

SYP71, a Plant-specific Qc-SNARE Protein, Reveals Dual Localization to the Plasma Membrane and the Endoplasmic Reticulum in Arabidopsis

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ABSTRACT. SNAREs ('Soluble N-ethyl-maleimide sensitive factor attachment protein receptors') play a critical role in the membrane fusion step of the vesicular transport system in eukaryotes. The number of the genes encoding SNARE proteins is estimated to be 64 in *Arabidopsis thaliana*. This number is much larger than those in other eukaryotes, suggesting a complex membrane trafficking in plants. The Arabidopsis SNAREs, the SYP7 group proteins, SYP71, SYP72, and SYP73, form a plant-specific SNARE subfamily with not-yet-identified functions. We have previously reported that the SYP7 subfamily proteins are predominantly localized to the endoplasmic reticulum in the Arabidopsis suspension cultured cells under transient expression condition. However, several proteomic analyzes indicated the plasma membrane localizations of one of SYP7 subfamily proteins, SYP71. In order to confirm the expression patterns and subcellular localization of SYP7, we performed combination analyses including promoter GUS analysis, a sucrose density gradient centrifugation analysis, as well as an observation on transgenic Arabidopsis plants expressing GFP-fused SYP71 under control of its native promoter. From these analyses, we concluded that one of the SYP7 subfamily proteins, SYP71, is predominantly expressed in all vegetative tissues and mainly localized to the plasma membrane. We also found that SYP71 is localized to the endoplasmic reticulum in the dividing cells of various types of tissues.

Key words: SNARE protein/plasma membrane/endoplasmic reticulum/endosome/membrane traffic/Arabidopsis

Introduction

Membrane trafficking is a process that maintains cellular homeostasis by delivering newly synthesized proteins to their correct destinations by transport vesicles. Transport vesicles carry cargo proteins from a donor compartment and discharge them by fusing with the membrane of the target compartment. Fusion between the membranes of specific transport vesicles and their target membranes is mainly regulated by the SNARE and Rab families of proteins (Sanderfoot *et al.*, 2000; Ungar and Hughson, 2003; Vernoud *et al.*, 2003; Uemura *et al.*, 2004; Sanderfoot, 2007). The SNARE family is divided into four groups: Qa-, Qb-, Qc-, and R-SNAREs; according to the basis of their similarities in particular amino acid sequence called the

SNARE motif. The functional SNARE complex, which drives specific membrane fusion process, consists of parallel hetero-oligomeric four-helix bundles. Each bundle contains one SNARE motif from each of the four distinct SNARE groups (Fasshauer *et al.*, 1998).

The higher plants have evolved a complex membrane transport system; namely, the higher plant cell has multiple pathways to deliver cargo proteins to different types of vacuoles and cell surface sub-domains (Jürgens, 2004). In fact, an excess number of SNAREs (64) and Rabs (57) have been identified in the genome of *Arabidopsis thaliana* compared with those in animal or yeast genomes (Sanderfoot *et al.*, 2000; Vernoud *et al.*, 2003; Sanderfoot, 2007). Particularly, the SNAREs found on the plasma membrane (PM) of Arabidopsis are of many different types: to date, 9 Qa-SNAREs, 3 Qb-SNAREs and 5 R-SNAREs have been identified (Uemura *et al.*, 2004). These PM-localized SNAREs seem to play a plant-specific role in higher-order functions such as pathogen resistance, cytokinesis and response to ABA (Collins *et al.*, 2003).

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Within the Arabidopsis SNARE molecules, the NPSN subfamily of Qb-SNAREs (consisting of NPSN11, 12, and 13) and the SYP7 subfamily in Qc-SNARE (consisting of SYP71, 72, and 73) appear to be unique to plants with no orthologs in other eukaryotes (Sanderfoot *et al.*, 2000). NPSN11 is localized to the cell plate in dividing cells, and interacts with the Qa-SNARE KNOLLE/SYP111, which is specifically expressed during mitosis and also localized on the cell plate. NPSN11 is thought to play a critical role in membrane fusion during cell plate formation (Zheng *et al.*, 2002; Surpin and Raikhel, 2004). The function of the SYP7 subfamily, on the other hand, is still largely unknown, although it is interesting to recall that the SYP7 subfamily proteins have also been found in the endoplasmic reticulum (ER) (Uemura *et al.*, 2004). However, proteomic studies have indicated that SYP71 protein exists on purified PMs from tobacco and Arabidopsis cells (Marmagne *et al.*, 2004; Morel *et al.*, 2006; Mongrand *et al.*, 2004; Alexanderson *et al.*, 2004). Furthermore, using an inhibitory SNARE fragment of SYP71 blocks secretion of fluorescent secretion marker (Tyrrell *et al.*, 2007). These results indicate that SYP71 functions in the secretion process in plants. In this study, we determined the detailed expression patterns of the SYP7 subfamily SNAREs and sub-cellular localization of one of SYP7s, SYP71, using promoter-*GUS* analysis and transgenic plant expressing the GFP-tagged SYP71, respectively. The results show that SYP71 is mainly localized to the PM and function as a Qc-SNARE on the PM. Interestingly, SYP71 is also localized to the ER in the dividing cells of various tissues. These results suggest that SYP71 may be involved in multiple membrane fusion steps during the secretion process in Arabidopsis.

