**Arabidopsis ABCB21 is a Facultative Auxin Importer/Exporter Regulated by Cytoplasmic Auxin Concentration**

Yoshihisa Kamimoto¹, Kazuyoshi Terasaka², Masafumi Hamamoto¹, Kojiro Takanashi¹, Shoju Fukuda¹, Nobukazu Shitan¹, Akifumi Sugiyama¹, Hideyuki Suzuki³, Daisuke Shibata³, Bangjun Wang⁴,⁶, Stephan Pollmann⁵,⁷, Markus Geisler⁴,⁶ and Kazufumi Yazaki¹,*

¹Laboratory of Plant Gene Expression, Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho Uji, 611-0011 Japan
²Graduate School of Pharmaceutical Sciences, Nagoya City University, Japan
³Kazusa DNA Research Institute, 2-6-7 Kazusakamatari, Kisarazu, Chiba, 292-0818 Japan
⁴Institute of Plant Biology, Basel–Zurich Plant Science Center, University of Zurich, CH-8008 Zurich, Switzerland
⁵Ruhr-Universität Bochum, Lehrstuhl für Pflanzenphysiologie, Germany
⁶Present address: University of Fribourg, Department of Biology, Plant Biology, CH-1700 Fribourg, Switzerland
⁷Present address: Centro de Biotecnologia y Genómica de Plantas, Campus de Montegancedo, 28223 Pozuelo de Alarcón, Madrid, Spain.

*Corresponding author: E-mail, yazaki@rish.kyoto-u.ac.jp; Fax, +81-774-38-3623.
(Received February 2, 2012; Accepted October 31, 2012)

The phytohormone auxin is critical for plant growth and many developmental processes. Members of the P-glycoprotein (PGP/ABCB) subfamily of ATP-binding cassette (ABC) transporters have been shown to function in the polar movement of auxin by transporting auxin over the plasma membrane in both monocots and dicots. Here, we characterize a new Arabidopsis member of the ABCB sub-family, ABCB21/PGP21, a close homolog of ABCB4, for which conflicting transport directionalities have been reported. ABCB21 is strongly expressed in the abaxial side of cotyledons and in junctions of lateral organs in the aerial part, whereas in roots it is specifically expressed in pericycle cells. Membrane fractionation by sucrose density gradient centrifugation followed by Western blot showed that ABCB21 is a plasma membrane-localized ABC transporter. A transport assay with Arabidopsis protoplasts suggested that ABCB21 was involved in IAA transport in an outward direction, while naphthalene acetic acid (NAA) was a less preferable substrate for ABCB21. Further functional analysis of ABCB21 using yeast import and export assays showed that ABCB21 mediates the 1-N-naphthylphthalamic acid (NPA)-sensitive translocation of auxin in an inward direction when the cytoplasmic IAA concentration is low, whereas this transporter mediates outward transport under high internal IAA. An increase in the cytoplasmic IAA concentration by pre-loading of IAA into yeast cells abolished the IAA uptake activity by ABCB21 as well as ABCB4. These findings suggest that ABCB21 functions as a facultative importer/exporter controlling auxin concentrations in plant cells.

**Keywords:** ABC protein • ABCB21 • Arabidopsis thaliana • Auxin transport • GUS expression • Pericycle cell • Transport direction.

**Abbreviations:** ABC, ATP-binding cassette; BA, benzoic acid; DTT, dithiothreitol; 5-FI, 5-fluoroindole; GUS, β-glucuronidase; LR, lateral root; NAA, naphthalene acetic acid; NBD, nucleotide-binding domain; NPA, 1-N-naphthylphthalamic acid; PGP, P-glycoprotein; PMSF, phenylmethylsulfonyl fluoride; RNAi, RNA interference; RT–PCR, reverse transcription–PCR.

**Introduction**

Higher plants develop various organs after germination; i.e. in aerial parts, the shoot meristem produces leaves and stems, while in underground parts, a branching root system is developed through the formation of lateral roots (LRs) from the primary root. LR formation is an important developmental process that contributes to the establishment of root conformation to enable the plant to absorb water and nutrients efficiently, and physically to sustain the aerial parts (Park et al. 2005). In Arabidopsis thaliana, for example, LRs are generated from several pericycle cells adjacent to the xylem parenchyma, which starts to divide in a highly regulated manner, to form new root tissue toward the lateral axis. The first trigger of cell division at the pericycle leading to LR formation is the event of auxin entry into the cell (Casimiro et al. 2001). Auxin is also deeply involved in vertical axis formation by the polar transport via cell-to-cell movement of IAA in xylem parenchyma cells in plants.

