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Novel type aquaporin SIPs are mainly localized to the ER membrane and show cell-specific expression in *Arabidopsis thaliana*

Fumiyoshi Ishikawa[a], Shinobu Suga[a], Tomohiro Uemura[b], Masa, H. Sato[b], Masayoshi Maeshima[a,*]

[a] Laboratory of Cell Dynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan, and

[b] Graduate School of Human and Environmental Studies, Kyoto University, Kyoto 606-8501, Japan

*Corresponding author: Masayoshi Maeshima, Fax: 81-52-789-4096

E-mail address:.maeshima@agr.nagoya-u.ac.jp

Abstract **We investigated the fourth subgroup of *Arabidopsis* aquaporin, small and basic intrinsic proteins (SIPs). When they were expressed in yeast, SIP1;1 and SIP1;2, but not SIP2;1, gave water-channel activity. The transient expression of SIPs linked with green fluorescent protein in *Arabidopsis* cells and the subcellular fractionation of the tissue homogenate showed their ER localization. The SIP proteins were detected in all of the tissues, except for dry seeds. Histochemical analysis of promoter-β-glucuronidase fusions revealed the cell-specific expression of SIPs. SIP1;1 and SIP1;2 may function as water channels in the ER, while SIP2;1 might act as an ER channel for other small molecules or ions.**

Key words: Aquaporin; ER; SIP; Arabidopsis; Water channel

Abbreviations: AHA3, *Arabidopsis* plasma membrane H^+-ATPase 3; Bip, binding protein; GFP, green fluorescent protein; GUS, β-glucuronidase; NIP, Nod26-like intrinsic proteins; PIP, plasma membrane intrinsic protein; SIP, small and basic intrinsic protein; TIP, tonoplast intrinsic protein; VHA, vacuolar H^+-ATPase.

1. Introduction

Water is a ubiquitous and indispensable molecule. Its transport across the membranes of various organisms is facilitated by aquaporins [1, 2]. Aquaporins that are located on the plasma membranes of animal cells have been extensively investigated and shown to be involved in the transcellular transport of water. Knockout experiments have revealed that some such aquaporins are essential for physiological activities of cells, while many others are not [1].

In plants, aquaporins are thought to be involved in plant growth and water relations [see reviews in 3, 4]. Plant aquaporins are present in various tissues, and play a role in water transport, cell differentiation and cell enlargement. Some are expressed in differentiated cells, such as motor cells [5, 6], guard cells [7] and reproductive organs [8, 9]. Studies with mutant aquaporins have demonstrated the involvement of these proteins in plant water relations [10]. In addition, the expression of aquaporins in response to various environmental stresses and

hormones has been reported in several plant species [3, 4]. Furthermore, some types of plant aquaporin have been shown to transport not only water but also various small molecules, including glycerol, urea, ammonia and CO_2 [3, 11-15], similar to animal and bacterial aquaporins [16-18].

Plant aquaporins comprise a large protein family. *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) and *Zea mays* have 35 and 33 aquaporin-encoding genes, respectively [19, 20]. Plant aquaporins have been classified into four major subfamilies, which are referred to as plasma-membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nod26-like intrinsic proteins (NIPs), and small and basic intrinsic proteins (SIPs). PIPs and TIPs have been extensively studied, whereas information about SIPs and NIPs is limited.

Arabidopsis has three SIPs, which are the smallest group of plant aquaporins. In the present study, we focused on these proteins using two independent approaches; namely, examining the expression of fusions of the SIPs with green fluorescent protein (GFP) in suspension-cultured cells, and immunoblotting the fractionated tissue homogenates. All of the SIP proteins were present in the ER, but were not detected in the plasma or vacuolar membranes. To our knowledge, this is the first report on ER membrane aquaporins. Furthermore, the SIPs were expressed in a tissue- and cell-specific manner. By expressing the SIPs in yeast and measuring the water-channel activity of membrane vesicles by stopped-flow spectrophotometry, we found that SIP2;1 could be distinguished from SIP1;1 and SIP1;2.

2. Materials and methods

2.1. Plant materials

Seeds of *Arabidopsis* ecotype Columbia 0 were germinated on sterile MS-salt plates and grown at 22 ˚C under continuous light. In some cases, plants germinated on agar plates were grown in vermiculite pots under continuous light. *Arabidopsis* (Columbia 0) suspension-cultured cells (also known as 'Deep' cells) were a kind gift from Dr. Masaaki Umeda of Tokyo University, Japan. The cells were cultured at 22 ˚C dark in Murashige-Skoog medium under dark conditions [21].

2.2. Subcellular fractionation

The stems of 6-week-old *Arabidopsis* plants were used to prepare membrane fractions. The tissues were homogenized in a medium containing 50 mM Tris-acetate (pH 7.5), 250 mM sorbitol, 2 mM EGTA, 2 mM dithiothreitol and 20 μM *p*-(amidinophenyl) methanesulfonyl fluoride hydrochloride. The homogenate was filtered and centrifuged at 10,000×*g* for 10 min. The supernatant was centrifuged at 100,000×*g* for 30 min and the pellet was suspended in Tricine-KOH (pH 7.5), 5% (w/w) sucrose, 1 mM EGTA and 2 mM EDTA. The crude membrane suspension (5 mg, 0.30 ml) was layered on a sucrose-density gradient (10.4 ml, 15 to 45%), centrifuged at 77,000×*g* for 19 h in a swing rotor, and collected in 0.45-ml fractions. The sucrose solution consisted of 10 mM Tricine-KOH (pH 7.5), 1 mM EGTA, and 2 mM EDTA.

