African Journal of Biotechnology Vol. 10(76), pp. 17409-17423, 30 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1771
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

A new putative plasmodesmata-associated protein, At1g19190, in *Arabidopsis*

Ping Cao, Jing Sun, Peng Zhao, Heng Liu* and Guochang Zheng

Institute of Cell Biology, Life Science School, Lanzhou University, Lanzhou, 730000, Gansu, P.R. China

Accepted 28 October, 2011

Plasmodesmata are channels that traverse cell walls to establish a symplastic continuum through whole plants. A plasmodesmal-associated protein, AtRGP2, is delivered to the plasmodesmata via the Golgi apparatus. In this paper, GFP:At1g19190 fusion protein was constructed and compared to AtRGP2:YFP. GFP:At1g19190 displays fluorescence patterns along the cell periphery and in a punctate pattern that co-localizes with aniline blue-stained callose. Cytoskeleton disturbance drugs were used to treat transgenic GFP:At1g19190 Arabidopsis seedlings. Colchicine, which disturbs the microtubule network, was found capable of disturbing the localization of At1g19190, but cytochalasin B (CB) and brefeldin A (BFA) did not affect the localization of At1g19190. Micrografting methods revealed that the transfer of GFP:At1g19190 fluorescence from transgenic *Arabidopsis* to non-fluorescent wild-type was absent during AtRGP2 grafting. At1g19190 appeared to be a potentially important protein in the plasmodesmata (Pd) structure and may play a critical role in substance transport, although its exact mechanism is still unknown.

Key words: Plasmodesmata, AtRGP2, At1g19190, cytoskeleton, micrografting.

INTRODUCTION

Plasmodesmata (Pds) are junctions between plant cells that are comparable to gap junctions in animal cells. Almost two centuries ago, Bernhardi (1805) proposed the existence of such channels and the possibility of their permitting intercellular communication. Tangl (1879) was the first to observe Pds in 1879, describing them as “offene communication.” In 1901, Strasbuger christened these junctions “plasmodesmata,” from the Latin “plasma” for fluid and the Greek “desma” for bond, which accurately describes the function of these structures (Zambryski, 2008). Pds form symplast passages that interconnect adjacent plant cells. They are composed of plasma membrane (PM)-lined channels with a central axial component, called the desmotubule (DM), and are derived from the endoplasmic reticulum (ER) (Thomas et al., 2008; Maule, 2009). The outer PM is continuous from one cell to another, whereas the inner DM is continuous with the ER (Ehlers and Kollmann, 2001; Heinlein and Epel 2004). Pds are channels that provide the potential for the exchange of informational molecules acting as non-cell autonomous proteins (NCAPs), small RNAs, pathogenic and some non-pathogenic RNAs. They regulate developmentally important molecules into the phloem and mediate particular cell types and tissues during development. Despite the important role of Pds in plant growth and development, however, very little is known about how these molecules transport through them. One aspect that has received attention recently is the protein composition of Pds. To date, a number of proteins that associate with Pds (Maule, 2009; Lucas et al., 2009) have been identified, including cytoskeletal elements (actin and myosin VIII) (Ding et al., 1996; Blackman and Overall, 1998; Reichelt et al., 1999), ER proteins (calreticulin) (Baluska et al., 1999), the plasmodesmal- associated protein kinase (PAPK) in tobacco and its ***Arabidopsis thaliana*** PAPK homolog (PAPK1) (Lee et al., 2005), plasmodesmal-associated protein (ppap) (AtBG_ ppap) (Levy et al., 2007), a class of reversibly glycosylated polypeptide (RGPs) (Sagi et al., 2005; Zavaliev et al., 2010), a family of Pd-located proteins (PDLPs) (Thomas et al., 2008) and Pd-callose

*Corresponding author. E-mail: hengliu@lzu.edu.cn. Tel: +8613239642690. Fax: +8609318912565.

binding proteins (PDCBs) (Simpson et al., 2009). Micrografting methods for young ***Arabidopsis*** seedlings are described by Turnbull et al. (2002). Micrografting has been a useful technique in the improvement, rejuvenation and virus-freeing of fruit crops. The technique can provide evidence of long-distance signal transport in plant processes because it maintains contact between scions and the rootstock until fusion is complete. This study investigates a new putative Pd-associated protein, At1g19190, in ***Arabidopsis***. Fusion GFP:At1g19190 was created to localize proteins in wall sheaths surrounding the Pds. Further analysis using cytoskeleton-disturbing drugs were also performed to determine the factors that affect At1g19190 localization. Transgenic ***Arabidopsis*** plants expressing GFP:At1g19190 were grafted into wild-type (WT) plants (***A. thaliana*** ecotype ***Columbia***) through micrografting. Separate experiments with transgenic ***Arabidopsis*** as scions and rootstock were performed, and fluorescence studies were used for analysis.

MATERIALS AND METHODS

Plant material, growth condition and grafting

Prior to their growth, ***A. thaliana*** ecotype ***Columbia*** samples were vernalized for three days at 4°C. The samples were then grown in tissue cultures under long-day conditions (16-h-light/8-h-dark cycles) at 21°C. They were cultivated in a greenhouse in 6 cm pots containing a mixture of equal volumes of potting mixture and vermiculite at 20 to 24°C. ***Nicotiana benthamiana*** and ***N. tobaccum*** were grown in 10 cm pots containing a potting mixture at 25°C under long-day conditions.

The Arabidopsis were grafted using butt grafts (Colin et al., 2002). For grafting, seed of ***Arabidopsis thaliana*** were surface-sterilized in 70% ethanol for 1 min, then in sodium hypochlorite solution for 10 min, followed by extensive washes in sterile distilled water. Seeds were sown under axenic conditions in 9 cm Petri dishes. Plates were placed at 4°C in the dark for two to three days to vernalize seedling. Seedlings were then grown with plates under long-day conditions (16-h-light/8-h-dark cycles) at 21°C. Grafts were all performed under a stereomicroscope, generally using seedlings between three or four days old. Cuts were most easily made with small blades in hypocotyl (Figure 8). Images were taken after grafting three to six days under confocal microscopy (LSM 510; Zeiss; Germany).

Plasmid constructs

Transgenic ***Arabidopsis*** plants expressing GFP-tagged At1g19190 were generated from their native sequences by fluorescence tagging of full-length proteins (Tian et al., 2004). At1g19190 was amplified from cDNA using the primer set LP5′ - GTCGACATGGATTCCGAAATCGCATT- 3' and RP 5' –CGGGGT ACCAAATGTTTCTTCTTTAAGAAACTCTG- 3'. The resulting DNA fragment was recombined into pCM1307-nGFP using SalI and KpnI restriction sites.

Plant transformation

Wild-type (WT) seedlings (***A. thaliana ecotype Columbia***) were transformed by an infiltration method using the ***Agrobacterium tumefaciens*** strain GV3101 carrying the appropriate constructs (Clough and Bent, 1998). Five to ten independent lines were analyzed. The lines were amplified, and the T3 plants were used for further experiments.

Transient expression

Transient expression was induced using ***A. tumefaciens*** leaf injections (Lavy et al., 2002). The abaxial side of ***N. benthamiana*** and ***N. tobaccum*** leaves was injected with the ***Agrobacterium*** strain GV3101 harboring the indicated plasmids using a 1 ml syringe without a needle. Observation of transient expression was limited to epidermal cells at the lower face of the leaves. GFP fluorescence was examined 24 to 48 h after injection.

Transgenic expression

Arabidopsis plants were transformed according to the flower-dip method (Bechtold et al., 1993). For the expression of GFP:At1g19190 in plants, transgenic ***Arabidopsis*** plants containing progeny were selected after surface sterilization of the seeds in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 15 mg/L hygromycin.

Reverse transcription polymerase chain reaction (RT-PCR)

Rosette leaves, stems and shoots were cut from newly flowering ***Arabidopsis*** plants, whereas cauline leaves, roots, flowers and siliques were cut from fully flowering plants. For each organ type, two samples with two repeats were collected and stored at 80°C. RNA was purified from 50 to 100 mg frozen tissue using the RNAiso^{TM} Plus total RNA isolation system kit (Takara) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using the Revert Aid^{TM} First Stand cDNA Synthesis kit (Fermentas). The At1g19190 and Actin2 coding sequences were amplified by PCR reactions using specific primers. For At1g19190, the LP and RP used were as described earlier. For Actin2, the forward primer was 5' - TACTGTGCCAATCTACGAGGGT -3', and the reverse primer was 5' -GGACCTGCCTCATCATACTCG -3'. Amplification of At1g19190 was performed using the following program: 1 cycle of 95°C for 2 min, followed by 33 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final elongation step of 72°C for 5 min. Actin2 was amplified using the following program: 1 cycle of 95°C for 2 min, followed by 24 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final elongation step of 72°C at 5 min. To calculate the transcript levels of At1g19190, the image processing software IMAGEJ version 1.31 (http://rsb.info.nih.gov/ij) was used.

Inhibition treatments

A. thaliana plants were cultivated on MS medium containing 3% sucrose and 1% agar (pH 5.8) in 12-well plates, with each well containing four plants. For the treatment of ***Arabidopsis***, stock solutions were prepared in dimethyl sulfoxide (DMSO) in concentrations of 0.1 M colchicine, 5 mM CB, and 10 mg/ml BFA. Stock solutions or DMSO was diluted in 1:1000 liquid MS media, 1 ml of which was applied to each well, followed by vacuum infiltration (0.08 mPa) for 5 min. Plants were kept in the tissue culture as earlier described.