Materials and Methods

Expression analysis based on microarray data

Absolute signal intensity values as micro-array data were obtained from the Bio-Array Resource for Arabidopsis Functional Genomics (BAR) (<http://bar.utoronto.ca/>). Data corresponding to the developmental stages of *Arabidopsis thaliana* were normalized for gray scale such that the signal corresponding to intensity of 500 was assigned to the value of 100% (black) and absence of signal (white).

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Columbia was used in this study. The plant was grown at a constant temperature of 25°C under continuous light. Arabidopsis suspension-cultured cells "Deep" (Glab *et al.*, 1994) were cultured in the Murashige-Skoog (MS) medium at 23°C with continuous agitation in the dark.

Antibody production and western blotting

The cytoplasmic regions of the SYP7 group proteins (Supplemental Fig. 1) were amplified from their cDNA and then were subcloned into pGEX 5X-1 expression vector (GE Healthcare, Chalfont St. Giles, Bucks, UK). The sets of primers used for the PCR amplification were as follows: for the SYP71 gene: 5'-GTGGCGGGATCC-TTATGACTGTGATCGATA-3' and 5'-GTGAGGCCCGGGGCTAGATCTCAGCTGG-3' (equivalent to 241 amino acids from the N-terminal amino acid being inserted into the BamHI-SmaI sites); for the SYP72 gene: 5'-GTGGCGGGATCC-TTATGCCGGTCAT-TGATA-3' and 5'-GTGAGGCCCGGGGCTGGATCGCATCTGC-3'; (equivalent to 242 amino acids from the N-terminal amino acid being inserted into the BamHI-SmaI sites); for the SYP73 gene: 5'-CGCGGCGTCTCGACCAATGGGCGTAATTGATT-3' and 5'-GTG-GAAGTCGACGCTGGATCTCAACTTT-3' (equivalent to 238 amino acids from the N-terminal amino acid being inserted into the SalI site). Each GST-fused protein was expressed in *E. coli* (BL21) under IPTG induction, and purified on a glutathione-Sepharose 4B column (GE Healthcare, Chalfont St. Giles, Bucks, UK). Due to high similarity among SYP71, SYP72, and SYP73 (Supplemental Fig. 1), all purified recombinant proteins then mixed and were used as antigens for immunizing a rabbit. The antiserum produced was confirmed to recognize all GST tag-SYP7s proteins (data not shown).

The anti-SYP7s antiserum was used for detection of endogenous SYP7 family proteins in several tissues of *Arabidopsis thaliana*. Total proteins of the plant tissues were extracted with a grinding buffer (50 mM HEPES-KOH, pH 7.5, 5 mM MgCl₂, 5 mM MEGTA, 250 mM Sorbitol, 1 mM DTT, 1% polyvinylpyrrolidone, 1% ascorbic acid and a protease inhibitor mix (Roche, Basel, Switzerland). One µg of the total protein was subjected to 12% SDS/PAGE gel for separation, and then transferred into PVDF membrane for western blotting.

Analysis of the expression patterns of SYP71, SYP72, and SYP73

We analyzed the gene expression patterns of SYP7 genes based on GUS expression under the control of each SYP7 promoter. Firstly, we created transgenic plants in which GUS was fused to the promoters regions of SYP7 genes, by cloning the 2000-bp upstream regions of the SYP71 and SYP73 genes in the pBI101 vector. We also created a similar chimera using the 1000-bp promoter region of SYP72. We then introduced the constructed plasmids into each plant with Agrobacterium-mediated transformation methods (Clough and Bent, 1998). We detected GUS expression by staining with X-gluc (5-bromo-4-chloro-3-indoleyl-β-D-glucuronide) as described elsewhere (Jefferson *et al.*, 1987).