2.3. Preparation of isoform-specific antibodies

For antibody production, we synthesized peptides corresponding to an internal region of *Arabidopsis* SIP1;1 (positions 109 to 122, MEFIPEKYKHMIGG) and the C-terminal region of SIP2;1 (positions 223 to 237, KPLTEEQEKPKAKSE). These peptides were linked with carrier protein and injected into rabbits. Antibody to *Arabidopsis* γ-TIP (TIP1;1) was prepared by injecting an authentic peptide (the 20 N-terminal residues) into a rabbit. The peptides and antibodies were prepared by Operon Biotechnology (Tokyo, Japan). The anti-peptide

antibodies to RsPIP1 (sequence of the antigen peptide, GKEEDVRVGANKFPERQPIGTSA) and RsPIP2;1 (MAKDVEAVSGEGFQTRDYQDP) were prepared as described previously [22]. Anti-peptide antibodies to plasma membrane H^+-ATPase (AHA3, TISKDRVKPSPTPDS), subunit-a of vacuolar H^+-ATPase (VHA-a, ELVEINANNDKLQRSYNEL) and the ER luminal binding protein (BiP, SKDNKALGKLRRE) of *Arabidopsis* have been described elsewhere [21]. Protein samples were subjected to SDS-PAGE and immunoblotting. The blots were visualized with horseradish peroxidase-coupled protein A and Western blotting detection reagents (Amersham Biosciences).

2.4. Expression of Arabidopsis aquaporins in yeast

*Kpn*I-*Sal*I and *Eco*RI-*Sal*I fragments of aquaporin cDNA (SIP1;1, *Kpn*I-*Sal*I; SIP1;2 and SIP2;1, *Eco*RI-*Sal*I) were inserted into the yeast expression vector pKT10 [23, 24]. The resulting plasmids were introduced into *Saccharomyces cerevisiae* strain BJ5458 (*Mat a*, *ura3-52*, *trp1*, *lys2-801*, *leu2Δ1*, *his3Δ200*, *pep4::HIS3*, *prbΔ1.6R can1* and *GAL*), which is deficient in the major vacuolar proteases. Ura^+ colonies were selected and crude membranes were prepared from yeast cultured for 16 h at 30 °C [25]. They were suspended in 50 mM Tris-HCl (pH 7.5), 0.3 M sorbitol, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml peptstatin, and used for testing antibody specificity.

2.5. Determination of the osmotic water permeability of membranes

Yeast cells expressing *Arabidopsis* aquaporin were cultured for 14 h at 30 °C and collected by centrifugation at 4500×*g* for 5 min. Crude membrane fraction was prepared from yeast and suspended in 20 mM Tris-HCl (pH 7.2), 450 mM mannitol, 90 mM KCl and 1 mM EDTA as described previously [24, 25]. The osmotic water permeability of membranes was measured with a stopped-flow spectrophotometer (Model SX18MV, Applied Photophysics) at 20 °C, as described previously [25]. Yeast membrane vesicles (0.5 mg/ml) in the 0.45 M mannitol buffer were mixed rapidly with an equal volume of 0.1 M mannitol solution in the same buffer. Initial rate constants in the first 20 msec were calculated from more than 9 independent experiments.

2.6. Transient transformation of Arabidopsis cultured cells with GFP-tagged SIP

In order to prepare GFP fusions, SIP cDNAs were generated by PCR using KOD plus DNA polymerase and a pENTR Directional TOPO Cloning Kit (Invitrogen). An artificial sequence, CACC, was added to the 5′ terminuses of the SIP cDNAs. The DNA constructs obtained were inserted into a Gateway vector, New-pUGW5 (developed by Dr. Tsuyoshi Nakagawa), which contains GFP DNA, using the pENTR Directional TOPO Cloning Kit (Invitrogen). These plasmids were transiently expressed in *Arabidopsis* suspension-cultured cells as described previously [26]. Cells cultured for 5 days were incubated in enzyme solution for 80 min at 30 ˚C with gentle agitation to prepare the protoplasts and then passed through a nylon mesh (pore size, 41 μm). Protoplasts containing the plasmid were incubated at 23 ˚C for 16 h in the dark. Transformed cells were viewed using a confocal laser microscope (LSM510 META, Zeiss) [26].

2.7. Promoter-GUS constructs and histochemical analysis

Genomic sequences for SIP1;1 (-2,012 to +18 from the fist ATG), SIP1;2 (-1,997 to +12) and SIP2;1 (-1,937 to +12) were isolated from genomic DNA by PCR using KOD plus. The resulting fragments were digested with *Xba*I and ligated into the *Xba*I–*Sma*I site of the binary vector pGWB203 (developed by Dr. Tsuyoshi Nakagawa), which contains the DNA sequence for β-glucuronidase (GUS), in order to produce a translational fusion product. The chimeric

constructs were introduced into *Agrobacterium tumefaciens* strain GV3101::PM90 by electroporation and used to transform *Arabidopsis* plants. Transformants were selected on plates containing 40 μg/ml Benlate (Sumitomo Chemical Co.), 0.20 μg/ml Cefotax (Chugai Pharmaceutical Co.), 20 μg/ml hygromycin, and 30 μg/ml kanamycin.

T_1 plants were used for the GUS analysis. Plant tissues were incubated in 1.92 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Re(CN)_6$ and 0.3% (w/v) Triton X-100 at 37°C until blue histochemical staining was confirmed (6 to 24 h). The stained tissues were incubated sequentially in 30, 50 and 70% ethanol for 1 h, followed by further incubation in 99.5% ethanol for 24 h. In some experiments, thin sections of the stained tissues were cut.

3. Results

3.1. Expression of Arabidopsis SIP Proteins in Yeast

Arabidopsis SIP1;1, SIP1;2 and SIP2;1 have 240, 243 and 237 amino acid residues, respectively. SIP1;1 and SIP1;2 share 69.6% identity. SIP1;1 and SIP2;1, and SIP1;2 and SIP2;1 are 25.7 and 25.3% identical, respectively. To express SIP aquaporins in yeast, we prepared three constructs (Fig. 1A) and transformed them into *S. cerevisiae* strain BJ5458, which lacks endogenous functional aquaporin [25]. To detect the translation products in the yeast transformants, we prepared antibodies against an internal segment of SIP1;1 and the C-terminal region of SIP2;1. Antibody to SIP1;1 recognized both SIP1;1 and SIP1;2, and was named anti-SIP1s. Anti-SIP2;1 recognizes its own antigen but not SIP1s (data not shown). Each SIP protein was detected as a band of around 27 kDa in yeast crude membrane fractions (Fig. 1B). Immunocompetition with the corresponding peptide confirmed the specificity of the antibodies.

The SIP proteins were examined in a large-scale preparation of yeast membranes (Fig. 2A). Two immunostained bands corresponding to the monomeric (30 kDa) and dimeric (55 kDa) forms of radish aquaporin RsPIP2;2 were seen after SDS-PAGE despite the presence of detergent; however, SIP1s and SIP2;1 gave only single monomer bands. The empty vector gave no reactive band.