FM4-64 membrane staining and plasmolysis

GFP:At1g19190 transgenic ***Arabidopsis*** leaves were stained with

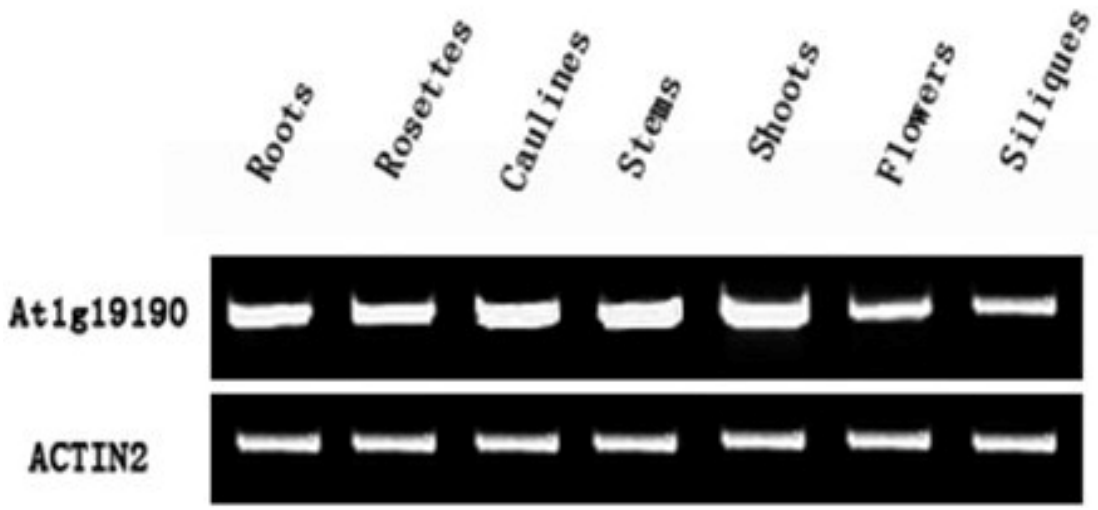

Figure 1. RT-PCR of At1g19190. The transcription levels of At1g19190 in different ***Arabidopsis*** tissues were determined using RT-PCR. Gray intensities were used to calculate the transcription levels. Actin2 served as the internal reference.

(N-[3-triethylammoniumpropyl]-4-[p-die-thylaminophenylhexatrienyl] pyridinium dibromide), (FM4-64) a plasma membrane (PM marker) (Bloch et al., 2005). An FM4-64 stock solution was prepared in DMSO at a concentration of 2 mg/ml. The working solution was 5 µM. For PM staining, the cotyledons of seven-day old transgenic GFP:At1g19190 ***Arabidopsis*** plants were incubated in FM4-64 at 30°C for 1.5 h. For plasmolysis, the FM4-64 stained cotyledons were transferred to 30% glycerol for about 4 h. Images were taken when the separation of the protoplasts from the cell walls was visible under confocal microscopy.

Callose staining

Callose staining for co-localization with GFP:At1g19190 was performed by incubating transgenic GFP:At1g19190 ***Arabidopsis*** leaf segments for 10 min in a mixture of 0.1% aniline blue in double-distilled water and 1 M glycine, pH 9.5, at a volume ratio of 2:3.

Microscopy

Fluorescence microscopy

Fluorescence was viewed with a Nikon Eclipse Ti-U. Aniline blue-stained callose fluorescence was measured with a band-pass 330–380 nm excitation filter, a DM 400 dichromatic mirror, and a long-pass 420 nm emission filter. GFP and YFP fluorescence was viewed with a band-pass 450 to 490 nm excitation filter, a DM505 dichromatic mirror, and a long-pass 505 nm emission filter. For better visualization, a negative image was generated using ADOBE PHOTOSHOP CS4.0 software.

Confocal fluorescence microscopy

Fluorescence was viewed with a CLSM (LSM 510; Zeiss; Germany). GFP fluorescence was excited with a 488 nm argon laser, and emission was detected with a 500 to 550 nm band-pass filter combination. YFP fluorescence was excited with a 514 nm argon laser, and emission was detected with a 535 to 590 nm band-pass filter combination. FM4-64 fluorescence was excited with a 543 nm argon laser, and emission was detected with a 565 to 615 nm band-pass filter combination.

RESULTS

At1g19190 is a member of the AtCXE family

Analysis by NCBI revealed a 3' untranslated region and a 957 bp long coding region (Figure 11). Semi-quantitative interpretation of the At1g19190 transcript levels in ***Arabidopsis*** tissues using RT-PCR revealed that the gene is not expressed equally in different tissues (Figure 1). IMAGEJ was used to calculate the protein transcript levels from gray intensities. Transcription of At1g19190 was highest in the flowers, shoots and roots, whereas the rosette leaves and siliques were of intermediate gray intensity. Stems and cauline leaves had the lowest transcription levels (Figure 12).

The gene translates into a 318-aa protein that is predicted to have no transmembrane regions (Figure 13) according to the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/; Hirokawa et al., 1998; Levy et al., 2007). An abhydrolase_3 domain (pfam07859) was found between 70 and 290 aa (Figure 4, CD-Search in http://www.ncbi.nlm.nih.gov/cdd/; Marchler-Bauer and Bryant, 2004), and it is a member of AtCXE family (Marshall et al., 2003).

At1g19190 targets the cell wall

Fusion between the protein and GFP was created to identify the localization of At1g19190. Analysis of

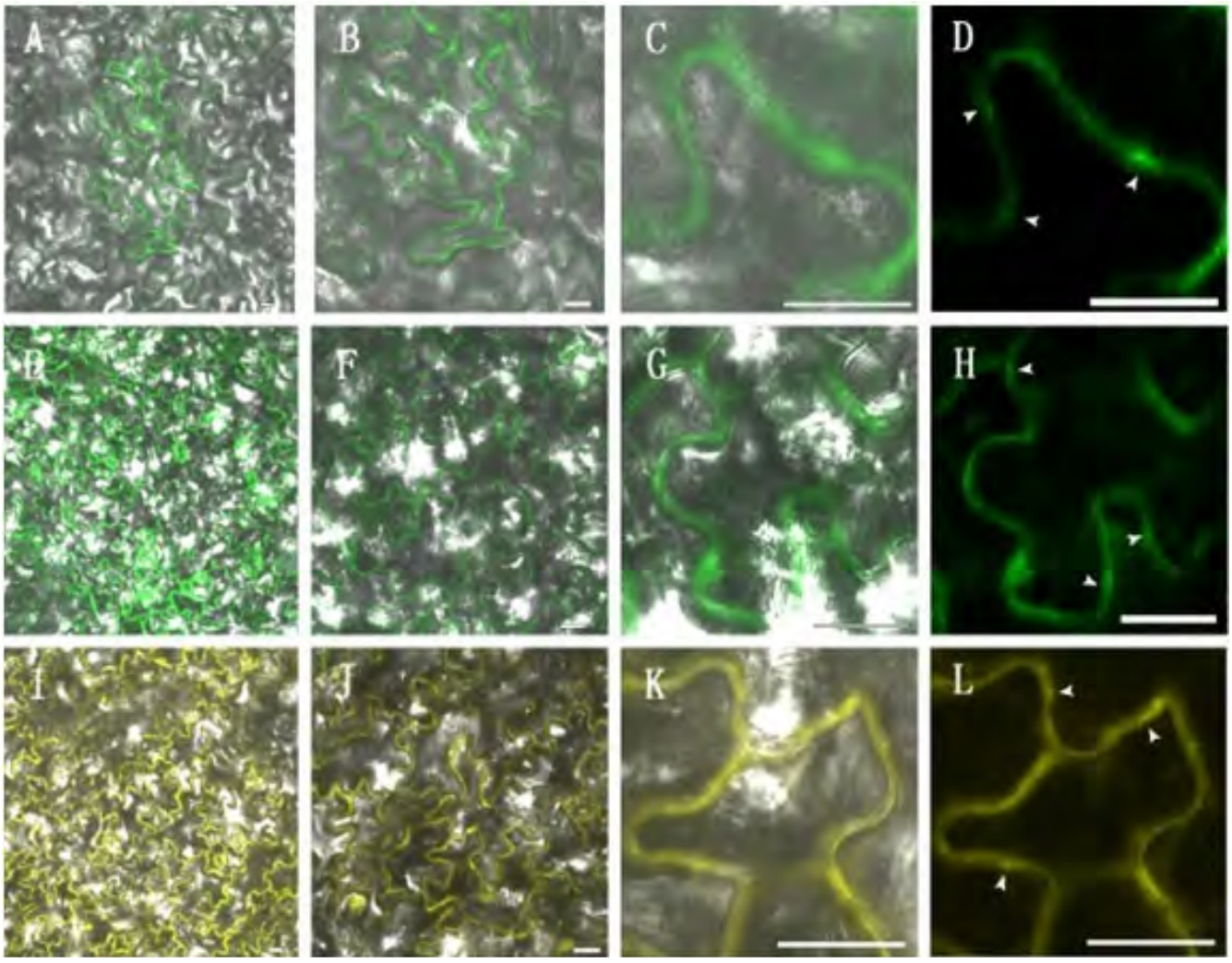

Figure 2. Cellular localization of GFP:At1g19190. A to D, Transiently expressed GFP:At1g19190 in the epidermis of ***Nicotiana benthamiana*** 48 h after microprojectile bombardment; E to H, stably expressed GFP:At1g19190 in ***Arabidopsis*** leaf epidermis; I and J, stably expressed AtRGP2:YFP in ***Arabidopsis*** leaf epidermis; D, H and L Nomarsky differential interference showing Pds. Bar = 20 µM.

GFP:At1g19190 with ***Arabidopsis*** mesophyll protoplast expression showed no fluorescence. Control experiments verified that GFP fluorescence was expressed throughout the cell (data not shown). GFP:At1g19190 was transiently expressed in ***N. benthamiana*** by microprojectile bombardment (Figure 2A to D) and stably expressed in ***Arabidopsis*** by ***Agrobacterium*** transformation (Figure 2E to H), which appears similar to the AtRGP2:YFP located in Pds (Figure 2I and L).

From observations of the abaxial leaves of tobacco and ***Arabidopsis***, At1g19190 was found to be located in the wall sheath surrounding Pds. To prove that the GFP:At1g19190 fluorescence inside cell walls indeed represented Pds, we stained callose with aniline blue. Callose is widely used as a plasmodesmal marker (Baluska et al., 1999; Bayer et al., 2004; Gorshkova et al., 2003; Sagi et al., 2005; Levy et al., 2007).

When overexpressed ***Arabidopsis*** source leaves that express GFP:At1g19190 were stained by aniline blue, GFP:At1g19190 co-localized with the aniline blue-stained callose present in the cell wall sheath (Figure 3). Control experiments verified that no GFP fluorescence existed under aniline blue conditions, and no aniline blue fluorescence existed under GFP conditions.

