi (Figure 1C). The growth of these abnormal buds ceased at this stage and they soon died, because water and nutrients might not have been sufficiently supplied between the cells, due to the large gap between the cells during the development of shoot and leaves. However, the transformed pale green leaves were not transparent and were considerably thicker than the control leaves. Although we tried to maintain these buds in the same state of a callus, as with the *nolac* mutants (Iwai et al. 2001), the buds soon died. Thus, the DEX-inducible *N/LRR-Myc* construct was introduced into *N. tabacum* leaf discs and normal adventitious buds were obtained in the absence of DEX. When the buds were transferred to DEX-containing medium, the adventitious buds showed a similar morphology to those driven by the 35S promoter (data not shown).

Compared with the control buds, the size and shape of cells in these transgenic leaves were irregular and large gaps were present between cells (Figure 1F–H). Because the thickness of the epidermal cells was not constant, the leaf epidermis was partially very thin or broken. On the other hand, defects were not observed in transgenic buds in which the full-length *NpLRX1* was expressed (Figure 1D, 1I). Additionally, transformation with *Arabidopsis* N/LRR (N/AtLRR), the truncated protein which lacks the extensin-domain of *Arabidopsis LRX1* (*AtLRX1*) that had the highest similarity to *NpLRX1* within the *Arabidopsis* LRXs family, did not affect the morphology of adventitious buds at all (Figure 1E).

The surface of the transgenic leaves was not smooth and had some defects in the cuticle layer (Figure 1G, 1H). The toluidine blue (TB) test that detects plant cuticle formation defects (Tanaka et al. 2004) showed that the buds in which *N/LRR-Myc* was expressed in the presence of DEX were strongly stained in most parts of the buds (Figure 1K), whereas control buds in the absence of DEX did not stain with TB because of the presence of the intact cuticle layer on the outer surface of the plant (Figure 1J).

Morphological features of transgenic BY-2 cells

When tobacco BY-2 cells were transformed with *35S promoter::N/LRR-Myc*, the cells frequently swelled, branching and the direction of cell division was out of order (Figure 2B), similar to *NpLRX1*-RNAi-transformed BY-2 cells (Figure 2C). On the other hand, *35S promoter::NpLRX1*-transformed BY-2 cells over-expressing full length *NpLRX1* showed no morphological change versus control BY-2 cells (Figure 2D).

To investigate the effects of the expression of *N/LRR-Myc* on the arrangement of the cortical microtubules, BY-GT-16 cells, in which GFP-fused tubulin was constitutively expressed (Kumagai et al. 2001), were transformed with the *35S promoter::N/LRR-Myc*. Control BY-GT-16 cells showed the cylindrical shape and nearly the same diameter, and the arrangement of the microtubules was vertical against the long axis and parallel with each other (Figure 3E, F). Although no apparent degradation of microtubules was observed, the direction of the cortical microtubules was irregular on the surface at the point where cells branched and on the surface of the cells in which a part was enlarged (Figure 3A–D).

Subcellular localization of the NpLRX1 protein

To examine the subcellular localization of the NpLRX1 protein, a NpLRX1-GFP fusion protein was expressed in BY-2 cells under the control of the 35S promoter (Figure 4). The GFP signals were observed at the cellular boundary and the peripheral region of the nucleus in *35S promoter::NpLRX1-GFP*-transformed BY-2 cells