Membrane vesicles prepared from the yeast cells expressing SIPs were assayed for osmotic water channels by stopped-flow light-scattering spectrophotometry. The swelling rate of vesicles in the hypotonic solution was monitored by the decrease in light scattering. The vector control showed a slow influx of water into the membrane vesicles (Fig. 2, B and C). SIP1;1 and SIP1;2 enhanced the permeability by 170 and 200%, respectively, while radish aquaporin RsPIP2;2 showed the highest activity [25]. By contrast, SIP2;1 did not enhance the permeability. This effect was not due to the absence of SIP2;1 in the membranes, as its presence was confirmed by immunoblotting (Fig. 2A).

3.2. Detection of SIP-GFP fusion proteins on the ER membrane

In order to determine their subcellular location, we prepared six fusion constructs of the SIPs with GFP (Fig. 3). These constructs were transiently expressed in *Arabidopsis* suspension-cultured cells, because they are efficiently transformed by the polyethylene glycol method [26]. With both the GFP-SIP1;1 and SIP1;1-GFP constructs (Fig. 3, A and B), the green fluorescence pattern had a tubular/reticular shape in the cells, whereas the fluorescence of free GFP was dispersed throughout the cytosol (Fig. 3I). The fluorescence patterns of SIP1;2 and SIP2;1 tagged with GFP at both the N- or C-termini also had tubular/reticular/sheet shapes (Fig. 3, C to F). This shape is typical of the plant ER, especially in suspension-cultured cells [27]. In a similar experimental system, AtSec22 and AtSYP81

were shown to be localized to the ER membrane [26]; the fluorescence patterns of the GFP-tagged SIPs were identical to those of AtSec22 and AtSYP81 (Fig. 3, G and H). The present results therefore strongly suggest that the three SIP isoforms are located in the ER membrane.

3.4. Subcellular localization of SIPs in plant tissues

To examine the subcellular location of SIP proteins without tags, crude membrane fractions were prepared from homogenates of the stems of 6-week-old *Arabidopsis* plants and subjected to equilibrium sucrose density-gradient centrifugation (Fig. 4). Plasma membrane H^+-ATPase (AHA3) was recovered in fractions 15 to 17. The subunit-a of vacuolar H^+-ATPase (VHA-a) was detected in fractions 3 to 8. In the presence of Mg^{2+} (Fig. 4A), SIP1 and SIP2;1 were recovered in fractions 11 to 16, together with the ER luminal protein BiP. When the membrane-bound ribosomes were removed from the rough ER by treatment with EDTA (Fig. 4B), the SIP peak moved to lower-density fractions 4 to 10, as did the BiP peak. The release of ribosomal proteins, such as L13 and S13, by the treatment with EDTA was confirmed by immunoblotting with their antibodies (data not shown). These results support the ER localization of the SIP proteins.

3.5. Levels of SIP proteins in different organs

We quantified the relative amounts of SIP proteins in various organs immunochemically, as shown in Figure 5. Both SIP1;1 and SIP1;2 were detected in all of the organs, except for in dry seeds. Substantial levels of SIP2;1 protein were present in the membranes of 2-week-old roots and in the flowers. Germinating seedlings contained SIP1s and SIP2;1, but no signal for the SIPs was detected in the dry seeds. The levels of PIP1 and TIP1 in the various organs were similar to those in the radish plant [22]. It should be noted that the suspension-cultured *Arabidopsis* cells (Deep cells), which were used for expression of GFP-SIP fusion proteins, accumulated SIPs and SIP2;1, but not PIP1s or TIP1;1.

3.6. Cell-specific expression of SIP genes

We performed promoter-GUS analysis of the three SIP genes in order to examine their tissue-specific expression. Constructs containing the putative promoter regions (about 2 kb in length) were fused in-frame with the GUS gene. *Arabidopsis* plants were grown for 2 weeks and stained for GUS. The pattern of GUS staining varied with the isogenes. There was strong GUS activity from the SIP1;1 construct in the roots and rosette leaves (Fig. 6A). Within the latter, activity was especially high in trichome cells (Fig. 6, B and C). GUS activity from SIP1;2 was observed particularly in the cotyledons (Fig. 6H), as well as in the minor veins and hydathode cells of the rosette leaves (Fig. 6, I and J). Strong staining was also observed in the cotyledons of plantlets expressing the SIP2;1::GUS construct (Fig. 6O). In contrast to SIP1;2, this construct gave rise to strong activity in the main veins of the rosette leaves (Fig. 6P). For roots, GUS activity was especially high in the dividing cells and elongating regions of the root tips (Fig. 6, D, K and Q), and in the emerging lateral roots (Fig. 6R). GUS activity was, in all cases, detected in the stele, which is the central vascular cylinder of the roots (for example, see Fig. 6R).

GUS activities in the flowers and siliques prepared from 7-week-old plants are also shown. For SIP1;1 (Fig. 6, E and F), strong GUS activity was detected in the vascular tissues of the flower petal, stigma, stamens (anthers and filaments) and pollen. In the siliques, the top and bottom (receptacle) were also strongly stained (Fig. 6G). For SIP2;1 (Fig. 6, S, T and U), the same tissues were stained, although the intensity was weaker than for SIP1;1. In contrast to these two genes, weak GUS activity from SIP1;2 was detected in the filaments of the stamens, the upper part of the styles, the vascular tissue of the petals, and the receptacles of the siliques, but not in the anthers or pollen (Fig. 6, L, M and N).

4. Discussion

A stopped-flow spectrophotometric assay of the membrane vesicles from yeast cells harboring one or other of the three *Arabidopsis* SIPs revealed that SIP1;1 and SIP1;2 had water-channel activity (Fig. 2), whereas SIP2;1 did not, although the protein was clearly present in the membrane vesicles. At the present we could not exclude a possibility that interaction of SIP2;1 with SIP1;1 or SIP1;2 modulates the water channel activity of SIP2;1, since it has been reported that heteromerization of maize PIPs altered the water channel activity [28].