Sucrose density gradient analysis

In order to obtain clear separation pattern between the ER and the PM membranes, we performed a sucrose density gradient separation in buffers with or without Mg²⁺. We homogenized 5 g of

Arabidopsis root in an extraction buffer (–Mg) (50 mM Tris-HCl, pH 7.5, 250 mM Sorbitol, 2 mM EGTA, 2 mM EDTA, a protease inhibitor mix (Roche, Basel, Switzerland)) or in an extraction buffer (+Mg) (50 mM Tris-HCl, pH 7.5, 250 mM Sorbitol, 2 mM MgCl₂, the protease inhibitor mix). After it was homogenized in 10 ml extraction buffer, it was then filtered through four layers of Miracloth, and centrifuged at 10,000×g for 10 min at 4°C. The crude membrane was then precipitated from the supernatant fraction by centrifugation at 100,000×g for 30 min at 4°C. The pellet was resuspended in a resuspension buffer (–Mg) containing 250 mM Tris-HCl pH 7.5, 5% sucrose and 5mM EDTA or a resuspension buffer (+Mg) containing 250 mM Tris-HCl, pH 7.5, 5% sucrose and 2 mM MgCl₂. The crude membrane fraction of each treatment then was layered on the top of the sucrose density gradient (15%–45%). The separation was performed by centrifugation on 110,000×g for 14 hr using a swing-out rotor. Sample was collected in each 0.5 ml fraction, and then the sucrose concentration was determined by using a hand-refractometer. The samples then were subjected to a SDS-PAGE followed by western blotting analysis using several antibodies. Quantitative analyses of the amounts of detected proteins were performed using the Image-J software.

Generating transgenic plants expressing the GFP-fused SYP71 protein

The translational fusions between GFP and the SYP71 was generated using the fluorescent tagging of full-length protein (FTFLP) method described by Tian *et al.* (2004) with some modifications. Briefly, about 2.3 kbp of the upstream sequence of *SYP71* with 5'-CACC sequence, a GFP sequence with GGSG-linker, and 1.0 kbp of the downstream sequence of *SYP71*, were separately amplified by PCR with following primer sets (upstream fragment, 5'-CACCAATTTGGGAATGTATAAACCATC-3' and 5'-TCGCCCTTGCTCACCATCTTCTTCCAAATCTATCACAAGAAGC; GFP fragment; 5'-GGTGAGCAAGGCGAGG-3' and 5'-GCCACTACCTCCCTGTACAGCTCGTCCATGCC-3'; downstream fragment, 5'-CTGTACAAGGGAGGTAGTGGCATGACTGTGATCGATATCTGACTAGAG and 5'-AGTTGTCTCTATGTTTGCTTCGATATG-3'). Then the three DNA fragments were conjugated by the TT-PCR method. The fragment was subcloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA), and then transferred into a binary vector pGWB1 (Nakagawa *et al.*, 2007) according to the manufacture's instruction. These constructs were introduced into *Agrobacterium tumefaciens* strain C58 Rif^r/pGV2260 in order to transform *Arabidopsis* wild type plants (Col-0) by the floral dipping method (Clough and Bent, 1998). Screening of transgenic plants were performed on the MS plates containing 50 µg mL⁻¹ hygromycin. T₂ lines, which showed a segregation ratio of 3:1 for antibiotic resistance, were used for further experiments.

Results

SYP71 is predominantly expressed in the vegetative tissues in Arabidopsis

SYP7 subfamily SNAREs consist of three related genes, *SYP71*, *SYP72*, and *SYP73*, which share high amino acid sequence identity (>53% identity to each other) (Supplemental Fig. 1). According to the global microarray gene expression analysis throughout *Arabidopsis* development (Toufighi *et al.*, 2005), only *SYP71* is expressed in almost all vegetative tissues including root, shoot, leaf, and flower. Meanwhile, *SYP72* is restrictedly expressed in mature pollen and *SYP73* is less expressed through all tissues but weak expressions were observed in dry seeds, mature pollen and bicellular pollen (Supplemental Table 1).

In order to examine the detailed expression pattern of each gene, we generated transgenic *Arabidopsis* harboring a 1000–2000 bp promoter region of each SYP7 gene fused to the GUS reporter (Fig. 1). The histological GUS analysis showed that *SYP71* was highly expressed in sepals, the filaments of stamen, and short styles of pistils, but less expressed in mature pollen (Fig. 1A, B, C, and J). A strong expression was also observed in the vascular bundles of various tissues including leaf and root in the mature and seedling stages (Fig. 1L and M). No expression was observed in young pollen and ovules (data not shown).