GFP:At1g19190 is not a membrane protein

When GFP:At1g19190 is transiently expressed by ***Agrobacterium*** leaf injection, it can be observed that fluorescence accumulate in wall sheath (Figure 4A and D). We suppose At1g19190 is a putative plasmodesmal-associated protein Likewise, as predicted, GFP: At1g19190 do not localize to the plasma membrane

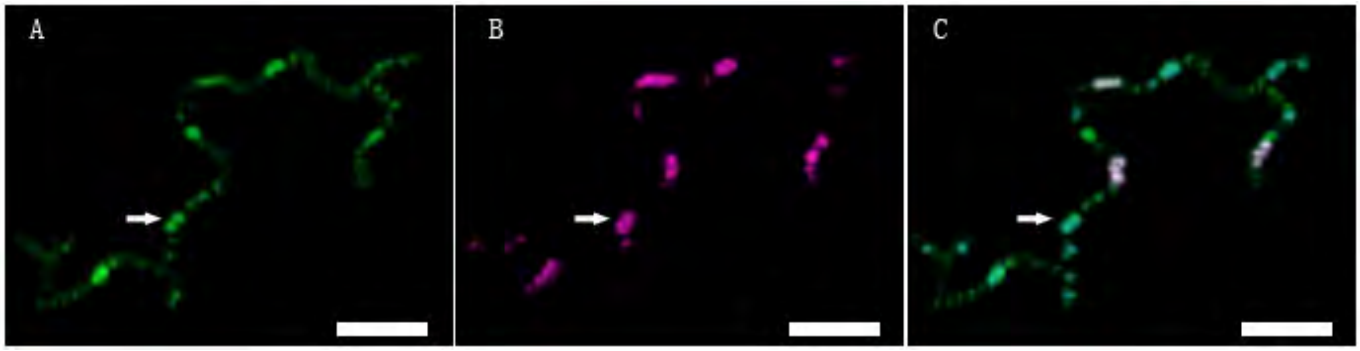

Figure 3. Aniline blue-stained callose with GFP:At1g19190. A, GFP:At1g19190 is shown in green; B, aniline-blue stained callose is shown in purple; C, GFP:At1g19190 and aniline-blue stained callose overlay. Bar = 20 µM.

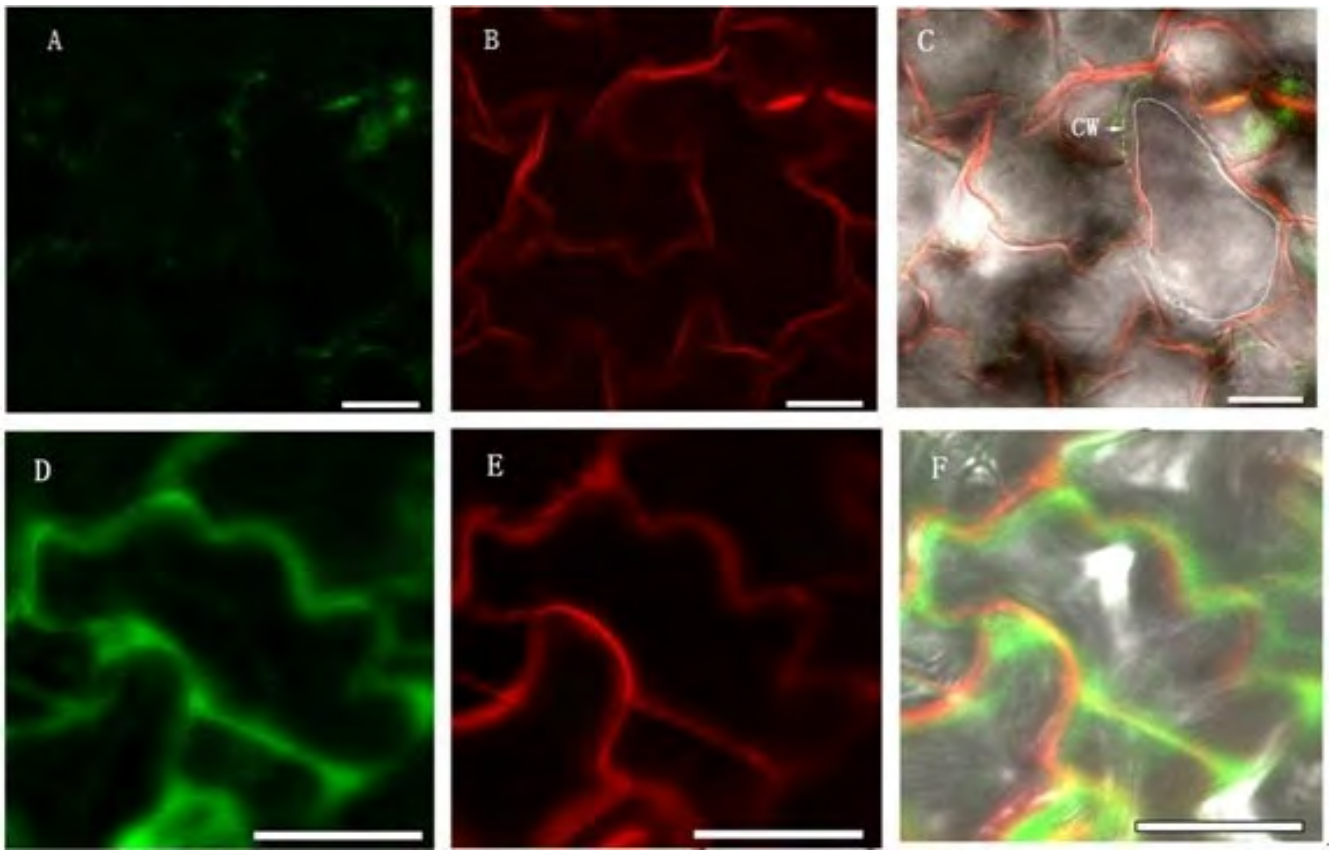

Figure 4. Fluorescence of GFP:At1g19190 and FM4-64. A and D, Stably expressed GFP:At1g19190 in ***Arabidopsis*** (shown in green); B and E, FM4-64-stained plasma membrane (shown in red) in transgenic ***Arabidopsis*** expressing GFP:At1g19190; C and F, DIC and FM4-64 fluorescence and GFP fluorescence overlay, overlay of all channels showing that GFP:At1g19190 not co-localized with FM4-64; C, upon plasmolysis, plasma membrane (PM) withdraws from the cell wall (see arrows), indicating that GFP:At1g19190 is not a plasma protein. Bar = 20 µM.

(Figure 4C and F). GFP:At1g19190 transgenic leaves were stained with FM4-64, a plasma membrane marker, and cells were plasmolysed. Following plasmolysis, GFP: At1g19190 could not be seen to recede from the cell wall along with the FM4-64 fluorescently stained plasma membrane (Figure 4A to C).

Disruption of the actin skeleton does not change the localization of GFP:At1g19190

CB is known to disorganize microfilaments, but not microtubules (MTs) (Wagner et al., 1972; Williamson and Hurley, 1986; Tominaga et al., 1997). To determine the

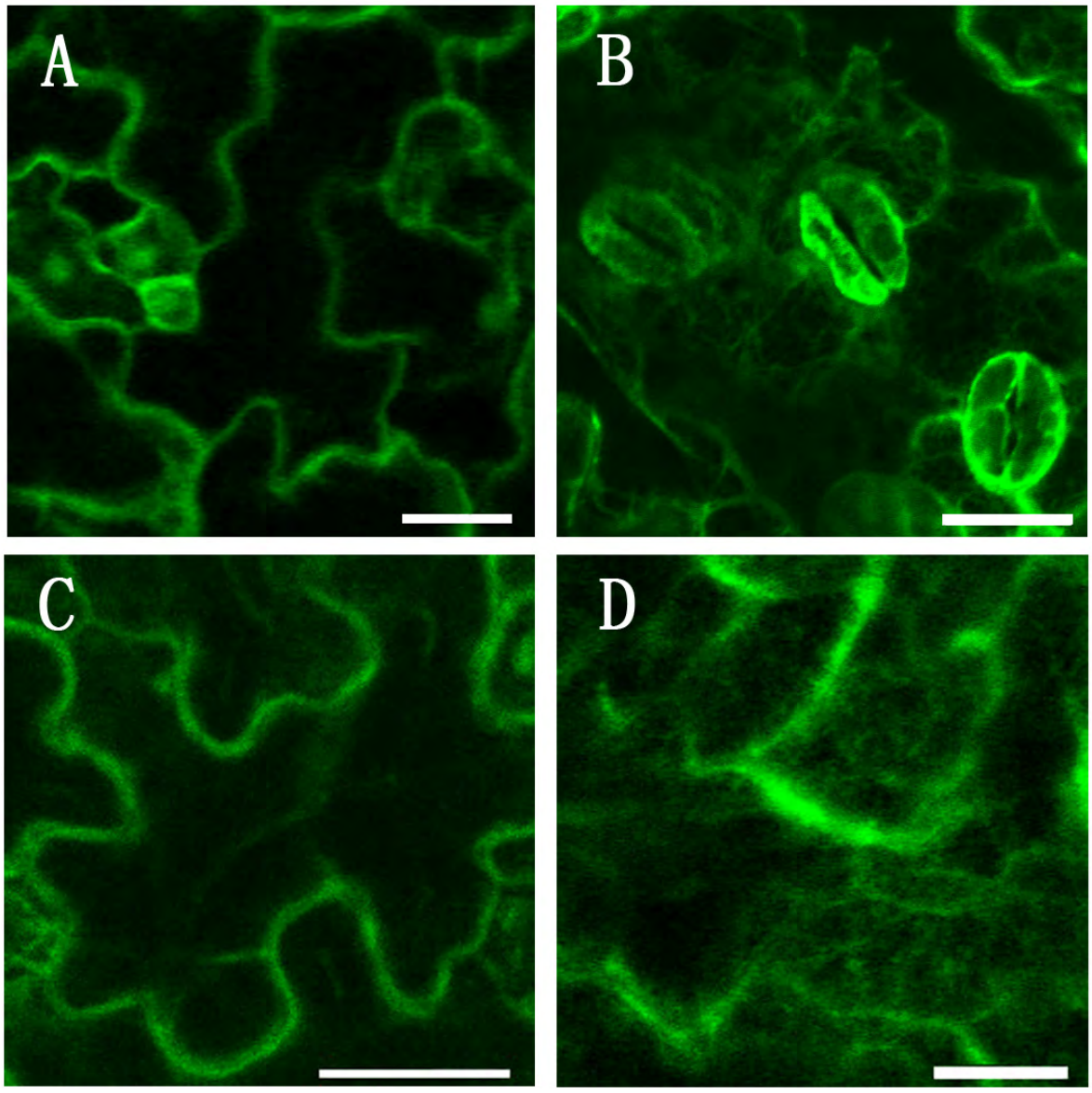

Figure 5. Effects of CB on the Pd-localization of GFP:At1g19190. (A) Cotyledons of seven-day-old GFP:At1g19190 seedlings treated with DMSO as control; (B) GFP:FABD2 showing actin filaments of epidermal and guard cells treated with DMSO; (C) GFP:At1g19190 treated with CB, which can specifically inhibit actin; (D) GFP:FABD2 treatment with CB, showing actin confusion. Bar = 20 μM.

involvement of microfilaments in the transport and position of At1g19190, the actin skeleton was disrupted by CB. ***Arabidopsis*** GFP: FABD2 plants verified that CB treatment works efficiently (Voigt et al., 2005) and showed the successful occurrence of the GFP:FABD2-mediated labeling of F-actin. 24 h after CB incubation, the GFP: FABD2-labeled actin network was completely disrupted (Figure 5B and D). The treatment of ***Arabidopsis*** expressing GFP:At1g19190 with CB resulted in no detectable accumulation of the protein in its leaves (Figure 5A and C). These results indicate that the GFP:At1g19190 localization in Pds is independent of actin influence.