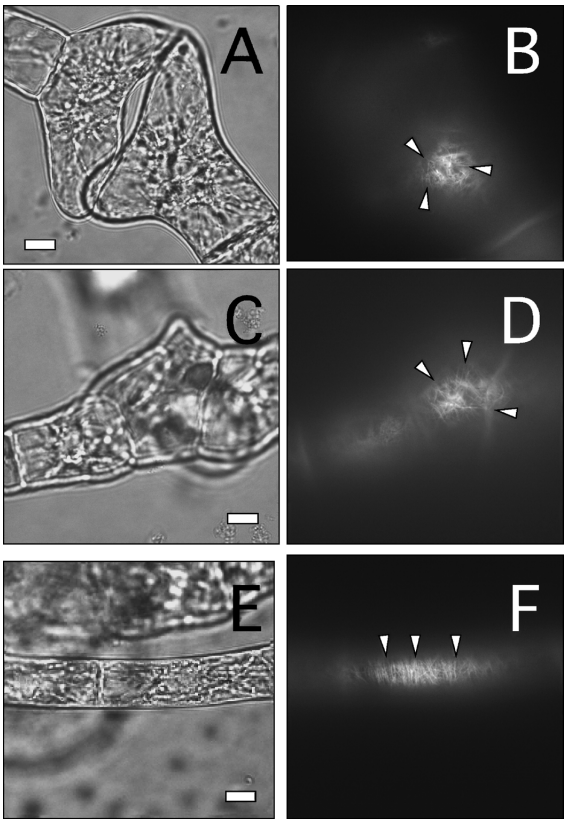

Figure 3. Observation of microtubules in BY-GT-16 cells and BY-GT-16 cells transformed with the *35S promoter::N/LRR-Myc* construct by confocal laser scanning microscopy. (A)–(D) BY-GT-16 cells expressing *N/LRR-Myc*. (E)–(F) BY-GT-16 cells. (A), (C), (E) Bright-field image. (B), (D), (F) Fluorescence image. Arrows indicate the microtubules. Bars: 10 μm.

(Figure 4B), whereas signals were detected throughout the cytoplasm with strong fluorescence in the whole nucleus in *35S promoter::GFP*-transformed BY-2 cells (Figure 4D). To determine whether the NpLRX1-GFP protein was localized in the symplast and/or on the cell membrane or in the apoplast with binding to the cell wall, the transformed cells were plasmolysed by treatment with 0.5 M mannitol. The fluorescence derived from NpLRX1-GFP remained at the cell surface, possibly on the cell membrane, but was not detected in the cell wall after plasmolysis (Figure 4F). When the microscopic focus was adjusted on the cell surface, the fluorescence was not detected equally on the cell membrane, but was detected in patch- or string-like arrangements (Figure 4J).

To confirm whether the extensin domain of *NpLRX1* was necessary for localization on the cell membrane, we tried to analyze the distribution of N/LRR-GFP fusion proteins in BY-2 cells, but no transformants were obtained, possibly indicating lethality. Thus, N/LRR-GFP fusion proteins were expressed transiently in onion epidermal cells using a gold-particle biolistic delivery system. The fluorescence in *35S promoter::GFP-*

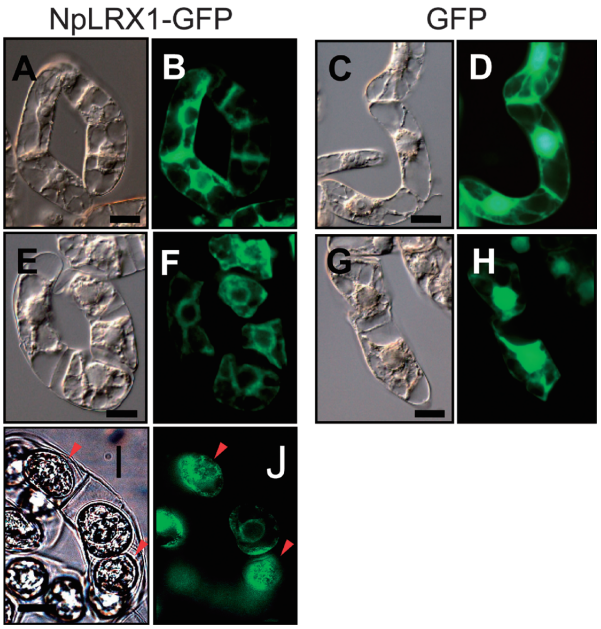

Figure 4. Subcellular localization of the NpLRX1-GFP fusion proteins in BY-2 cells. (A)–(B), (E)–(F), (I)–(J) BY-2 cells expressing NpLRX1-GFP fusion protein. (C)–(D), (G)–(H) BY-2 cells expressing GFP alone. The expression of both genes was controlled by the 35S promoter. The cells were incubated in PIPES buffer (pH 7.0). The image was observed with a fluorescence microscope (A)–(D) under normal conditions and (E)–(J) under plasmolysis condition with 0.5 M mannitol. Left panels show bright-field and right panels show fluorescence image. Bars: 10 μm. (I)–(J) Focus was adjusted with regard to the cell membrane. Red arrows indicate the surface of the cell membrane.

injected cells was localized to the cytosol and nuclear matrix (Figure 5D), but the fluorescence in *35S promoter::NpLRX1-GFP*- and *35S promoter::N/LRR-GFP*-injected cells was localized at the cell membrane (Figure 5E, F).