Several PIP proteins have been shown to be localized to plasma membranes [29]. Members of the TIP subfamily have been reported to be located in vacuolar membranes, and have been examined further with respect to the functional and morphological heterogeneity of the plant vacuolar system, which comprises the central vacuole, protein storage vacuole, tannin vacuole and prevacuolar compartments. Several groups have reported the specific localization of particular TIP isoforms to individual specialized vacuoles. These studies raise the possibility that TIPs reflect the identity of particular types of vacuole [30, 31]. However, in contrast to the PIP and TIP subfamilies, the subcellular location of NIPs and SIPs has not been reported.

The present study was carried out in order to determine the intracellular location of three SIP isoforms in *Arabidopsis*. Two experiments demonstrated the main localization of the SIPs in the ER membranes. Firstly, both N- and C-terminal GFP-SIP fusions were located in the ER membrane (Fig. 3). The ER consists of a three-dimensional network of continuous tubules and flattened sacs, which underlies the plasma membrane, courses through the cytoplasm and connects to the nuclear envelope, but remains distinct from the plasma membrane [32]. No SIP-GFP fluorescence was detected in the nuclear envelope, plasma membrane, vacuolar membrane, Golgi bodies or plastids (Fig. 3).

The ER of plant cells is a highly differentiated organelle, which contains a large number of discrete structural domains, including the vacuole-attachment domain, plasma membrane-anchoring domain, nuclear envelope-ER gate, transitional ER, rough ER, smooth ER and protein body ER [32]. Despite this morphological diversity, all ER membranes are physically linked and enclose a single continuous lumen. GFP fluorescence was observed in the ER underlying the plasma membrane, which is known as the cortical ER, the ER surrounding the nuclei, and the ER pervading the cytoplasm. The flat lamellar cisternae are known to correspond to the rough ER domain, while the tubular cisternae coincide with the smooth ER region [32]. The fluorescence images of the SIPs, especially SIP2;1, seem to reflect the distribution of the flat lamellar cisternae. Hence, the SIPs might be located in the rough ER.

The ER localization of the SIP proteins was confirmed by sucrose density-gradient fractionation of tissue homogenates (Fig. 4). Recently, immunohistochemical and biochemical analyses have revealed that the inner mitochondrial membrane of rat hepatocytes contains the Hg^{2+}-sensitive aquaporin-8 [33]; this has been suggested to be involved in the rapid expansion of mitochondria under normal conditions and during apoptosis. We did not detect SIP proteins in either the mitochondria or the plastids. SIP1s and SIP2;1 co-sedimented with the ER luminal protein BiP in the presence of Mg^{2+}, and were displaced to a lighter fraction when the polysomes were removed by EDTA. It should be noted that a part of the SIP1s was detected in the lighter fractions (8 and 9 in Fig. 4A) in addition to fractions 12 to 16, suggesting the presence of SIP1s in the rough ER and other ER subdomains. To our knowledge, the present study is the first demonstration of the ER localization of any aquaporin. Techniques with higher resolving power are needed to determine the precise location of the SIP proteins in the

ER.

The SIP1s and SIP2;1 were detected in all of the tissues and suspension-cultured cells tested, although their levels differed (Fig. 5 and 6). In the leaves, SIP1;1 was expressed in the trichomes, SIP1;2 was expressed in the minor veins and hydathode, and SIP2;1 was expressed in the main leaf veins. In *Arabidopsis,* trichomes are single large cells that provide physical protection against herbivores. They also contain a wide variety of secondary metabolites, which are toxic and cause allergic and irritant responses in herbivores. In the trichomes, the ER might be related to the biosynthesis of the thick cell wall and of secondary metabolites.

Our results showed that both SIP1s have water-channel activity, whereas SIP2;1 does not. As shown in Figure 3, the ER appears as a sheet-like network that extends throughout the cytoplasm surrounding the vacuoles. These sheet-like membranes might be barriers to intracellular water transport, similar to giant vacuoles. It is possible that the water channels facilitate water transport across the double sheets of the ER membranes and so promote intercellular water movement. Another possibility is that the SIP1s facilitate the export of water from the ER lumen to maintain the sheet- or cisternae-like shape.

In the roots, the three SIP isogenes were highly expressed in the differentiated and elongating regions (Fig. 6). Our GUS-promoter analysis also revealed strong expression in the central cylinder, which consists of the pericycle, cambium, metaxylem, protoxylem and xylem parenchyma cells. The central cylinder is the main waterway, and a dense distribution of SIPs within it might permit the rapid dispersion of water from the phloem to the outside cortex. In the flowers, SIP1;1 and SIP2;1 were expressed in the stigma of the carpel, as well as the petals and pollen (Fig. 6). The results suggest the involvement of SIPs in seed maturation and fertilization.

The present study firstly demonstrates the presence of the ER localized aquaporins. The actual physiological functions of the SIP proteins remain to be determined. The knockout and overexpression of these genes should provide insights into their roles. In addition, the specific substrate of each SIP protein must be established. Recently, several substances (such as glycerol, urea, CO_2, NH_3, NO and purine) have been reported to act as transport substrates of aquaporins in various organisms [3, 11-18, 34]. It has been proposed that plant SIP aquaporins belong to the subfamily referred to as super-aquaporins, similar to the human aquaporins AQP11 and AQP12 [35]. It is possible that the SIPs are involved in regulating the volume and morphology of the ER lumen, and the concentration of ions within it. Single-cell organisms possess no members of this super-aquaporin subfamily. Thus, SIPs and related super-aquaporins may be involved in the cell differentiation and/or intercellular communication through plasmodesmata in plants. Further investigation in conjunction with the present results of this unique aquaporin SIP subfamily should provide insights into the functions of aquaporins in the ER.