Inhibition of the ER-Golgi secretion system does not affect the Pds-localization of GFP:At1g19190

BFA is often used to inhibit the ER-Golgi secretion systems of plant cells (Sciaky et al., 1997; Ritzenthaler et al., 2002). Previous studies reported that AtRGP2 localizes in both Pds and the Golgi apparatus, indicating that AtRGP2 is a plasmodesmal-associated protein delivered to the former via the latter (Sagi et al., 2005). In this experiment, transgenic ***Arabidopsis*** plants expressing AtRGP2:YFP were treated with DMSO as controls (Figure 6B). The 24 h BFA treatment of AtRGP2:YFP plants resulted in fluorescence accumulation,

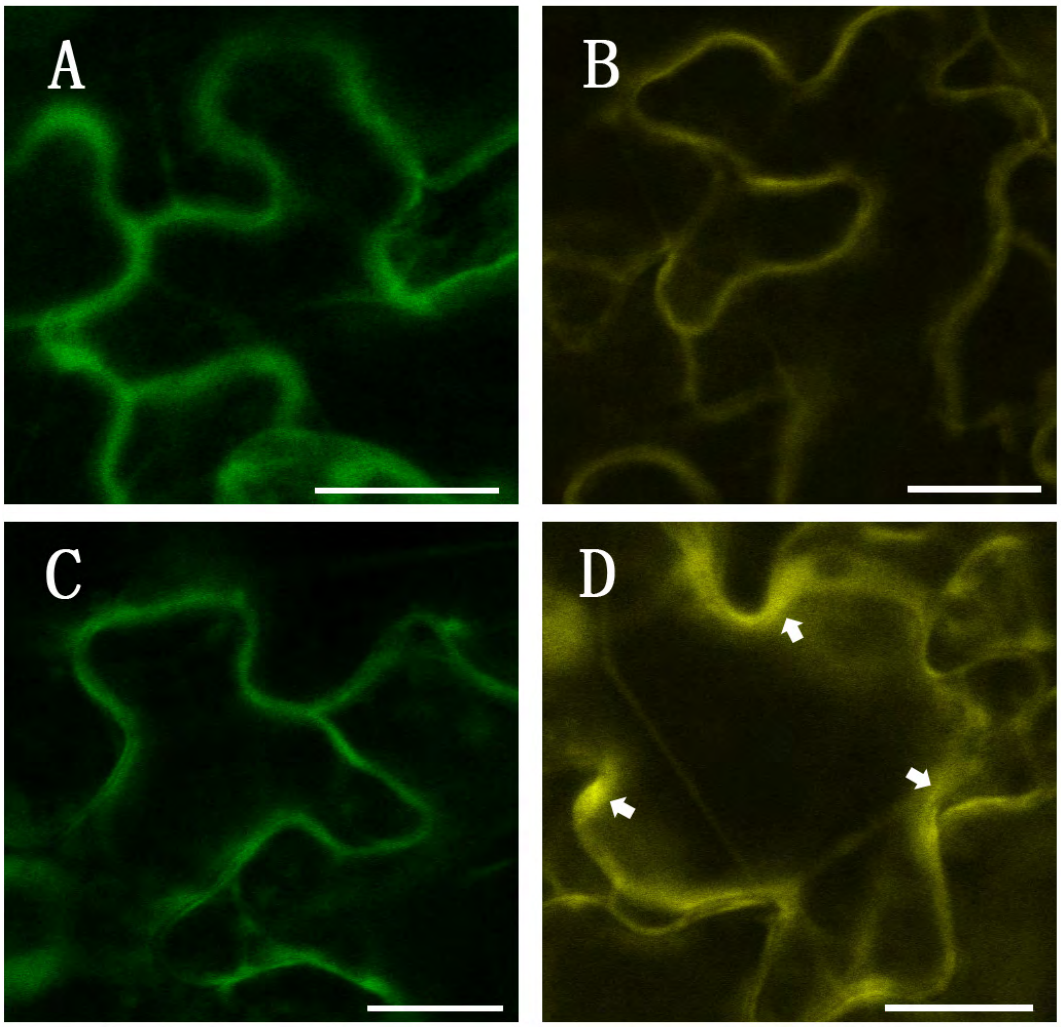

Figure 6. Treatment of transgenic ***Arabidopsis*** plants with BFA. (A) Cotyledons of 7-days-old GFP:At1g19190 seedlings treated with DMSO as control; (B) AtRGP2:YFP reported to be involved in Golgi apparatus transport to Pds was treated with DMSO; (C) GFP:At1g19190 was treated with BFA, which can specifically inhibit AtRGP2 delivered to plasmodesmata via the Golgi apparatus(arrow show fluorescence accumulation); (D) AtRGP2:YFP treatment with BFA, showing aggregation of AtRGP2. Bar = 20 μM.

demonstrating that the Pds-labeling of AtRGP2 is dependent on Golgi functions (Figure 6D). In contrast, GFP:At1g19190 plants treated with BFA did not differ from those treated with DMSO (Figure 6A and C). Pds-localization of GFP:At1g19190 is independent of ER-Golgi secretion.

Disruption of microtubules results in the accumulation of GFP:At1g19190 in *Arabidopsis* leaves

Disruption of the MTs network with colchicine was performed to determine the importance of MTs in protein localization. Colchicine treatment of ***Arabidopsis*** plants expressing TUA6: GFP showed complete depolymerization of the network within 24 h (Figure 7D and H). When the same treatment was applied to transgenic ***Arabidopsis*** plants expressing GFP: At1g19190 for 24 h, visible fluorescence accumulation in the leaves, compared with DMSO treated plants, was observed (Figure 7A to G). These findings suggest that disrupted MTs could change the Pds-localization of GFP: At1g19190. Thus, At1g19190 is possibly an MT-dependent Pds-associated protein.

At1g19190 could make long-distance transport

The micrografting technique was used to test the movement of At1g19190. After grafting overexpressed GFP:At1g19190 scion to WT rootstock, the fluorescence

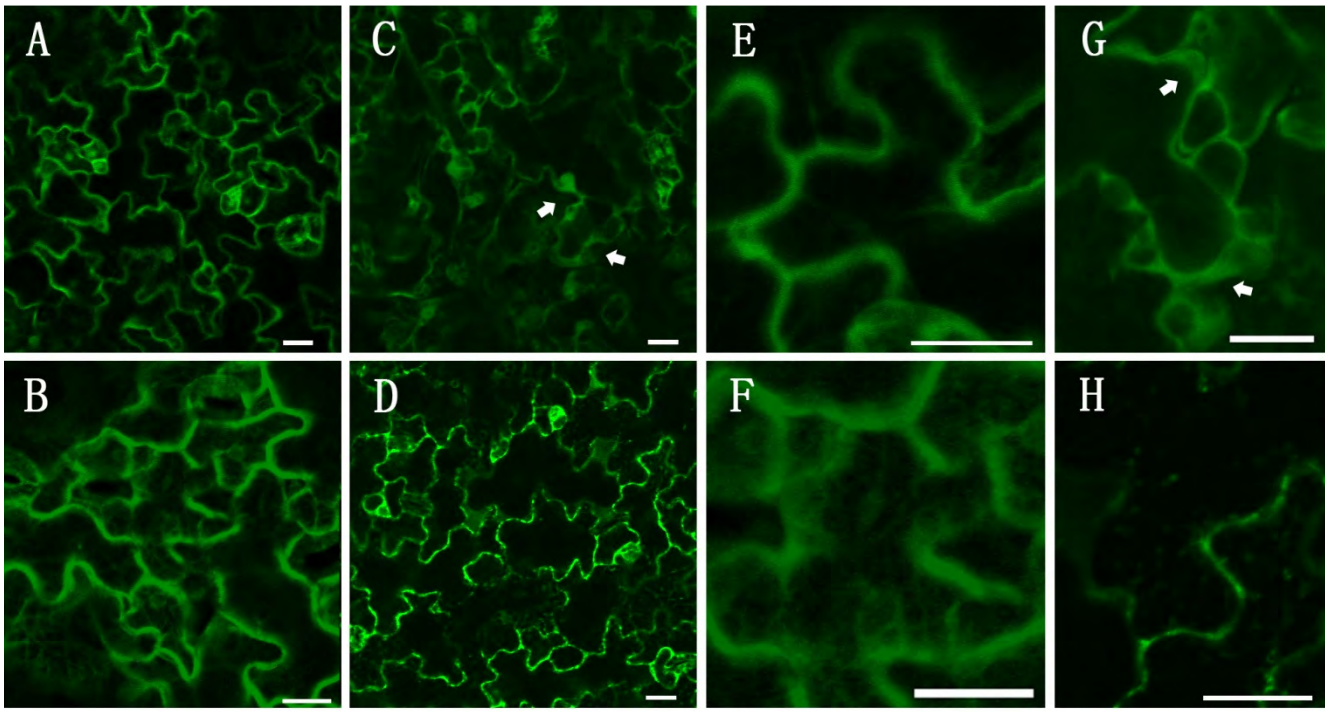

Figure 7. Treatment of colchicine results in the accumulation of GFP:At1g19190 in ***Arabidopsis*** leaves. (A) Cotyledons of seven-days-old GFP:At1g19190 seedlings treated with DMSO as control; (B) TUA6:GFP, showing microtubule network, treated with DMSO; (C) GFP:At1g19190 was treated with colchicine, which can specifically disrupt the microtubule network (arrow shown); (D) TUA6:GFP treatment with colchicine showing disruption of the microtubule, resulting in disruption of GFP:At1g19190 localization; E to H show the amplifications of (A), (B), (C), and (D), respectively. Bar = 20 μM.