The transgenic plant harboring the *SYP72* and *SYP73* promoter *GUS*-reporter constructs showed strong *GUS* expression during pollen development, although the expressions of two genes had different profiles. The expression of *SYP72* increased during pollen development, and maintained high levels in the mature pollen (Fig. 1F). The *SYP72* promoter activity was also detected in stigmatic papillae and short styles of pistils (Fig. 1N), but not in leaves or roots (Fig. 1P and Q), nor during the seedling stage of plant development (data not shown). In contrast, the expression of *SYP73* was observed in the early developmental stages of embryo and pollen development (Fig. 1G, H, I, R and S). Weak expression was observed in the root tip but not in the leaf (Fig. 1T, U). The expression of *SYP73* during seed development is consistent with the microarray data of the molecule.

In summary, *SYP71* is mainly expressed during vegetative growth, whereas *SYP72* is mainly expressed in pollen development and *SYP73* plays a role in the developmental processes of pollen and embryo.

Due to high similarity among the SYP7 subfamily proteins (Supplemental Fig. 1), it is difficult to generate antibodies which can distinguish each SYP7 protein. Therefore, in this study, we generated an anti-SYP7 antibody to immunize a rabbit with a mixture of three recombinant SYP7 proteins in order to increase the sensitivity of the antibody. As shown in Fig. 2, the anti-SYP7 antibody detected a 32.5-kDa polypeptide in all *Arabidopsis* vegetative tissues.

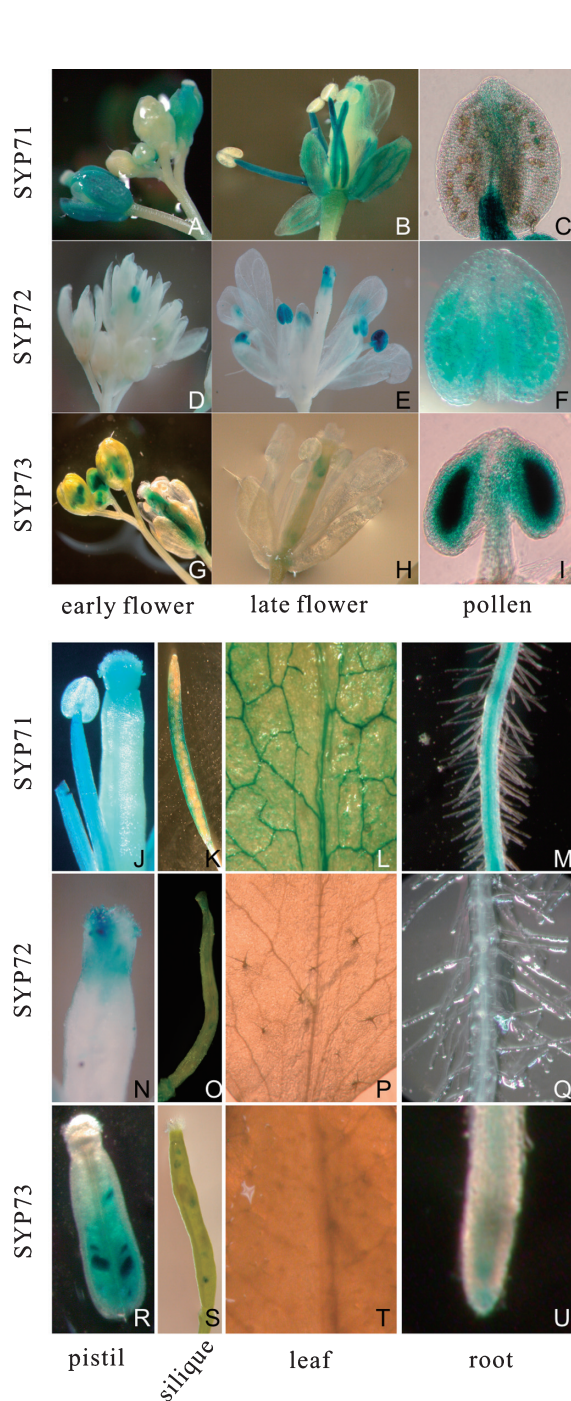


Fig. 1. Promoter GUS expression patterns of *SYP71*, *SYP72* and *SYP73*. GUS expression under the control of *SYP71* promoter (A, B, C, J, K, L, and M), *SYP72* promoter (D, E, F, N, O, P, and Q), and *SYP73* promoter (G, H, I, R, S, T, and U) were detected on various plant tissues: early stage of flower development (A, D, and G), late stage of flower development (B, E, and H), anther (C, F, and I), pistil (J, N, and R), silique (K, O, and S), leaf (L, P, and T), and root (M, Q, and U).