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References

[1] King, L.S., Kozono, D. and Agre, P. (2004) From structure to disease: the evolving tale of

aquaporin biology. Nature Rev. Mol. Cell Biol. 5, 687-698.
[2] Takata, K., Matsuzaki, T., and Tajika, Y. (2004) Aquaporins: water channel proteins of the cell membrane. Prog. Histochem. Cytochem. 39, 1-83.
[3] Maurel, C., Javot, H., Lauvergeat, V., Gerbeau, P., Tournaire, C., Santoni, V. and Heyes, J. (2002) Molecular physiology of aquaporins in plants. Int. Rev. Cytol. 215, 105-148.
[4] Tyerman, S. D., Niemietz, C.M. and Bramley, H. (2002) Plant aquaporins: multifunctional water and solute channels with expanding roles. Plant Cell Environ. 25, 173-194.
[5] Moshelion, M., Becker, D., Biela, A., Uehlein, N., Hedrich, R., Otto, B., Levi, H., Moran, N. and Kaldenhoff, R. (2002) Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. Plant Cell 14, 727-739.
[6] Frangne, N., Maeshima, M., Schäffner, A. R., Mandel, T., Martinoia, E. and Bonnemain, J. L. (2001) Expression and distribution of vacuolar aquaporin in young and mature leaf tissues of *Brassica napus* in relation to water fluxes. Planta 212, 270-278.
[7] Sarda, X., Tousch, D., Ferrare, K., Legrand, E., Dupuis, J. M., Casse-Delbart, F., and Lamaze, T. (1997) Two TIP-like genes encoding aquaporins are expressed in sunflower guard cells. Plant J. 12, 1103-1111.
[8] Bots, M., Feron, R., Uehlein, N., Weterings, K., Kaldenhoff, R. and Mariani, T. (2005) PIP1 and PIP2 aquaporins are differentially expressed during tobacco anther and stigma development. J. Exp. Bot. 56, 113-121.
[9] O'Brien, M., Bertrand, C. and Matton, D.P. (2002) Characterization of a fertilization-induced and developmentally regulated plasma-membrane aquaporin expressed in reproductive tissues, in the wild potato *Solanum chacoense* Bitt. Planta 215, 485-493.
[10] Ma, S., Quist, T.M., Ulanov, A., Joly, R. and Bohnert, H.J. (2004) Loss of TIP1;1 aquaporin in *Arabidopsis* leads to cell and plant death. Plant J. 40, 845-859.
[11] Gaspar, M., Bousser, A., Sissoëff, I., Roche, O., Hoarau, J. and Mahé, A. (2003) Cloning and characterization of *ZmPIP1-5b*, an aquaporin transporting water and urea. Plant Sci. 165, 21-31.
[12] Hanba, Y. T., Shibasaka, M., Hayashi, Y., Hayakawa, T., Kasamo, K., Terashima, I. and Katsuhara, M. (2004) Overexpression of the barley aquaporin HvPIP2;1 increases internal CO_2 conductance and CO_2 assimilation in the leaves of transgenic rice plants. Plant Cell Physiol. 45, 521-529.
[13] Loqué, D., Ludewig, U., Yuan, L. and von Wirén, N. (2005) Tonoplast intrinsic proteins AtTIP2;1 and AtTIP2;3 facilitate NH_3 transport into the vacuole. Plant Physiol. 137, 671-680.
[14] Niemietz, C.M. and Tyerman, S. D. (2000) Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules. FEBS Lett. 465, 110-114.
[15] Uehlein, N., Lovisolo, C., Siefrits, F. and Kaldenhoff, R. (2003) The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. Nature 425, 734-737.
[16] Ikeda, M., Bitz, E., Kozono, D., Guggiono, W.B., Agre, P. and Yasui, M. (2002) J. Biol. Chem. 277, 39873-39879.
[17] Liu, K., Kozono, D., Kato, Y., Agre, P., Hazama, A. and Yasui, M. (2005) Conversion of aquaporin 6 from an anion channel to a water-selective channel by a single amino acid substitution. Proc. Natl. Acad. Sci. U.S.A. 102, 2192-2197.
[18] Yasui, M., Hazama, A., Kwon, T. H., Nielsen, S., Guggino, W. B. and Agre, P. (1999) Rapid gating and anion permeability of an intracellular aquaporin. Nature 402, 184-187.
[19] Chaumont, F., Barrieu, F., Wojcik, E., Chrispeels, M. J. and Jung, R. (2001) Aquaporins constitute a large and highly divergent protein family in maize. Plant Physiol. 125, 1206-1215.
[20] Johanson, U., Karlsson, M., Johansson, I., Gustavsson, S., Sjövall, S. Fraysse, L., Weig, A. R. and Kjellbom, P. (2001) The complete set of genes encoding major intrinsic proteins

in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol. 126, 1358-1369.
[21] Kobae, Y., Uemura, T., Sato, M. H., Ohnishi, M., Mimura, T. and Maeshima, M. (2004) Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. Plant Cell Physiol. 45, 1749-1758.
[22] Suga, S., Imagawa, S. and Maeshima, M. (2001) Specificity of the accumulation of mRNAs and proteins of the plasma membrane and tonoplast aquaporins in radish organs. Planta 43, 294-304.
[23] Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K. and Toh-e, A. (1990) IRA2, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein. Mol. Cell Biol. 10, 4303-4313.
[24] Nakanishi, Y., Saijo, T., Wada, Y. and Maeshima, M. (2001) Mutagenic analysis of functional residues in putative substrate-binding site and acidic domains of vacuolar H^+-pyrophosphatase. J. Biol. Chem. 276, 7654-7660.
[25] Suga, S. and Maeshima, M. (2004) Water channel activity of radish plasma membrane aquaporins heterologously expressed in yeast and their modification by site-directed mutagenesis. Plant Cell Physiol. 45, 823-830.
[26] Uemura, T., Ueda, T., Ohniwa, R. L., Nakano, A., Takeyasu, K. and Sato, M.H. (2004) Systematic analysis of SNARE molecules in *Arabidopsis*: Dissection of post-Golgi network pathways in plants. Cell Struc. Func. 29, 49-65.
[27] Takeuchi, M., Ueda, T., Sato, K., Abe, H., Nagata, T. and Nakano, A. (2000) A dominant negative mutant of sar1 GTPase inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus in tobacco and Arabidopsis cultured cells. Plant J. 23, 517-525.
[28] Fetter, K., Wilder, V.V., Moshelion, M. and Chaumont, F. (2004) Interactions between plasma membrane aquaporins modulate their water channel activity. Plant Cell 16, 215-228.
[29] Luu, D-T. and Maurel, C. (2005) Aquaporins in a challenging environment: molecular gears for adjusting plant water status. Plant Cell Environ. 28, 85-96.
[30] Neuhaus, J.-M. (2000) GFP as a marker for vacuoles in plants. *In* Vacuolar Compartments. Ed. Robinson, D.G. and Rogers, J.C., pp. 254-269, Sheffield Academic Press, Sheffield, UK.
[31] Jiang, L., Phillips, T.E., Hamm, C.A., rozdowicz, Y.M., Rea, P.A., Maeshima, M., Rogers, S.W. and Rogers, J.C. (2001) The protein storage vacuole: a unique compound organelle. J. Cell Biol. 155, 991-1002.
[32] Staehelin, L.A. (1997) The plant ER: a dynamic organelle composed of a large number of discrete functional domains. Plant J. 11, 1151-1165.
[33] Calamita, G., Ferri, D., Gena, P., Liquori, G.E., Gavalier, A., Thomas, D. and Svelto, M. (2005) The inner mitochondrial membrane has aquaporin-8 water channels and is highly permeable to water. J. Biol. Chem. 280, 17149-17153.
[34] Liu, L.H., Ludewig, U., Gassert, B., Frommer, W. B. and von Wiren, N. (2003) Urea transort by nitrogen-regulated tonoplast intrinsic proteins in Arabidopsis. Plant Physiol. 133, 1220-1228.
[35] Morishita, Y., Sakube, Y., Sasaki, S. and Ishibashi, K. (2004) Molecular mechanisms and drug development in aquaporin water channel diseases: aquaporin superfamily (superaquaporins): expansion of aquaporins restricted to multicellular organisms. J. Pharmacol. Sci. 96, 276-279.