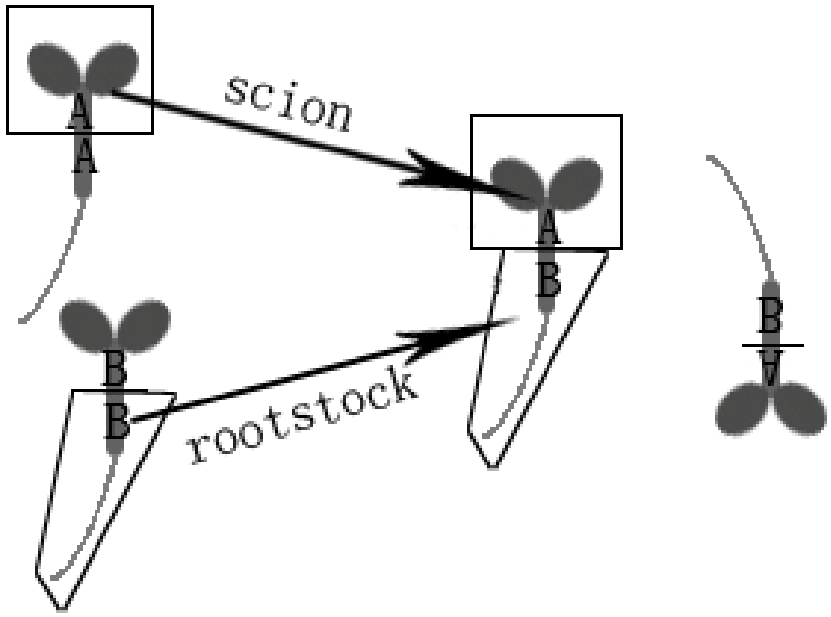

Figure 8. Micrografting model. The left-hand side shows the two plants, A and B, from which the scion and rootstock, respectively, were taken. The right-hand side shows the inversion of the plant when viewed with an LSM 510 (Zeiss, Germany).

signal in the rootstock was tested three and four days later (Figure 14A to H). The WT rootstock showed obvious

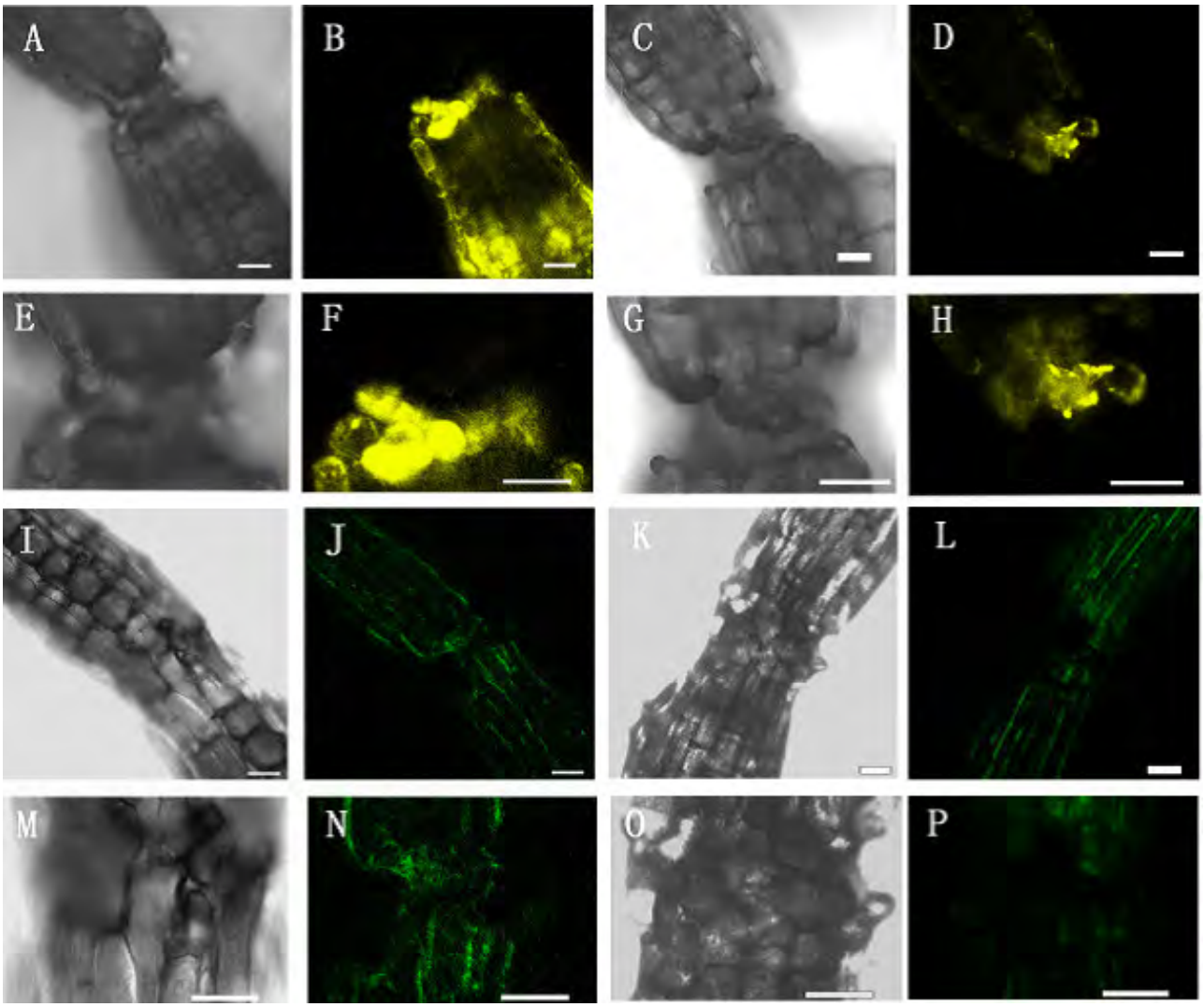

Figure 9. Transport of At1g19190 and AtRGP2 using the micrografting technique. A and B, Micrografting using AtRGP2:YFP plant as the scion and WT plants as the rootstock; (B) YFP fluorescence three days after micrografting; YFP fluorescence was not transported from the scion to the rootstock; C and D, micrografting with WT plants as the scion and AtRGP2:YFP as the rootstock; D, YFP fluorescence six days after micrografting: YFP fluorescence was not transported from the scion to the rootstock; I and J, micrografting using GFP:At1g19190 plants as the scion and WT plants as the rootstock; J, GFP fluorescence four days after micrografting: GFP fluorescence was transported from the scion to the rootstock; K and L, micrografting with WT plants as the scion and GFP:At1g19190 as the rootstock; L, GFP fluorescence two days after micrografting: GFP fluorescence was transported from the rootstock to the scion A, C, E, G, I, K, M and O show the DIC corresponding to each frame, respectively; E, F, G, H, M, N, O and P show the amplification of A, B, C, D, I, J, K and L, respectively. Bar = 20 μM.

fluorescence. Similar fluorescence signals were detected in WT scion after grafting onto the rootstock of ***Arabidopsis*** expressing GFP:At1g19190 (Figure 14I to P). This conferment of fluorescence to non-fluorescent rootstock or scion after grafting was not observed in grafts between WT plants and ***Arabidopsis*** ATRGP2:YFP (Figure 15). Instead, fluorescence accumulates in the callus of the graft incisions (Figure 9E and H). These

results indicated that the At1g19190, against AtRGP2, could make long-distance transport in grafted ***Arabidopsis***.

DISCUSSION

At1g19190 is a putative plasmodesmal-associated protein

Several lines of evidence presented here support the hypothesis that At1g19190 is a Pd-associated protein. First, the fusion of GFP:At1g19190 was used to explore the localization of the protein. Fluorescence studies showed that both transient and stable expressions of GFP:At1g19190 are localized at the wall sheath. In addition, the plasmid GFP:At1g19190 transformation of protoplasts in Arabidopsis leaves does not give off fluorescence. Secondly, GFP:At1g19190 fluorescence appears in a punctate pattern on the cell walls, a pattern similar to that of plasmodesmal marker the Pd-associated protein AtRGP2: GFP (Sagi et al., 2005). Lastly, GFP:At1g19190 fluorescence does not co-locate with FM4-64 but co-localizes with aniline-blue stained callose.

At1g19190 is associated with tubulin cytoskeleton

Pds are symplasmic channels that can establish dynamic intercellular delivery pathways for nutrients and developmental messengers. It is composed of the outer boundary of the PM of two neighboring cells, and an inner boundary formed by appressed ER, called the DM, running through to the axial core (Cilia et al., 2004; Oparka, 2004; Kim, 2005; Maule, 2009). Molecular flux is proposed to occur through the cytoplasmic sleeve between the PM and DM, and is believed to be regulated by callose deposition in the neck region of the wall. Knowing that the GPI-anchor protein PDCB1 and AtBG_ppap are bound to callose, these two molecules were used to locate Pds (Levy et al., 2007; Simpson et al., 2009). AtRGP2 is a plasmodesmal-associated protein delivered to Pds via the Golgi apparatus (Sagi et al., 2005; Zavaliev et al., 2010).

To determine the interaction between At1g19190 and the cytoskeleton, three cytoskeleton disturbance drugs were applied to the overexpressed GFP:At1g19190 ***Arabidopsis*** plants. CB and BFA treatments did not disturb At1g19190 targeting in any way. Only colchicine treatment, which destroys MTs, resulted in fluorescence accumulation, thus hinting that it plays a role in the Pds-localization of GFP:At1g19190.