On the other hand, localization of the fluorescent around the nucleus in transgenic BY-2 cells suggested that the NpLRX1 protein also existed in organelles, such as the endoplasmic reticulum (ER). To determine which organelle the NpLRX1-GFP protein was localized in, the cells were stained with ER-tracker (Villarejo et al. 2005). The GFP image did not match with that of the ER-tracker (Figure 6A). Furthermore, FM4-64, a styryl dye (Bolte et al. 2004), was used to monitor vesicle trafficking and organelle organization in living plant cells. FM4-64 is known to immediately stain the cell membrane by becoming inserted and anchored in the outer leaflet of the cell membrane lipid bilayer. Subsequently, FM4-64 is internalized to the inner leaflet of endocytic vesicles by endocytosis and transported to intercellular organelles, such as to the Golgi body and vacuoles, but not to ER (Bolte et al. 2004). The BY-2 cells expressing NpLRX1-GFP were stained with FM4-64 (Figure 6B). Soon after adding FM4-64, the cell membrane was strongly stained and the peripheral region of nucleus was weakly stained

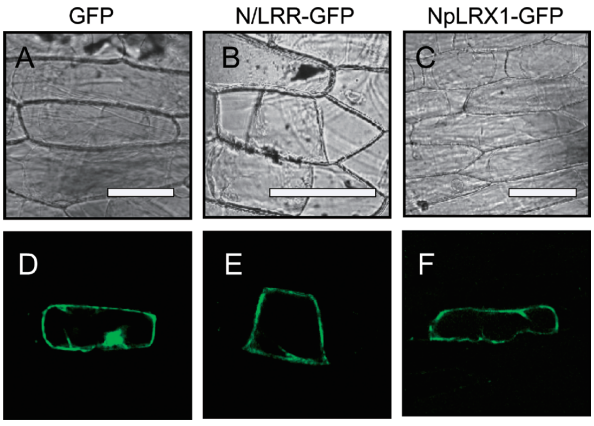

Figure 5. Subcellular localization of N/LRR-GFP and NpLRX1-GFP fusion protein in onion epidermal cells. Transient expression of GFP, N/LRR-GFP and NpLRX1-GFP was driven by the 35S promoter. The image was observed under plasmolysis condition with 0.5 M mannitol. (A)–(C) Bright-field image. (D)–(F) Fluorescence image. Bar: 10 μm.

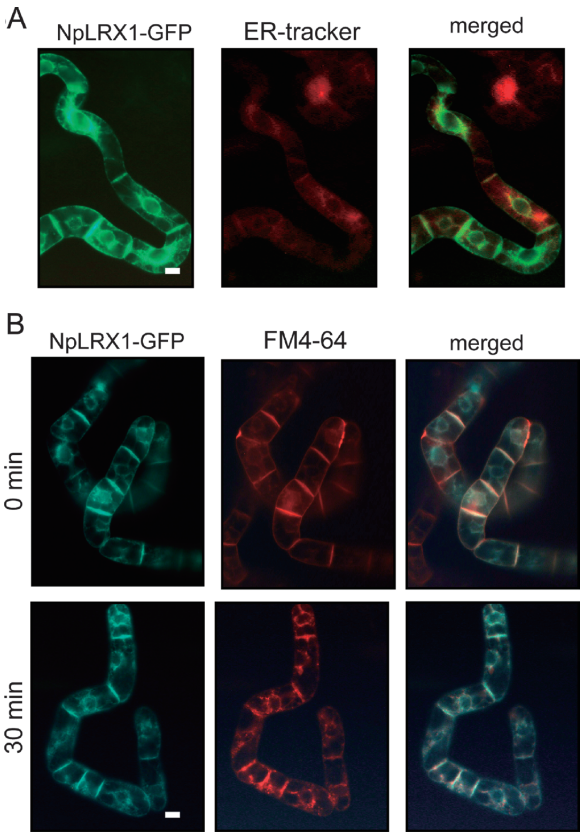

Figure 6. Localization of NpLRX1-GFP on the cell membrane and cell membrane-derived endocytic vesicles. (A) Cells were incubated with 1 μM ER-Tracker to label the ER; 7 h after the dye washed out, cells were observed with a fluorescence microscope. GFP signals did not co-localize with ER-tracker signals. (B) Cells were incubated with 32 μM FM4-64, after which the dye was washed out (0 min). Most of the dye was localized at the plasma membrane. After 30 min, the staining of the cell membrane was weak and the endocytic vesicles and peripheral region of the nucleus were also clearly labeled. GFP signals did co-localize with FM4-64 signals.

by FM4-64. The GFP signal and FM4-64 signal clearly overlapped in the cell membrane, but not inside the cells. However, the merged image of cells after 30 min showed significant co-localization of two fluorescent signals, both on the cell membrane and in the cells. These data suggested that NpLRX1-GFP was localized on the cell membrane and in cell membrane-derived endocytic vesicles.