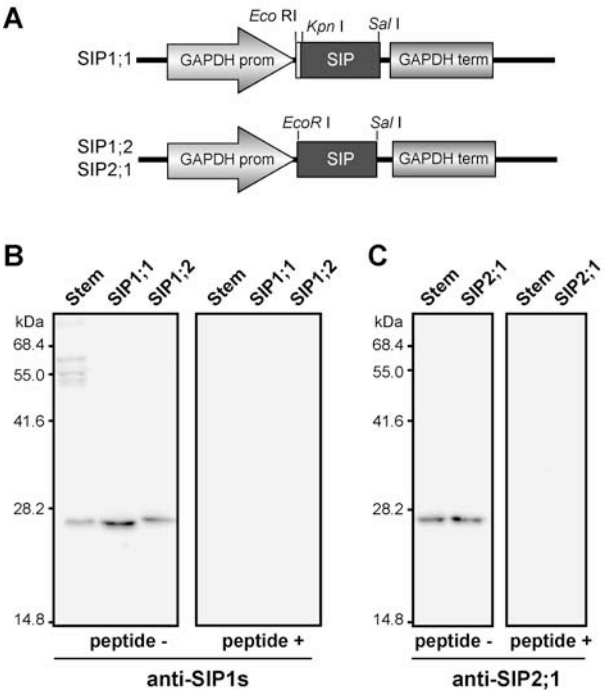

Fig. 1. Expression of *Arabidopsis* SIP proteins in yeast. A. Constructs used to express the SIPs. cDNAs for *SIP1;1*, *SIP1;2* and *SIP2;1* were inserted into the interface between the GAP promoter and the GAP terminator of pKT10. These constructs were introduced into *S. cerevisiae* BJ5458. B and C. Immunological specificity of antibodies. Crude membranes prepared from yeast cells (0.2 μg per lane) and the stems and leaves (10 μg per lane) of *Arabidopsis* were immunoblotted with anti-SIP1s (B) and anti-SIP2;1 antibodies (C). Immunoblotting was carried out in the presence (peptide +) or absence (peptide -) of the corresponding authentic antigen peptide.

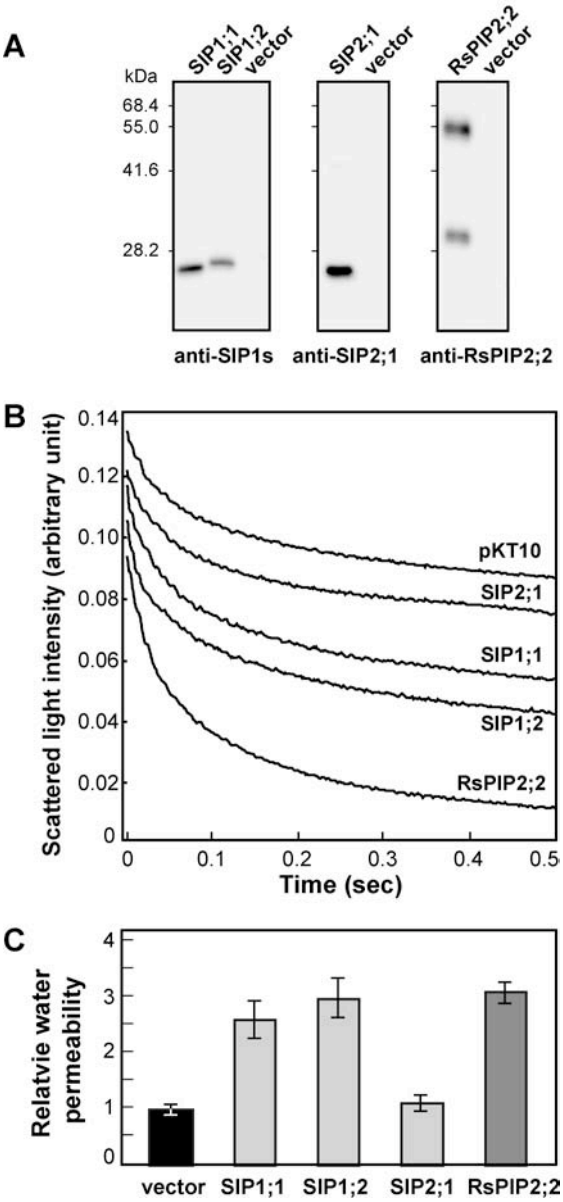

Fig. 2. Stopped-flow spectrophotometric assay of the osmotic water permeability of membrane vesicles from yeast expressing SIP proteins. A. Immunological detection of SIP proteins in yeast cells. Crude membranes (2 μg per lane) prepared from *S. cerevisiae* cells transformed with SIP1;1, SIP1;2, SIP2;1 or vector, were subjected to SDS-PAGE and immunoblotted with anti-SIP1s, anti-SIP2;1 or anti-RsPIP2;2 antibodies. B. Membrane vesicles (0.5 mg/ml) were suspended in 450 mM mannitol solution and mixed with 100 mM mannitol solution at 10 °C. The reaction curves show the average traces of 10 individual shots. C. Initial rates of decrease in scattered light intensity were calculated for the average trace of each membrane preparation and expressed relative to the empty vector control (closed bar). The data are presented as the mean ± standard deviation (S.D.) of multiple assay (n = 9, 11, 9, 11, 12 for vector, SIP1;1, SIP1;2, SIP2;1 and RsPIP2;2) .