At1g19190 may be accompanied with secondary Pds-transport

Arabidopsis grafting has generated persuasive evidence of the involvement of long-distance signals in many plant processes, including miRNA transportation (Hsieh et al., 2009), shoot branching and flowering time regulation (Notaguchi et al., 2008, 2009). In this study, the micro-grafting technique was used to determine whether or not At1g19190 is transported from cell to cell. Results show that GFP:At1g19190 fluorescence can be transported to WT plants regardless of whether GFP:At1g19190 acts as a scion or rootstock. This property is unique to GFP:At1g19190 and not to AtRGP2:YFP. Previous reports claimed that GFP (27 kDa) can move throughout the embryo, whereas 2xGFP (54 kDa) cannot pass through Pds that connect cells at the boundary between the shoot apex and the cotyledons (Kim et al., 2005). The molecular weight of GFP: At1g19190 is about 63 kDa, whereas that of AtRGP2:YFP is about 68 kDa. This difference shows why only GFP:At1g19190 is transported from fluorescent to non-fluorescent regions of the plant when it is only about the same size as AtRGP2:YFP. The grafting mechanism is not clear, so the incision is hypothesized to induce the formation of secondary Pds. At1g19190 may be transported by newborn secondary Pds because it is an MTs-dependent, and ER-Golgi independent process.

Conclusion

At1g19190 may be a very important protein in the Pds structure and may play a critical role in substance transport. According to the model of transport and degradation of potato leaf roll virus 17-kDa movement protein (MP17) in plant (Vogel et al., 2007) and our experimental results, the model was modified and we propose a theory for the transport of substances to Pds (Figure 10), where substances may attach to Golgi-derived vesicles, and AtRGP2, as a chaperone, assists substance transport to Pds. In some cases, the substances are distinguished by At1g19190 and translocated by MTs into proteasome complexes for proteolytic degradation before they are transported to neighboring cells. This process can be inhibited by the disruption of MTs, although the exact mechanism for this remains unknown. Further studies are necessary to explore its mechanism and realize its true potential in substance transport.

ACKNOWLEDGEMENTS

We are very grateful to Pengshan Zhao for providing the plasmid pCM1307-nGFP. We thank Dr. Bernard L. Epel for providing the AtRGP2:YFP seeds. This work was supported by grants received under the National Natural Science Foundation of China (No. 30771091&30970234), the Program for New Century Excellent Talents in University (NCET) and the Chunhui Program of Chinese Ministry of Education (2009).

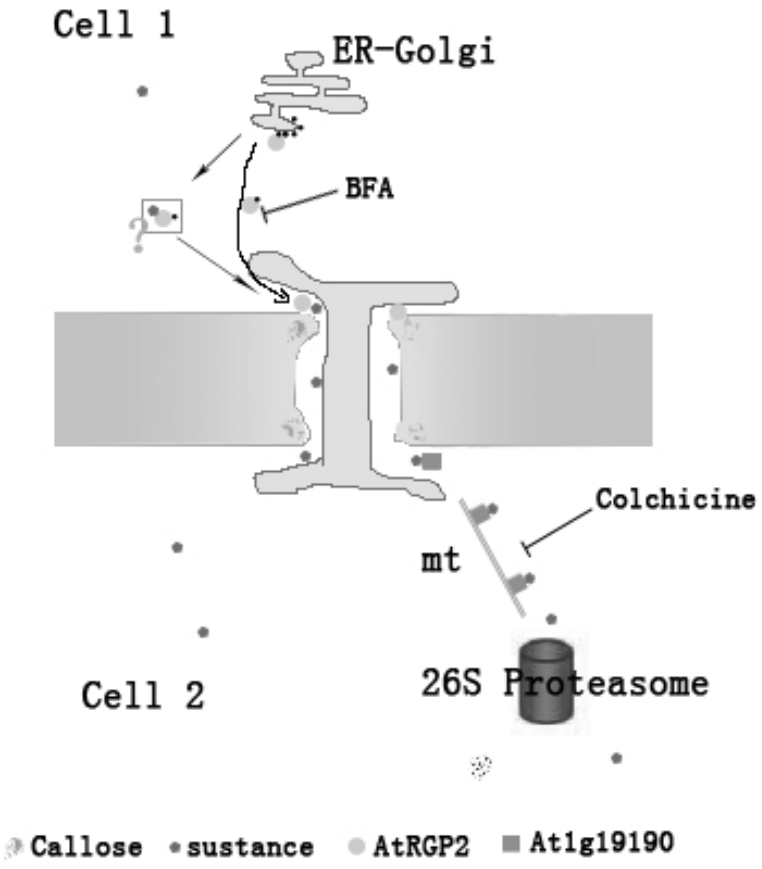

Figure 10. Theoretical model of transport and degradation of substance in plants. Substances may be attached to Golgi-derived vesicles, whereas AtRGP2 as a chaperone assists substance transport to Pds. This process can be inhibited by BFA. Substances are recruited to Pds, and a mechanism emerged that allowed for Pds-dilation. This mechanism can transport to neighboring cells. In some case, substances were distinguished by At1g19190 and translocated by MTs into proteasome complexes for proteolytic degradation. This process can be inhibited by the disruption of the MTs.

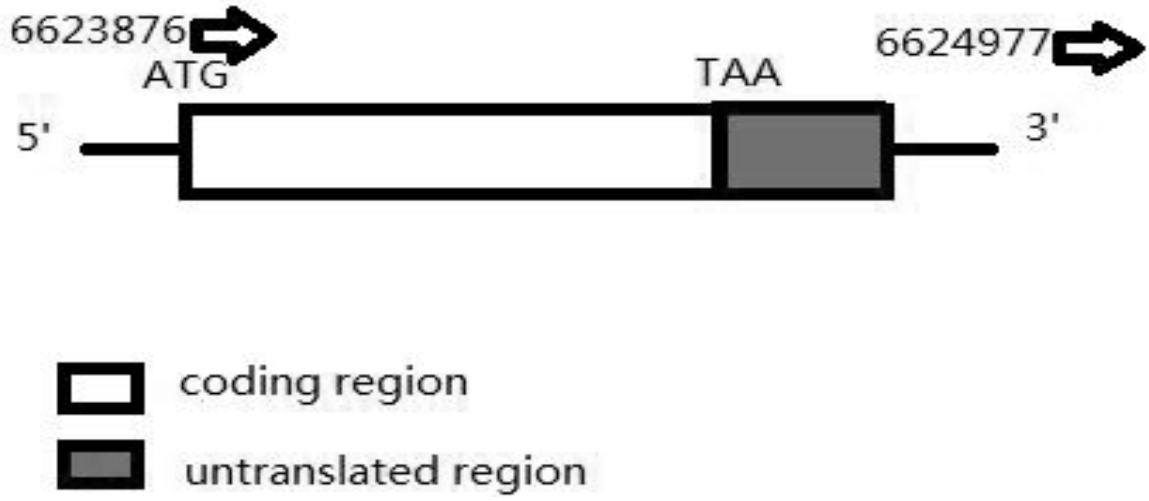

Figure 11. Schematic model of At1g19190. White represents the coding region, 957 bp long and gray represents the 3' untranslated region.

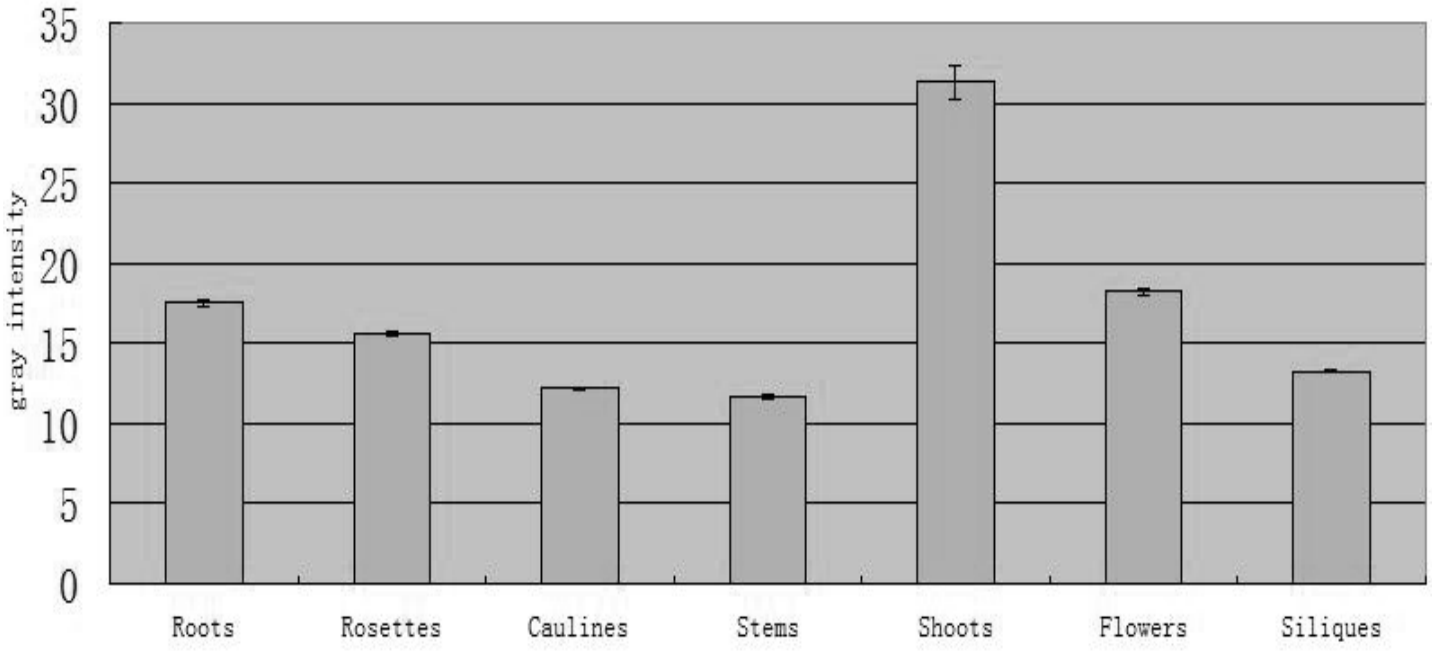

Figure 12. The transcription level of At1g19190 by IMAGEJ. Average values of gray intensity generated by IMAGEJ software. These were used to calculate the At1g19190 transcription levels in different tissues.