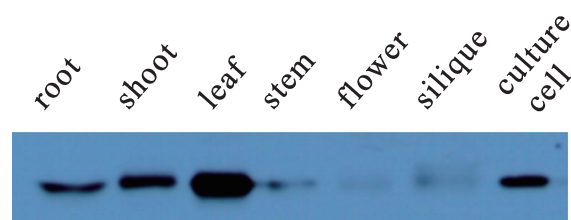


Fig. 2. Immunoblot analysis of SYP71 protein in various vegetative tissues. Total proteins (1 μ g) from different *A. thaliana* plant tissues and cultured cells were separated by SDS/PAGE, before western blotting detection using an anti-SYP7 antibody.

Since only *SYP71* is expressed in vegetative tissues, we concluded that the 32.5-kDa polypeptide recognized by the SYP7 antibody in vegetative tissues is SYP71. SYP71 detected almost all tissues including root, shoot, leaf, stem, flower, silique, and suspension cultured cells, with different levels of expression intensity. An especially strong expression was observed in rosette leaves. Intriguingly, less SYP71 protein was detected in flower, although strong expression was observed in flower tissues by the promoter-GUS and the microarray analyzes. We also found that two distinct bands existed in the blot of silique protein extract. The promoter-GUS and microarray analyzes indicated that SYP73 is expressed during seed maturation in addition to the SYP71 expression in silique. Therefore, it is likely that the two distinct bands detected in the silique fraction by the anti-SYP7 antibody are SYP71 and SYP73.

***SYP71* is localized not only to the PM but also to the ER in actively dividing cells**

To investigate the subcellular localization of endogenous SYP71 in Arabidopsis, we performed a sucrose density gradient centrifugation analysis using the crude membrane fraction prepared from the Arabidopsis root culture. As shown in Fig. 3, in the absence of Mg^{2+} , the peak of the ER marker protein, AtSar1, was shifted to the lighter fraction (Fraction 16), and was completely separated from the peak fraction of the PM marker protein, H^+ -ATPase. AtSar1 was also detected in the lighter fractions not only in the presence of Mg^{2+} , but also in the absence of Mg^{2+} . The lighter peak of AtSar1 is likely a soluble form of the protein.

In the separation without Mg^{2+} , SYP71 peak was recovered in two distinct peak fractions, one corresponding to the ER marker and the other corresponding to the PM marker. The results clearly indicate that SYP71 is localized both to the ER and PM membranes. Similar results were obtained from the same analysis using Arabidopsis suspension culture cell (data not shown).

Furthermore, we confirmed the subcellular distribution of SYP71 by using a transgenic plant expressing GFP-SYP71 under the control of its native promoter. The expression of SYP71 protein under its promoter was observed throughout