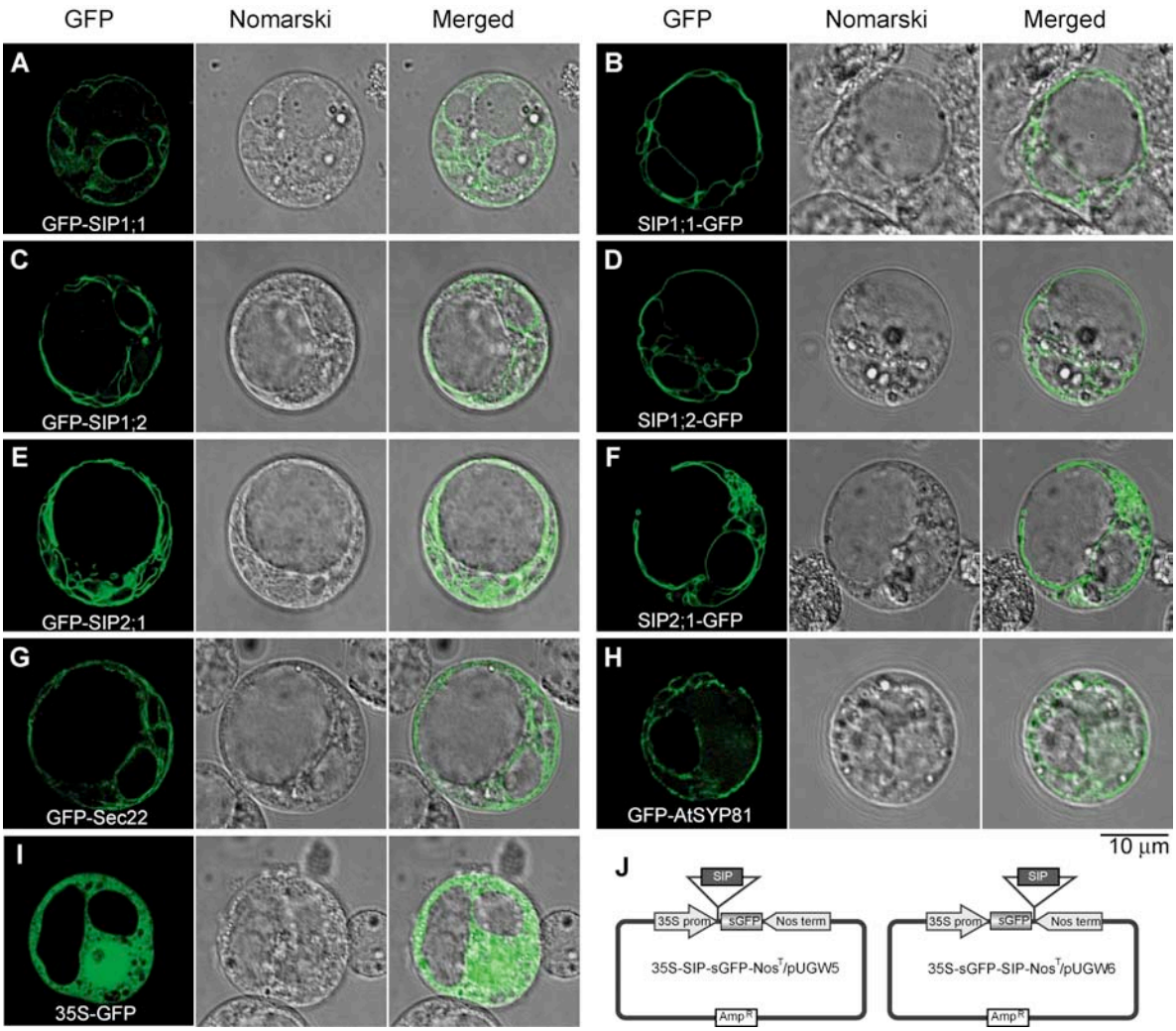

Fig. 3. Expression of SIP-GFP fusion proteins in protoplasts. Constructs GFP::SIP1;1 (A), SIP1;1::GFP (B), GFP::SIP1;2 (C), SIP1;2::GFP (D), GFP::SIP2;1 (E), SIP2;1::GFP (F), GFP::AtSec22 (G), GFP::AtSYP81 (H) and free GFP (I) were transiently expressed in *Arabidopsis* suspension-cultured cells. The green fluorescence of GFP-tagged proteins was viewed by confocal laser-scanning microscopy. Nomarski images were also recorded and then merged with fluorescence images. (J) Diagrams of constructs SIP::sGFP (left) and sGFP::SIP (right).