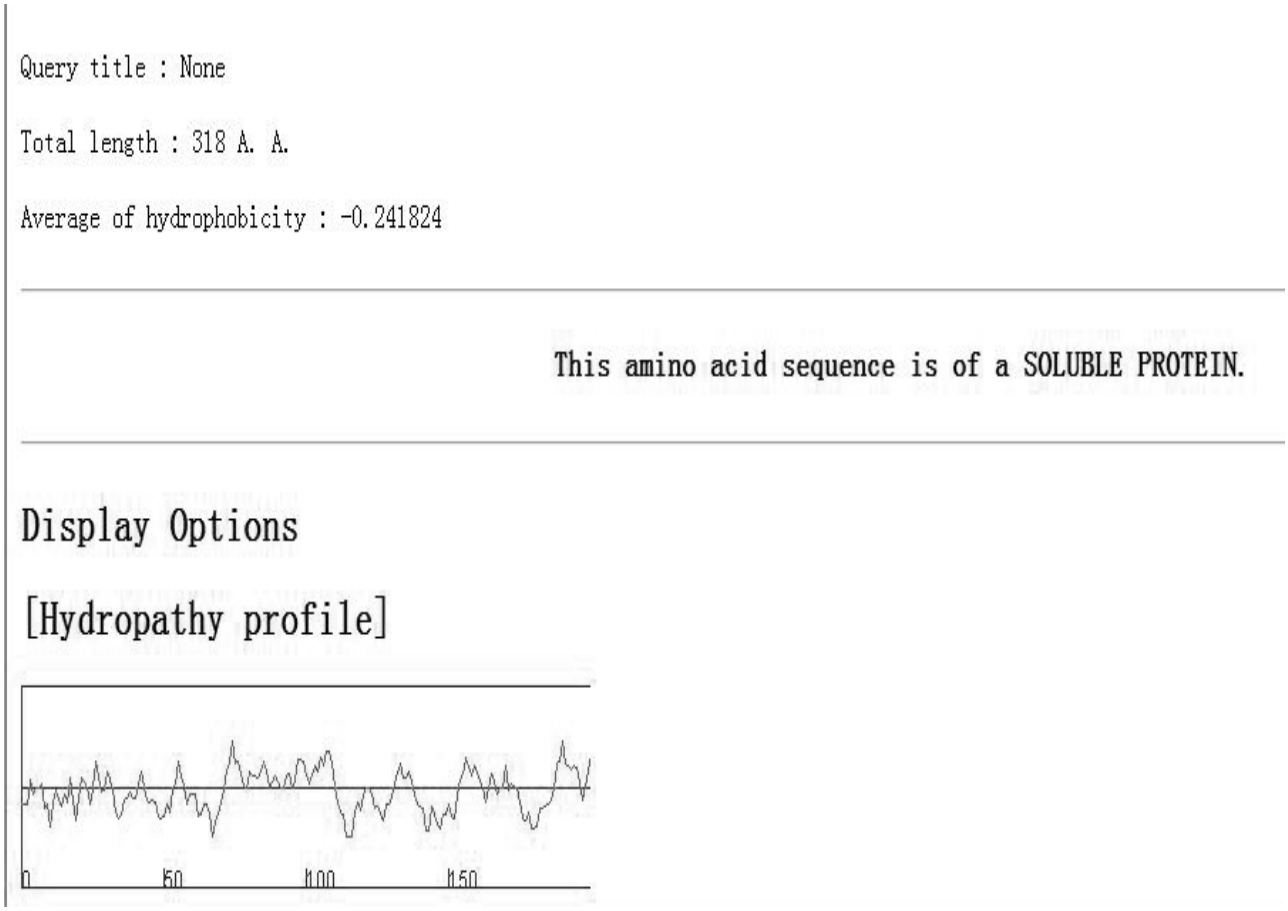

Figure 13. Transmembrane region predict of At1g19190. At1g19190 does not have any transmembrane regions

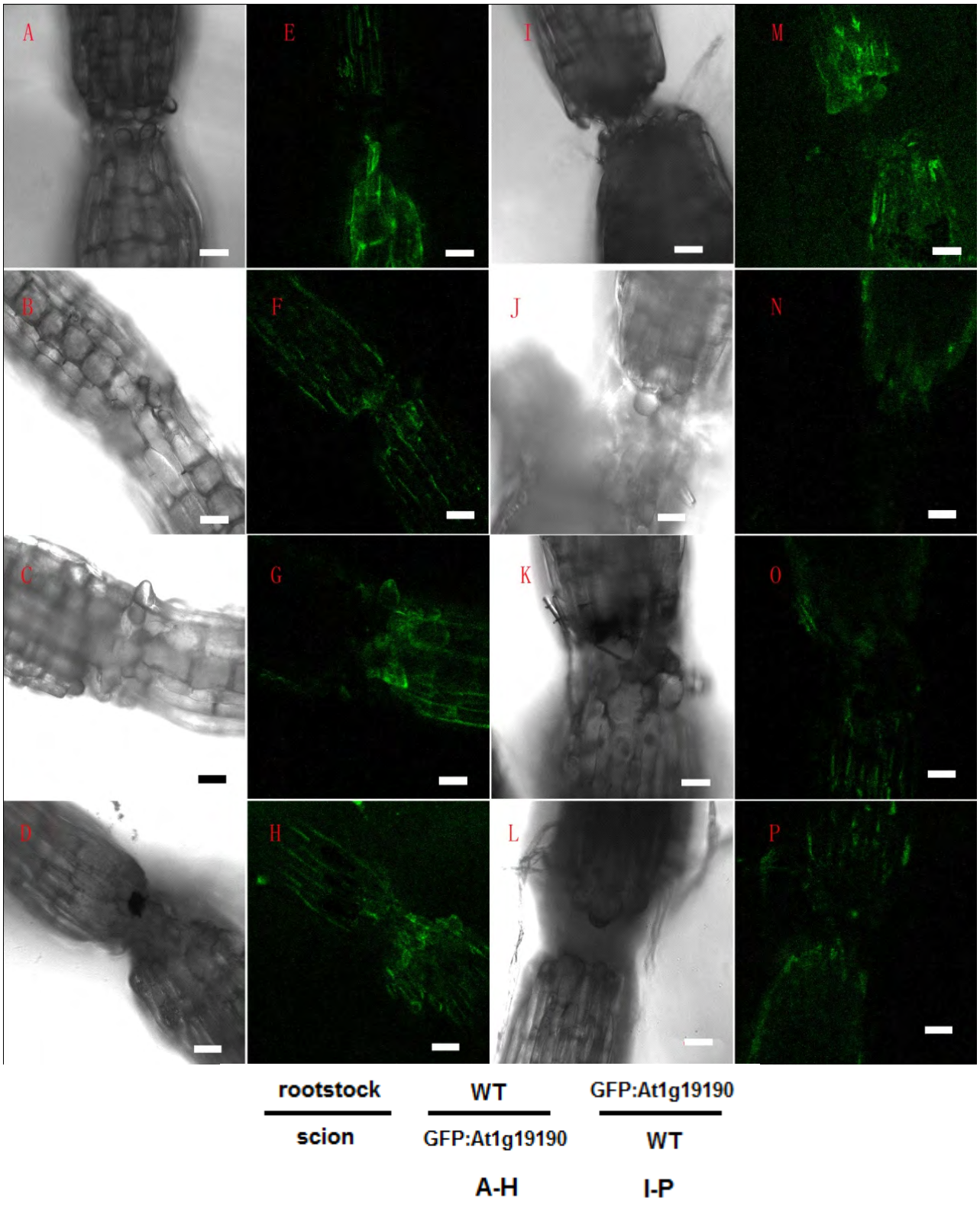

Figure 14. Long-distance transport of At1g19190. A to H, Micrografting using GFP:At1g19190 plants as the scion and WT plants as the rootstock; E to H, GFP fluorescence 3, 4, 5 and 6 days after micrografting, respectively. GFP fluorescence was transported from the scion to the rootstock. A to D show the DIC corresponding to each frame, respectively; I to P, show micrografting with WT plants as the scion and GFP:At1g19190 as the rootstock, I to L, GFP fluorescence 3, 4, 5 and 6 days after micrografting, respectively. GFP fluorescence was transported from the rootstock to the scion; M to P show the corresponding DIC to each frame. Bar = 20 μM.