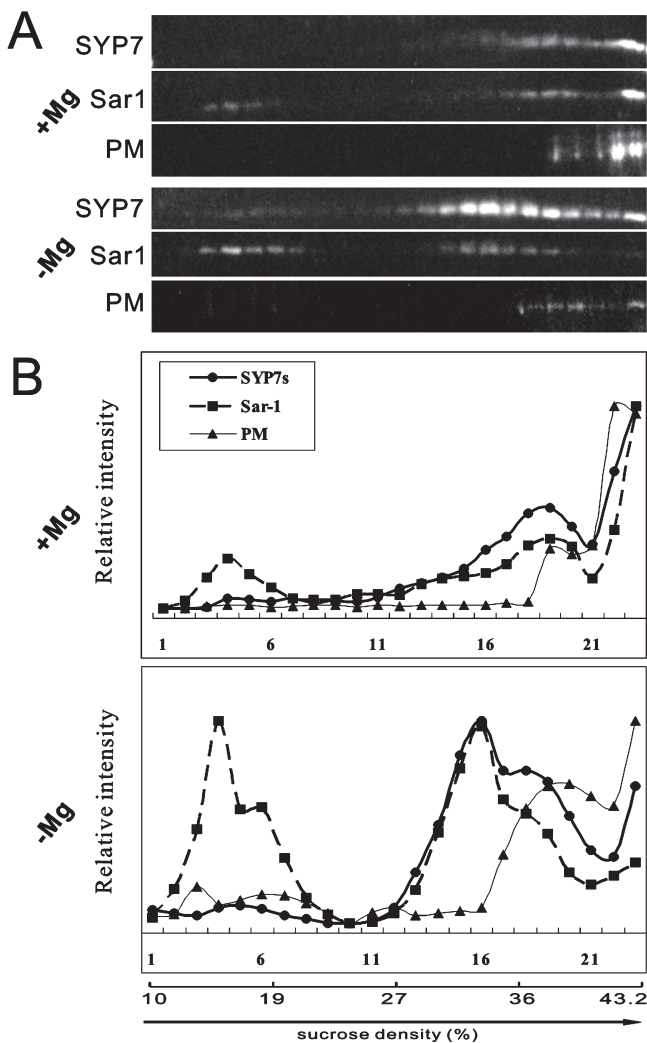


Fig. 3. ER localization of SYP71 shown by subcellular fractionation. (A) Microsomal membrane fraction was isolated from 5 g of Arabidopsis root and was separated on a sucrose density gradient (15%–45%) with or without Mg^{2+} . Twenty-three fractions were analyzed by immunoblotting, using antibodies against SYP7, AtSar1 and PM- H^{+} -ATPase. (B) Quantitative analysis of the detected proteins was performed with the Image J software.

all plant tissues including root, leaf, conductive tissue of root and stem, and flower with different fluorescent intensities (Fig. 4 and Supplemental Fig. 2). Especially, a weak fluorescence was observed in flower tissues such as stigma and stamen filament (Supplemental Fig. 2F and G). These weak expression intensities were inconsistent with those of transcriptions (Fig. 1A and J), but consistent with western blot data (Fig. 2). These results suggest that SYP71 proteins rapidly degraded in flower tissues in spite of the high level of transcription.

Close observation under CLSM microscope indicated that SYP 71 was mainly observed in the PM of mature cells

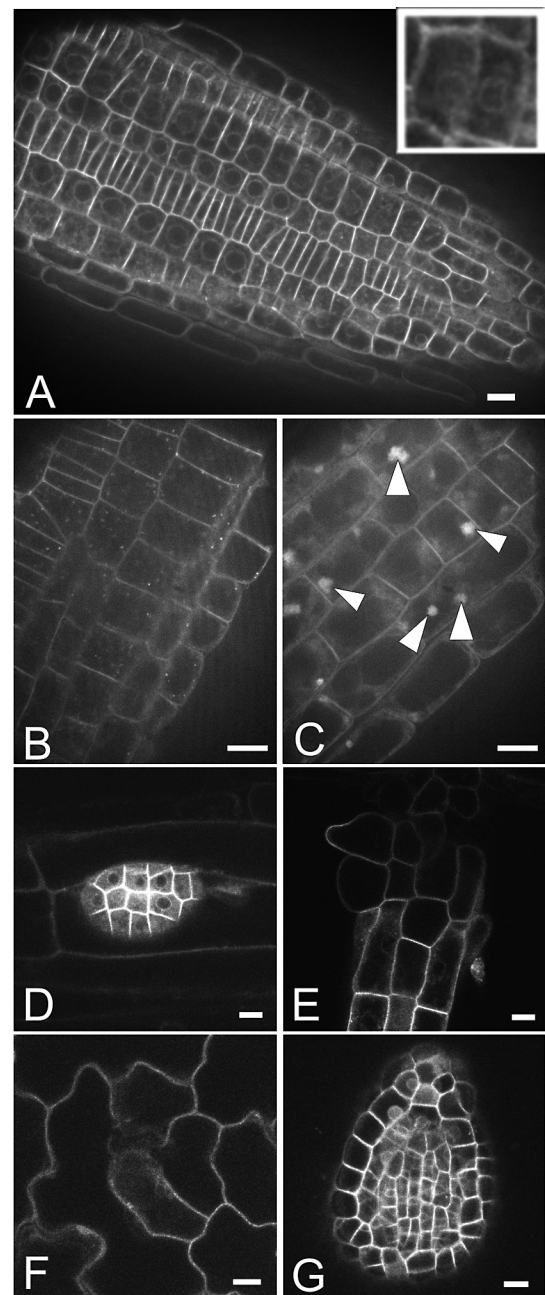


Fig. 4. GFP-SYP71 expression under its native promoter was detected on the ER, the PM and the endosomes. The fluorescence of GFP-SYP71 was detected mainly in the PM of epidermal cells of root (A, B, and C), primordial region of the lateral root (D), developing lateral root (E), leaf (F), and in a young developing seed (G). Close observation of the dividing region of the root tip revealed *GFP* fluorescence was also observed in the ER in addition to the PM (A inset) and the endosomes as punctate structures (B). When the cells were treated with Brefeldin A (BFA), the punctate structures became aggregated and formed the BFA compartments (arrowheads in C). Clear ER structure was also detected in the dividing cells of primordial region of the lateral root (D) and in the young developing seed (G). Scale bar is 10 μm .