Membrane transport of auxin is regulated by several types of transporters, e.g. PIN proteins (major facilitator superfamily; MFS) and AUX1/LAX H⁺ symporters (Schnabel and Frugoli 2004, Xu et al. 2005, Carraro et al. 2006, Wisniewska et al. 2006, Bainbridge et al. 2008). In addition, ATP-binding cassette
(ABC) transporters have been discovered as the third major family responsible for the membrane transport of auxin. Out of approximately 120 ABC transporters in a plant, mostly the P-glycoprotein (PGP)/ABCB (hereafter referred to as ABCB) subclass (Jasinski et al. 2003, Verrier et al. 2008) is reported thus far as being relevant for auxin transport (Noh et al. 2001, Luschnig 2002, Noh et al. 2003, Geisler et al. 2005, Terasaka et al. 2005). First, two ABCBs, ABCB1 and ABCB19, in A. thaliana were shown to bind specifically the auxin efflux inhibitor 1-N-naphthylphthalamic acid (NPA) (Murphy et al. 2002). T-DNA insertion mutants of abcb19 showed pleiotropic auxin-related phenotypes, and characterization of the double mutant abcb1/abcb19 and antisense abcb1 plants strongly suggested that these ABC transporters are necessary for the polar auxin transport.

Previously, we identified another plasma membrane ABCB-type transporter, (PGP4)/ABCB4, in A. thaliana, which was specifically expressed in the root epidermis, and demonstrated its involvement in basipetal shootward auxin transport, i.e. from the root tip to the root–shoot transition zone (Terasaka et al. 2005). Most plant ABCB members form pairs sharing high sequence similarity with each other (Geisler and Murphy 2006). The paralog of ABCB4 in the Arabidopsis genome is ABCB21 (68% nucleotide identity and 79% amino acid identity), but no biochemical and molecular biological characterization was made on this ABCB member. Besides, for ABCB4, conflicting transport directionalities, either inward or outward transport activity, have been reported, depending on the experimental systems (Santelia et al. 2005, Terasaka et al. 2005, Cho et al. 2007, Lewis et al. 2007, Kubes et al. 2012). In this report, we describe the biochemical characterization of ABCB21 comparing the transport property of ABCB4 as well as ABCB1, and suggest that ABCB21 functions as facultative transporter that mediates import or export of IAA depending on the cytoplasmic IAA concentration.

**Results**

**Expression analysis of ABCB21**

ABCB21 locates at chromosome 3 and is divided into 11 exons, and the deduced polypeptide consists of 1,293 amino acids (Fig. 1a). This ABC protein is grouped in cluster II of the ABCB subfamily, which is separate from cluster I where ABCB1 and ABCB19 are classified (Fig. 1b). Due to the high sequence similarity between ABCB4 and ABCB21, a possible transcriptional correlation was evaluated employing a microarray analysis, in which the abcb4 mutant and wild-type Arabidopsis were compared (Supplemental Fig. S1). In abcb4, 158 genes showed a \(>2\)-fold elevated expression (Supplementary Table S1a), whereas 21 genes revealed less than half that level of expression (Supplementary Table S1b), but no ABC protein genes, including ABCB21, were observed in these lists.

To evaluate the organ-specific expression of ABCB21, a real-time PCR analysis was conducted, and this indicated that the ABCB21 transcript was detectable in all Arabidopsis tissues examined (Fig. 2a). This expression pattern is different from that of ABCB4 that is almost solely expressed in the root, in particular in the epidermis and the root cap (Terasaka et al. 2005), while strong expression of ABCB21 was also seen in leaf tissues.

We analyzed the response of ABCB21 expression to various phytohormones and abiotic stresses using 14-day-old seedlings of Arabidopsis. While ABCB21 expression was decreased upon treatments with benzyladenine, ABA and gibberellic acid, as well as cold or dark treatments, no increase in the gene expression was observed even by auxins at a high concentration in this primary screening (Supplementary Fig. S2). However, a time course experiment with a low concentration (300 nM) of IAA showed that the seedlings responded to exogenously added IAA in a different pattern from that of ABCB4, i.e. the ABCB4 expression increased rapidly and then decreased gradually, whereas ABCB21 expression decreased transiently and then returned to a steady-state level gradually (Fig. 2b), suggesting a different physiological role for ABCB21 from that for ABCB4.