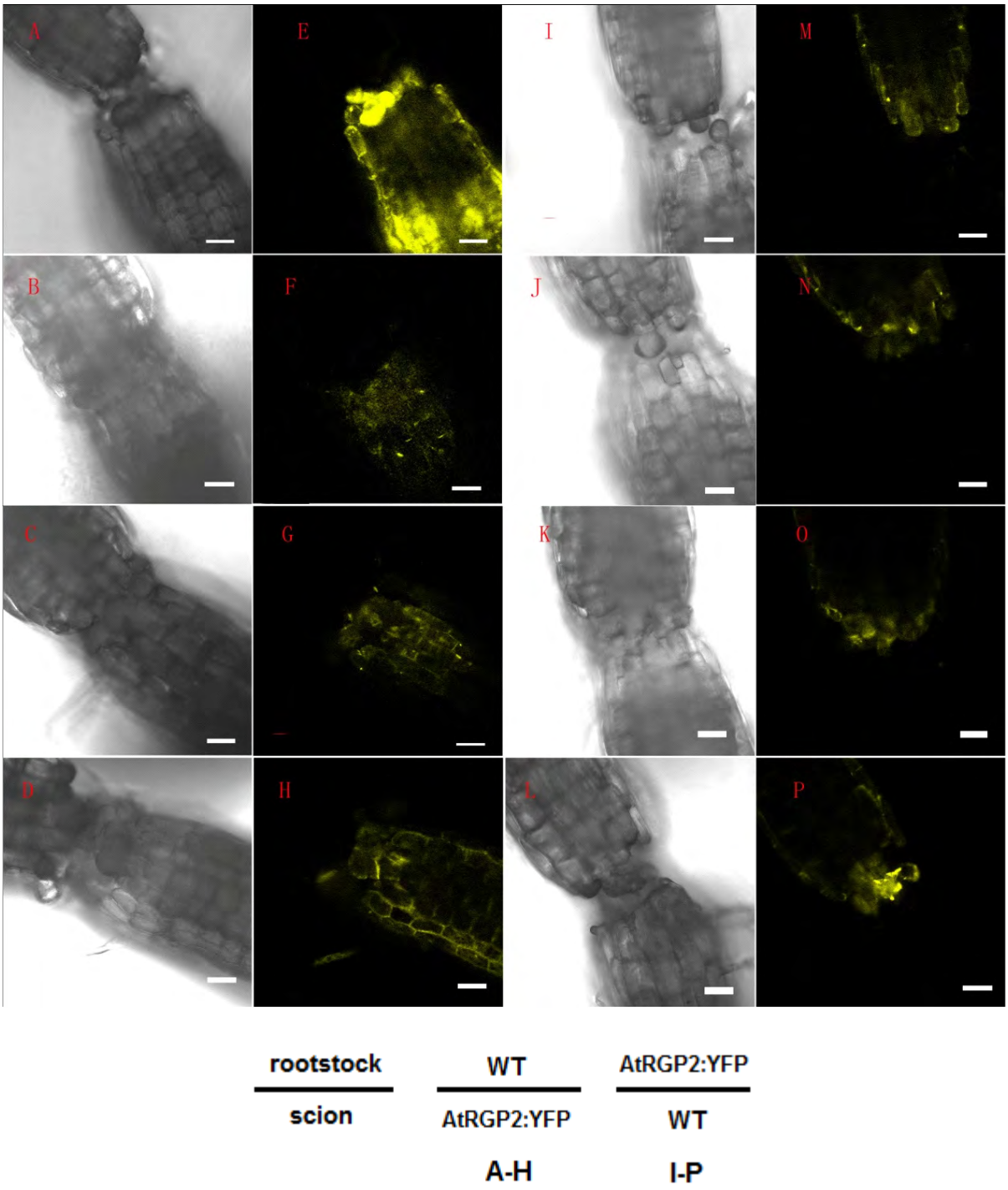

Figure 15. Transport of AtRGP2 using micrografting technique. A to H, Micrografting using AtRGP2:YFP plant as the scion and WT plants as the rootstock; E to H, YFP fluorescence at 3, 4, 5 and 6 days after micrografting, respectively. YFP fluorescence was not transported from the scion to the rootstock; A to D, show the DIC corresponding to each frame, respectively; I to P, show micrografting with WT plants as the scion and AtRGP2:YFP as the rootstock; I to L, YFP fluorescence at 3, 4, 5 and 6 days after micrografting, respectively. YFP fluorescence was not transported from the rootstock to the scion; M to P, show the DIC corresponding to each frame. Bar = 20 µM.

REFERENCES

Baluska F, Samaj J, Napier R, Volkmann D (1999). Maize calreticulin localizes preferentially to plasmodesmata in root apex. Plant J. 19: 481–488.

Bayer E, Thomas CL, Maule AJ (2004). Plasmodesmata in ***Arabidopsis***

thaliana suspension cells. Protoplasma. 223: 93–102.
Bechtold N, Ellis J, Pelletier G (1993) In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. C. R. Acad. Sci. Paris, Sciences de la vie/Life Sci. 316: 1194-1199.
Bernhardi JJ (1805). Beobachtungen u¨ber Pflanzengefa¨sse und eine neue Art derselben. Pages 66.
Blackman LM, Overall RL (1998). Immunolocalisation ofthe cytoskeleton to plasmodesmata of Chara corallina. Plant J. 14: 733–741.
Bloch D, Lavy M, Efrat Y, Efroni I, Bracha-Drori K, Abu-Abied M, Sadot E, Yalovsky S (2005) Ectopic expression of an activated RAC in Arabidopsis disrupts membrane cycling. Mol.Biol.Cell. 16: 1913–1927.
Cilia ML, Jackson D (2004). Plasmodesmata form and function. Curr. Opin. Cell Biol. 16: 500–506.
Clough SJ, Bent AF (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16: 735–743.
Colin G N Turnbull, Jon P.Booker, HM Ottoline Leyser (2002). Micrografting techniques for testing long-distance signaling in Arabidopsis. Plant J. 32: 255-262.
Ding B, Kwon MO, Warnberg L (1996). Evidence that actin filaments are involved in controlling the permeability of plasmodesmata in tobacco mesophyll. Plant J. 10: 157–164.
Ehlers K, Kollmann R (2001). Primary and secondary plasmodesmata: Structure, origin,and functioning. Protoplasma. 216: 1–30.
Gorshkova EN, Erokhina TN, Stroganova TA, Yelina NE, Zamyatnin AA, Kalinina NO, Schiemann J, Solovyev AG, Morozov SY (2003). Immunodetection and fluorescent microscopy of transgenically expressed hordeivirus TGBp3 movement protein reveals its association with endoplasmic reticulum elements in close proximity to plasmodesmata. J. Gen. Virol. 84: 985–994.
Heinlein M Epel BL (2004). Macromolecular Transport and Signaling Through Plasmodesmata. Int Rev Cytol. 235: 93-164.
Hirokawa T, Boon-Chieng S, Mitaku, S (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics, 14: 378–379.
Hsieh LC, Lin SI, Shih AC, Chen JW, Lin WY, Tseng CY, Li WH, Chiou TJ (2009). Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiol. 151: 2120-2132.
Kim I, Kobayashi K, Cho E, Zambryski PC (2005). Subdomains for transport via plasmodesmata corresponding to the apical-basal axis are established during Arabidopsis embryogenesis. Proc. Natl. Acad. Sci. U. S. A. 102: 11945–11950.
Kim JY (2005). Regulation of short-distance transport of RNA and protein. Curr. Opin. Plant Biol. 8: 45–52.
Lavy M, Bracha-Drori K, Sternberg H, Yalovsky S (2002). A cell-specific, prenylation-independent mechanism regulates targeting of type II RACs. Plant Cell. 14: 2431–2450.
Lee JY, Taoka K, Yoo BC, Ben-Nissan G, Kim DJ, Lucas WJ (2005). Plasmodesmal-associated protein kinase in tobacco and Arabidopsis recognizes a subset of non-cell-autonomous proteins. Plant Cell. 17: 2817-2831.
Levy A, Erlanger M, Rosenthal M, Epel BL (2007). A plasmodesmata-associated beta-1,3-glucanase in Arabidopsis. Plant J. 49: 669-682.
Lucas WJ, Ham BK, Kim JY (2009). Plasmodesmata - bridging the gap between neighboring plant cells. Trends Cell Biol. 19: 495-503.
Marchler-Bauer A, Bryant SH (2004). CD-Search: protein domain annotations on the fly. Nucleic Acids Res. 32: W327–W331.
Marshall SD, Putterill JJ, Plummer KM, Newcomb RD (2003). The carboxylesterase gene family from Arabidopsis thaliana. J. Mol. Evol. 57: 487-500.
Maule AJ (2009). Plasmodesmata: structure, function and biogenesis. Curr Opin. Plant Biol. 11: 680-686.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. 15(3): 472-497.
Notaguchi M, Abe M, Kimura T, Daimon Y, Kobayashi T, Yamaguchi A, Tomita Y, Dohi K, Mori M, Araki T (2008). Long-distance, graft-transmissible action of Arabidopsis FLOWERING LOCUS T protein to promote flowering. Plant Cell Physiol. 49: 1645-1658.
Notaguchi M, Daimon Y, Abe M, Araki T (2009). Graft-transmissible action of Arabidopsis Flowering Locus T protein to promote flowering. Plant Signal Behav. 4: 123-125.
Oparka KJ (2004). Getting the message across: how do plant cells exchange macromolecular complexes. Trends Plant Sc. 9: 33–41.
Reichelt S, Knight AE, Hodge TP, Baluska F, Samaj J, Volkmann D, Kendrick-Jones J (1999). Characterization of the unconventional myosin VIII in plant cells and its localization at the post-cytokinetic cell wall. Plant J. 19: 555–567.
Ritzenthaler C, Nebenfu¨ hr A, Movafeghi A, Stussi-Garaud C, Behnia L, Pimpl P (2002). Reevaluation of the effects of brefeldin A on plant cells using tobacco bright yellow 2 cells expressing golgi-targeted green fluorescent protein and COPI antisera. Plant Cell. 14: 237–261.
Sagi G, Katz A, Guenoune-Gelbart D, Epel BL (2005). Class 1 reversibly glycosylated polypeptides are plasmodesmal-associated proteins delivered to plasmodesmata via the golgi apparatus. Plant Cell. 17: 1788-1800.
Sciaky N, Presley J, Smith C, Zaal KJ, Cole N, Moreira JE, Terasaki M, Siggia E, Lippincott-Schwartz J (1997). Golgi tubule traffic and the effects of brefeldin A visualized in living cells. J Cell Biol. 139: 1137–1155.
Simpson C, Thomas C, Findlay K, Bayer E, Maule AJ (2009). An Arabidopsis GPI-anchor plasmodesmal neck protein with callose binding activity and potential to regulate cell-to-cell trafficking. Plant Cell. 21: 581-594.
Tangl E (1879). Ueber offene Communicationen zwischen den Zellen des Endosperms einiger Samen. Jahrb. Wiss. Bot. 12: 170–190.
Thomas CL, Bayer EM, Ritzenthaler C, Fernandez-Calvino L, Maule AJ (2008). Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. PLoS Biol. 6: 180-190.
Tian GW, Mohanty A, Chary SN, Li SJ, Paap B, Drakakaki G, Kopec CD, Li JX, Ehrhardt D, Jackson D, et al (2004). High-throughput fluorescent tagging of full-length Arabidopsis gene products in planta. Plant Physiol. 135: 25–38.
Tominaga M, Morita K, Sonobe S, Yokata E, Shimmen T (1997). Microtubules regulate the organization of actin filaments at the cortical region in root hair cells of Hydrocharis. Protoplasma, 199: 83-92.
Turnbull CG, Booker JP, Leyser HM (2002). Micrografting techniques for testing long-distance signalling in Arabidopsis. Plant J. 32: 255-262.
Vogel F, Hofius D, Sonnewald U (2007). Intracellular trafficking of Potato leafroll virus movement protein in transgenic Arabidopsis. Traffic, 8: 1205-1214.
Voigt B, Timmers AC, Samaj J, Müller J, Baluska F, Menzel D (2005). GFP-FABD2 fusion construct allows in vivo visualization of the dynamic actin cytoskeleton in all cells of Arabidopsis seedlings. Eur. J. Cell Biol. 84: 595-608.
Wagner, G, Haupt, W, Laux A (1972). Reversible inhibition of chloroplast movement by cytochalasin B in the green alga Mougeotia. Science, 176: 808–809.
Williamson RE, Hurley UA (1986). Growth and regrowth of actin bundles in Chara: bundle assembly by mechanism differing in sensitivity to cytochalasin. J. Cell Biol. 85: 21-32.
Zambryski (2008). Plasmodesmata. Curr Biol. 18: R324-325.
Zavaliev R, Sagi G, Gera A, Epel BL (2010). The constitutive expression of Arabidopsis plasmodesmal- associated class 1 reversibly glycosylated polypeptide impairs plant development and virus spread. J. Exp. Bot. 6: 1131-1142.