of various tissues (Fig. 4; Supplemental Fig. 2 B, D, and E). Additionally, the ER localization of GFP-SYP71 was obviously detected in the dividing regions including root tip (Fig. 4A inset, Supplemental Fig. 2A), lateral root primordial (Fig. 4D; Supplemental Fig. 2C), immature seed (Fig. 4G), and epidermis of ovules (Supplemental Fig. 2H). The ER localization of SYP71 of these tissues disappeared during cell maturation. These data suggested that SYP71 is mainly localized on the PM of the mature cells and is also localized on the ER in addition to the PM of the young cells under division or growth. Intriguingly, a closer observation revealed that SYP71 was localized not only on the PM but also in the punctate structures (Fig. 4B). When the cells were treated with Brefeldin A (BFA), which inhibits trafficking from the endosomes to the PM, the punctate structures aggregated and formed the so-called BFA compartment (Fig. 4C, arrowheads). These features were reminiscent of the endosomes.

Discussion

SYP71 showed predominant expression in vegetative tissues, but almost no expression was observed in reproductive tissues such as pollen and embryos. *SYP72* was specifically expressed at a late stage of pollen development, and *SYP73* was strongly expressed in an early stage of pollen and embryo maturation. These data suggest that each SYP7 protein functions in a specific stage during plant development with little expression redundancy. In fact, no homozygous T-DNA insertion mutant of *SYP71* was isolated from 123 progenies of *SYP71/syp71* heterozygote (data not shown), indicating that the function of *SYP71* in the vegetative tissues is essential for plant growth and development.

We demonstrated in this study that SYP71 was mainly localized on the PM/endosomes by a comparison of the subcellular localization obtained from different approaches including membrane fractionation analysis, and observations of transgenic plants expressing GFP-SYP7s driven by the native promoter.

In animals and yeasts, the PM-localized Qa-SNAREs form a SNARE complex by interacting with SNAP-25s (Qb/Qc-SNAREs) and the R-SNAREs called VAMPs or brevins (Bock *et al.*, 2001). In plants, a SNAP-25 homologue, AtSNAP33 (Qb/Qc-SNARE), interacts with the Qa-SNARE, SYP111 (KNOLLE) during cell plate formation (Heese *et al.*, 2001) or SYP121 in plant immune responses or general secretion event (Kwon *et al.*, 2008; Geelen *et al.*, 2002; Tyrrell *et al.*, 2007). Yet a plant-specific Qb-SNARE (NPSN11) also forms a SNARE complex with SYP111 (KNOLLE) (Zheng *et al.*, 2002), although it is not clear which Qc- and R-SNAREs are involved in this SNARE complex.

Our previous localization studies of Arabidopsis SNAREs have shown that 9 Qa-SNAREs, 5 VAMPs (R-SNARE), 3

Qb-SNAREs (NPSN11, NPSN12 and NPSN13) and 3 SNAP-25s (SNAP29, SNAP30 and SNAP33) are located on the PM, but no Qc-SNARE had been identified on the PM before the present study (Uemura *et al.*, 2004). In the present study, we show that SYP71, which is a type of Qc-SNAREs, exists in the PM, and that they might form a SNARE complex with the plasma-membrane Qa-SNAREs (SYP11, 12, or 13), NPSN-type Qb-SNAREs and VAMP 72-type R-SNAREs. Namely, two types of SNARE complex could be formed on the plant PM: one might be a conventional SNARE complex consisting of Qa-SNARE, SNAP-25 and R-SNARE, and the other might be a plant-specific SNARE complex consisting of Qa-SNARE, NPSN1s (Qb), SYP7s (Qc) and R-SNARE. Recent studies have clearly shown that the recycling pathways between the endosomes and the PM are essential for the polar localization of an auxin efflux carrier, PIN1, for polar auxin transport (Geldner *et al.*, 2001, Geldner *et al.*, 2003) and for the cell plate localization of KNOLLE (SYP111) for cytokinesis (Jürgens, 2004; Lukowitz *et al.*, 1996, Lauber *et al.*, 1997). These two different types of PM-SNARE complexes may confer a complexity upon the-membrane traffic to the PM to be involved in various membranes trafficking to the PM in higher plants.

Intriguingly, SYP71 is also localized on the ER membrane of dividing cells, and this ER localization pattern of SYP71 at the dividing cells raises the question of why SYP71 shows a dual localization pattern both to the ER and the PM at the dividing cells stage.