A detailed cell type-specific expression analysis of ABCB21 was performed using a ProABCB21:β-glucuronidase (GUS) transformant at the T\(_3\) generation, in which a 0.75 kb sequence upstream of the ABCB21 initiation codon was fused to a GUS reporter gene. The ProABCB21:GUS transformants were...
shown by \( C12 \) (top panel) and \( ABCB4 \) prepared from whole seedlings was probed with \( 32P \)-labeled peaks at the 32/38% fraction that coincides with that of the directed against \( ABCB21 \) was used. The \( ABCB21 \) polypeptide followed by Western blot, in which a specific peptide antibody 

Membrane localization of \( ABCB21 \)

Membrane localization of \( ABCB21 \) was analyzed by sucrose density gradient fractionation of microsomal membranes followed by Western blot, in which a specific peptide antibody directed against \( ABCB21 \) was used. The \( ABCB21 \) polypeptide peaks at the 32/38% fraction that coincides with that of the plasma membrane \( H^+\)-ATPase AHA, and the fractionation pattern is apparently different from those of the \( H^+\)-pyrophosphatase (AVP1) and of the endoplasmic reticulum luminal binding protein (BiP), which were used as markers of vacuolar and endoplasmic reticulum membrane proteins, respectively (Fig. 4). These results suggest that \( ABCB21 \) is located on the plasma membrane and not on vacuolar membranes or the endoplasmic reticulum.

\textbf{ABCB21 is a facultative auxin importer/exporter}

To analyze the transport function of \( ABCB21 \) in planta, we obtained an insertion (WiscDsLox1C2) mutant of the \( ABCB21 \) gene from the Wisconsin mutant collection. However, reverse transcription–PCR (RT–PCR) analysis of mutant seedlings indicated that the transcript corresponding to the 3’-truncated \( ABCB21 \) open reading frame was clearly detected and the coding sequence for the first ATPase domain remained (Supplementary Fig. S4). Adding to the absence of the \( P \)-value of this gene in the microarray analysis of the loss-of-function mutant (not shown) and its low germination rate, we did not use the strain for further studies.

As the second best option, we therefore generated \( ABCB21 \) RNA interference (RNAi) lines, in which an intron-spliced hairpin RNA (RNAi) construct targeted to the \( ABCB21 \) sequence was introduced (Fig. 1a). Among 18 kanamycin-resistant transformants, two lines, designated \( abcb21\)-ir1 and \( abcb21\)-ir2, showed strong silencing at the protein level and were used for further studies (Fig. 5a). Importantly, in these RNAi lines, \( ABCB4 \) expression was not significantly influenced.

Having the proposed import directionality for \( ABCB4 \) (Santelia et al. 2005, Terasaka et al. 2005), we performed auxin import and export assays in \( ABCB21 \) RNAi plant (line ir-1) mesophyll protoplasts in the presence of 100 nM external IAA (Fig. 5b). Surprisingly, in import assays, the \( ABCB21 \) RNAi line showed significantly increased IAA uptake compared with the vector control, indicating, together with the plasma membrane localization, that the transport directionality of \( ABCB21 \) may be pointing outward in Arabidopsis protoplasts (Fig. 5b). Using naphthalene acetic acid (NAA) double labeling in the same assay, we found no significant difference in NAA loading between the RNAi line (ir-1) and the vector control (Fig. 5c), which is in line with the chemical feature of NAA, unlike IAA, to be able to by-pass carrier-mediated uptake by diffusion (Marchant et al. 1999). However, reduced NAA export capacities have also been recently reported for \( ABCB4 \) using tobacco BY2 cells (Kubesˇ et al. 2012). In agreement with this proposed efflux directionality, the export of IAA by the RNAi line was reduced in export assays where outside concentrations of auxins were monitored (Fig. 5d), while the transport activity for NAA was not at a significant level (Fig. 5e).

The transport activity of \( ABCB21 \) for IAA was further investigated at the cellular level in yeast. As previously reported for \( ABCB1 \) (Geisler et al. 2005), the plasma membrane localization of \( ABCB21 \) expressed in yeast cells was confirmed by membrane 