To confirm the subcellular localization of the NpLRX1 protein, the organelles of NpLRX1-GFP-transformed BY-2 cells were fractionated by differential centrifugation followed by the separation of proteins with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the detection of NpLRX1 protein with anti-GFP antibody (Figure 7). In immunoblots of *35S promoter::NpLRX1-GFP*-transformed BY-2 cells, a comparatively strong band was detected at the size of 37 kDa and three weak bands were detected at the size of 55, 27, and 18 kDa in the 4,500×*g* precipitate fraction, which was rich in mitochondria (lane 8). The band was not detected in the 800×*g* precipitate fraction (lane 7, rich in nucleus), the 200,000×*g* precipitate fraction (lane 9, rich in microsomes, including the vesicles derived from the cell membrane, ER, Golgi body and vacuole), the 200,000×*g* supernatant fraction (lane 10, rich in cytosol), and the MiraCloth fraction (lane 6, rich in cell wall). It was expected that there was the NpLRX1-GFP protein to a microsome fraction by a result of microscopy, the signal was detected in the heavier fraction than microsome fraction. The sizes of detected bands were smaller than the expected size (102 kDa) of the NpLRX1-GFP-fused protein. These results suggest that NpLRX1-GFP fused proteins were partially degraded and localized in the vesicular component, including the cell membrane and endocytic vesicles. On the other hand, in *35S promoter::GFP*-transformed BY-2 cells, the band at 27 kDa was detected predominantly in the 200,000×*g* supernatant fraction (lane 5) and was comparatively stronger in the 800×*g* precipitate fraction (lane 2). Like a results of the microscopy (Figure 4D, H), the GFP proteins were mainly present in the cytosol and nucleus.

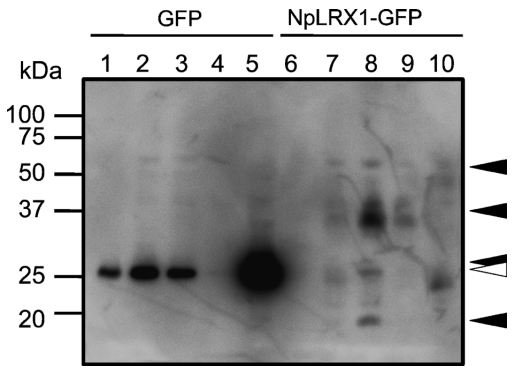

Figure 7. Subcellular fractionation of NpLRX1-GFP fusion proteins. Immunoblot analysis with anti-GFP antibody in transgenic BY-2 cells expressing GFP alone or NpLRX1-GFP fusion protein. 1 and 6: debris in MiraCloth (cell wall-rich fraction), 2 and 7: 800×*g* precipitate (nucleus-rich fraction), 3 and 8: 4,500×*g* precipitate (mitochondria-rich fraction), 4 and 9: 200,000×*g* precipitate (microsome-rich fraction), 5 and 10: 200,000×*g* supernatant (soluble fraction rich in cytosol). Sizes of protein markers are labeled on the left. Black arrows indicate the signals derived from the NpLRX1-GFP fusion protein. White arrow indicates the signal derived from the GFP protein.

Discussion

NpLRX1 was constitutively expressed in whole tobacco plants (Chida and Yazawa et al. 2007) and *35S promoter::N/LRR*-transformed adventitious buds formed abnormal leaves (Figure 1C) with distorted cells in shape and size, and with large spaces between cells (Figure 1F and 1H) and poor differentiation of the cuticle layer (Figure 1J, k). A more severe phenotype was observed in *NpLRX1*-RNAi-transformed adventitious buds (Figure 1B, G). The abnormal morphology, branching and enlargement of the root hair cells have been reported in the *Arabidopsis* in which *N/AtLRR* was ectopically expressed (Baumberger et al. 2001). Interestingly, the dominant negative phenotype was limited in root hair cells, even when *N/AtLRR* was expressed in a whole *Arabidopsis* plant under the control of the 35S promoter. In tobacco plants expressing *N/AtLRR*, no defect was observed in any tissue, including root hair (Figure 1E). This suggests that the peculiarity of the each LRX family protein is high, even though other LRX proteins may exist in the same tissues. The LRR of NpLRX1 may be recognized by a specific factor in tobacco cells.