We have previously shown that SYP71 is localized to the ER membrane in transient expression condition (Uemura *et al.*, 2004). This ER-localization might be due to an overexpression effect of strong 35S promoter. However, we could also observe the ER-localization of SYP71 in dividing cells even though SYP71 was expressed under the control of the native promoter. Furthermore, SYP71 is detected both in the ER and the PM membrane fractions of root tissues. These data indicate that authentic SYP71 localizes on the ER in dividing cells.

In yeast, Ufe1p (Qa), Sec20p (Qb), Use1p (Qc) and Sec22p (R) forms the SNARE complex on the ER membrane, involved in the retrograde transport to the ER (Burri *et al.*, 2003; Dilcher *et al.*, 2003). According to Sanderfoot (2007), the counterparts of the components of the ER SNARE complex are found in the Arabidopsis genome: SYP8 (Qa-SNARE, Ufe1p ortholog), AtSec20 (Qb-), AtUse1 (Qc-) and At Sec22 (R-), suggesting that the retrograde transport pathway to the ER is highly conserved across the eukaryotic kingdom. In plants, in addition to this conventional ER-resided SNARE complex, there might be an additional SNARE complex including SYP71 as another Qc-SNARE of the ER SNARE complex in order to confer complexity or flexibility to the membrane traffic during cell division.

The dominant negative SNARE fragment (Sp2) of

SYP71 inhibits the secretion of a fluorescent secretion marker, secGFP, suggesting that SYP71 functions in the membrane traffic to the PM as a PM-Qc-SNARE (Tyrrell *et al.*, 2007). Interestingly, the expressions of Sp2 fragment of SYP71 as well as the Sp2 fragments of SYP121 and SYP122 caused the ER-retention of the secretion marker. It is unknown why the Sp2 fragments of PM-localized SNAREs causes the ER-retention of the secretion marker. However, this observation might suggest that PM-resident SNAREs influence the transport pathway from the ER.

Generally, SNARE molecules are localized predominantly to specific subcellular compartment in order to achieve specific membrane trafficking processes. However, in order for the membrane fusion process to continue to cycle between the transport vesicles and the target membranes, it is necessary for SNAREs to be returned to their donor compartments via recycling pathways. Consequently, SNAREs reside not only in the target organelles, but also reside in the donor organelles (Jahn and Scheller, 2006). The dual localization pattern of SYP71 seen in the present study might be such a case, namely, SYP71 might be involved in the direct transport pathway from the ER to the PM.

In mammalian cells, the ER membranes are directly in contact with the PM and phagosomes during phagocytosis (Gagnon *et al.*, 2002). This membrane-traffic process is mediated by an ER-localized SNARE protein called syntaxin 18 (Hatsuzawa *et al.*, 2000). In transgenic Arabidopsis seed cells, the recombinant single-chain Fv-Fc antibodies are transported directly from the ER to the periplasmic space (Van Droogenbroeck *et al.*, 2007). The SYP71 might be involved in a similar direct pathway from the ER to the PM at least in certain condition/type of plant cells, such as rapidly elongating or dividing cells. Of course, we could not completely exclude the possibility that we just only observed the transient localization of SYP71 at the ER membrane on the way to the final destination. Nonetheless, we have never observed the ER localization of other PM-resident SNAREs under the transient and/or the transgenic expression conditions (Uemura *et al.* 2004 and our unpublished data).

The other possible explanation of this localization pattern is that SYP71 is involved in more than one fusion step within one type of cell. It was reported that Vti1 (Fisher von Mollard and Stevens, 1999), Sed5 (Tsui and Banfield, 2000) and VAMP8 (Antonin *et al.*, 2000; Wang *et al.*, 2004) function in multiple membrane fusion steps in yeast and mammals. SYP71 might form distinct SNARE complexes on the PM and the ER. Further experiments are needed to confirm the precise function of SYP71 in the complex membrane traffic in higher plants.

Acknowledgements. We thank Ms. Y. Hori for her technical assistance. We also thank Drs. Y. Niwa and K. Matsuoka for their kind gifts of vectors. We also thank Dr. Y. Kasahara and Dr. A. Nakano for providing

the *Oryza sativa* plasma membrane H⁺-ATPase and AtSar1 antibodies, respectively. This work was supported in part by grants from the Japanese Ministry of Education, Culture, Sport, Science and Technology, a grant-in-aid for Basic Science Research (C) and a grant-in-aid for Scientific Research on Priority Areas (MHS), as well as by a grant from the Yamada Science Foundation (MHS): a grant-in-aid for JSPS Research Fellowships for Young Scientists (TU). INS was also recipient to a scholarship from the Japanese Ministry of Education, Culture, Sport, Science and Technology.

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(Received for publication, July 18, 2008, accepted, September 9, 2008
and published online, October 1, 2008)