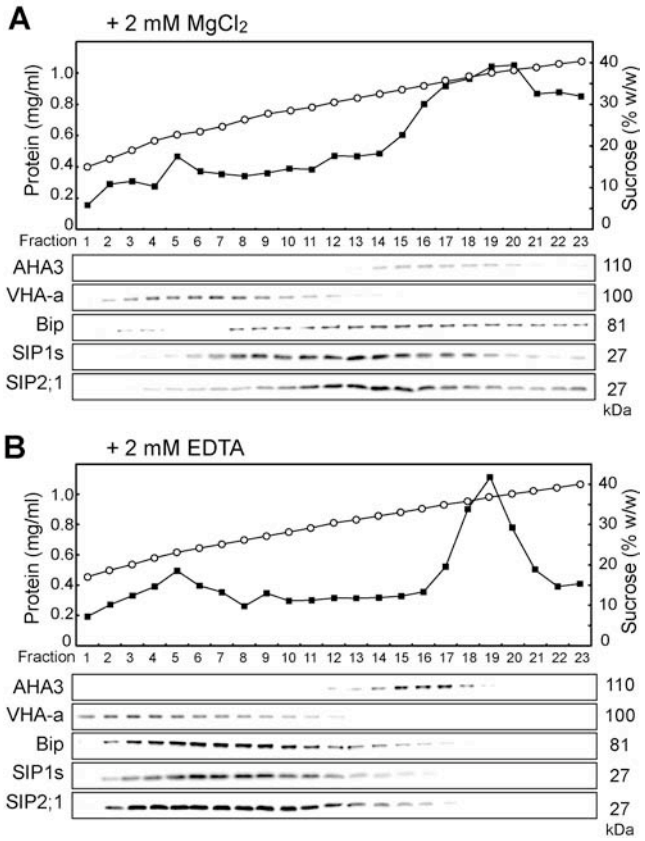

Fig. 4. Subcellular localization of SIP proteins in wild type Arabidopsis plants. Microsomal fractions were prepared from the stems of *Arabidopsis* in the presence of magnesium (A) or EDTA (B), and subjected to sucrose density-gradient centrifugation in the presence of 2 mM $MgCl_2$ (A) or 2 mM EDTA (B). Proteins in the fractions were electrophoresed, blotted and detected using antibodies against SIP1s, SIP2;1, plasma membrane H^+-ATPase (AHA3), subunit-a of the vacuolar H^+-ATPase (AtVHA-a) and BiP. Protein concentrations (closed squares) and sucrose (open circles). The molecular sizes (kDa) of the immunostained bands are shown on the right.

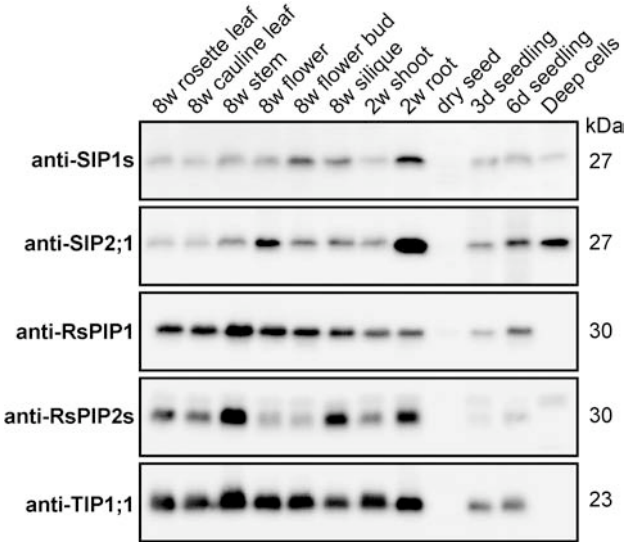

Fig. 5. Levels of SIP1, SIP2;1, PIP1, PIP2 and TIP1;1 in membranes from *Arabidopsis* plants and suspension-cultured cells. Crude membrane fractions were prepared from various organs at different growth stages: dry seed and plants at 3 days (3d), 6 days (6d), 2 weeks (2w) and 8 weeks (8w). Membrane fraction was also prepared from Arabidopsis suspension cells cultured for 6 days (Deep cells). Aliquots of the membrane fractions were immunoblotted with the indicated antibodies. The molecular sizes (kDa) of the immunostained bands are shown on the right.

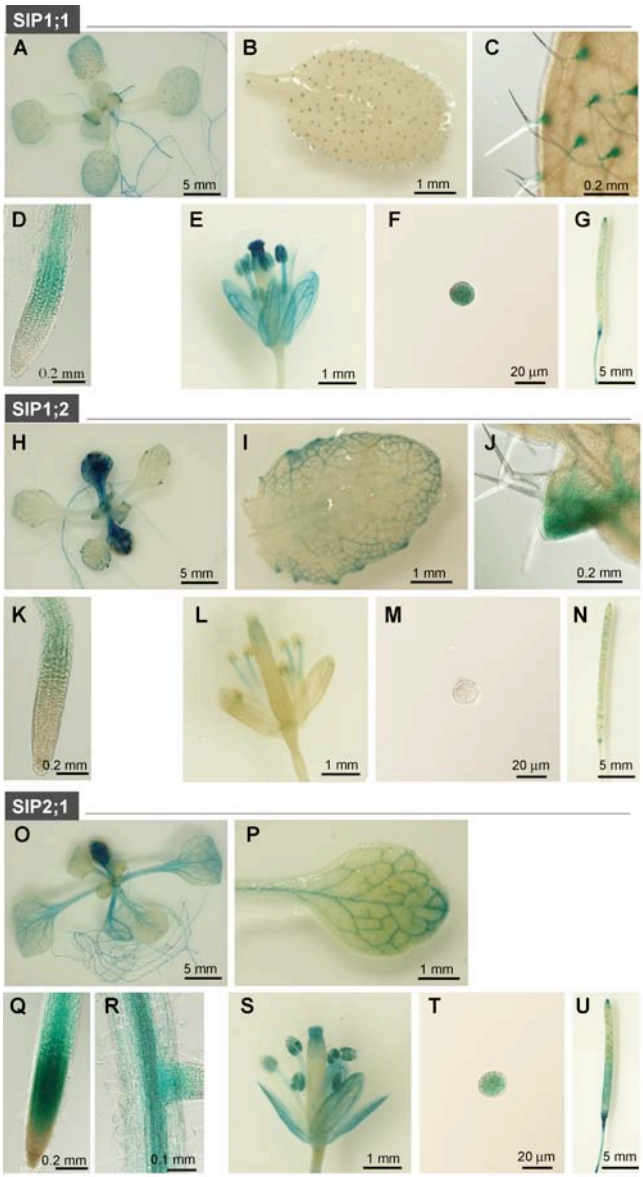

Fig. 6. Expression profiles of *SIP* promoter::*GUS* fusions in *Arabidopsis* plants. (A to G) *SIP1;1::GUS*. (H to N) *SIP1;2::GUS*. (O to U) *SIP2;1::GUS*. Plantlets grown for 2 weeks were incubated in staining solution for 6 h (A to D, H to K, and O to R). Flowers and siliques from 8-week-old plants were incubated in staining solution for 18 h (E, F, G, L, M, N, S, T and U). (B, I and P): rosette leaf. (C and J): magnified images of rosette leaf. (D, K, Q and R): root tip. (E, L and S): flower. (F, M and T): pollen. (G, N and U): silique.