NpLRX1 protein has the predicted cell external secretion signal peptide and the extensin domain (Chida et al. 2007). *Arabidopsis* LRX1 was immunologically confirmed to be localized in the cell wall of root hairs (Baumberger et al. 2001). PEX1, a pollen-specific maize LRX, was immunolocalized in the intine layer of the pollen grain and the callosic sheath of the pollen tube (Rubinstein et al. 1995). Thus, we expected that NpLRX1 would also be localized in the cell wall, but its association with the cell membrane, not the cell wall, was observed when the BY-2 cells expressing the NpLRX1-GFP fusion protein were plasmolysed (Figure 4). Additionally, most of the fluorescence was observed on the cell membrane when NpLRX1-GFP or N/LRR-GFP was transiently expressed in onion epidermal cells (Figure 5).

The fluorescence of NpLRX1-GFP was also detected inside of the cell along the circumference of the nucleus (Figure 4F) and co-localization of GFP with FM4-64, a dye for monitoring vesicle trafficking and organelle organization in living plant cells (Bolte et al. 2004), at 0 and 30 min of staining (Figure 6B) indicated that the NpLRX1-GFP protein was taken into the cell, with the cell membrane, by endocytosis. Although it has been reported that the apparent molecular mass of *Arabidopsis*

LRX1 was larger than the calculated size, as a result of the glycosylation of the extensin domain (Baumberger et al. 2001), glycosylation of NpLRX1 is as yet unknown. Detection of the bands at the smaller molecular mass than that was expected in immunoblotting for NpLRX1-GFP (Figure 7) suggests that the NpLRX1-GFP protein may be degradated *in vivo*, or during the subcellular fractionation by the proteinases which are resistant to the proteinase inhibitor cocktail.

LRX family proteins that have already been reported were thought to be located in the cell wall and to bind tightly to cell wall components via the extensin domain (Baumberger et al. 2001). On the other hand, NpLRX1 might form a complex with factors on the cell membrane via the LRR domain and associate weakly to the cell wall using the extensin domain. This indicates that the NpLRX1 protein might have different functions from other previously reported LRX family proteins (Baumberger et al. 2001; Rubinstein et al. 1995). This is also supported by the fact that the phenotype was observed in a *nolac*-K4 mutant of *N. plumbaginifolia*, which lacks only *NpLRX1* among the *LRX* family of genes (Chida et al. 2007). This indicates that NpLRX1 apparently has no functionally redundant protein in this role in the control of the shape of leaf cells.

BY-2 cells generally show cylindrical morphology with a linear arrangement of cells (Figure 2A). The BY-2 cells expressing *NpLRX1*-RNAi showed a phenotype with some parts of the cell enlarged and extruded and the size of the individual cells and the direction of cell division was irregular (Figure 2C). *NpLRX1* may regulate proper cell wall expansion. BY-2 cells expressing *N/LRR* also showed similar phenotypes (Figure 2B), but cells expressing full-length *NpLRX1* showed no difference in the shape of the cells (Figure 2D). The over-expressed *N/LRR* might block the interaction sites as a competitor on the cell membrane, which had been used by the endogenous NpLRX1 protein.

In the BY-GT-16 cells expressing *N/LRR*, the orientation of microtubules, known to control cell shape, was disorganized in the part of the cell where the shape was abnormal (Figure 3). Interestingly, on the surface of the cell membrane in plasmolysed cells, the GFP fluorescence was observed in patch- or string-like arrangements (Figure 4J). These data lead to the hypothesis that the localized protein on the cell membrane with NpLRX1 attached may be associated with the cytoskeleton to control cell shape.

Now, we try to identify the putative NpLRX1-interacting cell-membrane protein. To understand the function of NpLRX1 in cell morphogenesis, it is important to clarify the relations with this unknown protein and microtubules.

Acknowledgements

The authors greatly thank Dr. T. Sano and Dr. T. Higaki (Graduate School of Frontier Sciences, University of Tokyo) and Dr. H. Chida (University of Tsukuba) for providing helpful advice on examination using and technical assistance with microscopy, and Dr. T. Nakagawa (Shimane University) for the generous gift of pGWB vectors. This work was supported in part by a Grant-in-Aid for Science Research on Priority Areas (Grant No. 18075004 and No. 21027004).

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