![Fig. 2 Expression profile of \( ABCB21 \) in Arabidopsis. (a) Organ-specific expression of \( ABCB21 \) monitored by real-time RT–PCR. The experiment was repeated three times, and error bars indicate the standard deviation. (b) Time course of auxin response of \( ABCB21 \) and \( ABCB4 \) gene expression. Seedlings were treated with 300 nM IAA, and sampled at 0, 0.5, 1, 3 and 7 d after treatment. Total RNA (10 μg) prepared from whole seedlings was probed with \( 32P \)-labeled \( ABCB21 \) (top panel) and \( ABCB4 \) fragments (middle panel). Loading controls are shown by \( \beta\)-actin (bottom panel). The experiment was repeated twice to yield similar results.](https://academic.oup.com/pcp/article-abstract/53/12/2090/1870174?locale=en)
fractionation using sucrose density gradient centrifugation followed by Western blot analysis (Fig. 6a). The IAA-sensitive strain gef1 (for Glycerol/ethanol Fe-requiring) lacks a putative chloride channel protein. In a growth test using 5-fluoroindole (5-FI), a toxic analog of a potential IAA precursor, which provides a useful tool for evaluating auxin transport (Gaxiola et al. 1998), ABCB21, like ABCB4 (Santelia et al. 2005), showed a significant hypersensitivity compared with the vector control (Fig. 6b), supporting the assumption that ABCB21, like its closest homolog, might function as an auxin analog importer in yeast.

In order to clarify these seemingly conflicting transport data in more detail, we performed yeast IAA transport studies for ABCB21, in direct comparison with ABCB1 and ABCB4, as both import and export assays. As explained in detail in the Materials and Methods, in import assays external IAA is added at time point 0 resulting in high external IAA but low cytoplasmic auxin concentrations during this assay. In export assays, prior to export, IAA loading followed by removal of external IAA is performed, resulting in low external but high cytoplasmic auxin levels. In import assays (Fig. 7a), as reported, ABCB1 clearly reveals efflux activity for IAA, while ABCB21 as well as
ABCB4 show uptake activity in this assay. Use of the unspecific diffusion control, benzoic acid (BA), employed as double labeling in the same assay, demonstrated transport specificity of this assay. However, surprisingly, in export assays (Fig. 7c), all three ABCBs were capable of exporting IAA, resulting in lower IAA retentions, which again was not found with the diffusion control, BA.

To test the idea that the cytoplasmic IAA concentration indeed alters the transport directionality of ABCB4 and ABCB21, we repeated the import assays after pre-loading with IAA. Strikingly, pre-loading with 10 μM IAA at pH 4.5 completely blocked the ABCB4/ABCB21-mediated IAA import to the vector control level (Fig. 7b). IAA pre-loading also reduced ABCB1-catalyzed IAA export, which is, however, most probably caused by competition of unlabeled IAA with radiolabeled IAA. These findings strongly support our hypothesis that internal IAA regulates the transport directionalities of ABCB21 and ABCB4. Interestingly, BA export seems to be reduced in ABCB21- and ABCB4-expressing yeast, despite this not being significant, arguing that internal IAA might influence the specificity of transport substrates for ABCB21 and ABCB4 (Fig. 7b).

Finally, we also tested NPA binding of those ABCB members. Fig. 7d and e reveal that all three can bind to NPA to a similar extent, and that NPA suppressed the IAA transport function of

---

**Fig. 4** Membrane localization of ABCB21. Fractionation of total microsomes from wild-type Arabidopsis roots was done on a non-continuous sucrose gradient consisting of 0, 20, 32, 38 and 45% (w/v) sucrose. Membrane fractions were collected from the interfaces between different sucrose concentrations and analyzed via SDS–PAGE and protein gel blotting. Blots were probed with antisera against ABCB21, plasma membrane H\(^{+}\)-ATPase (AHA), vacuolar H\(^{+}\)-PPase (AVP1) and endoplasmic reticulum BiP.

**Fig. 5** Transport assay of ABCB21 knock-down protoplast cells. (a) Protein gel blot analyses of the abcb21 RNAi line. The experiment was repeated three times and yielded similar results. (b) Import assay: IAA uptake into protoplasts of the abcb21 RNAi line compared with that of the vector control. Values are mean activities ± SEs, four individual measurements (n = 4). (c) Import assay: NAA uptake into protoplasts of the abcb21 RNAi line compared with that of the vector control. Values are mean activities ± SEs, four individual measurements (n = 4). (d) Export assay: IAA export from protoplasts of the abcb21 RNAi line compared with that of the vector control. Values are mean activities ± SEs, four individual measurements (n = 4). (e) Export assay: NAA export from protoplasts of the abcb21 RNAi line compared with that of the vector control. Values are mean activities ± SEs, four individual measurements (n = 4).
dependent transformants. We have carefully looked for morphological differences in abcb21 RNAi lines compared with wild-type plants, especially in the root phenotype. These RNAi lines tend to show retardation of the germination process, but they grow normally and revealed basically no significant morphological differences (Supplementary Fig. S5). In 10-day-old seedlings of the T3 generation, for example, the main root length of the wild-type and RNAi line was 22.3 ± 4.8 and 21.7 ± 5.5 mm (n = 15), respectively, whereas the number of LRs was 2.2 ± 1.4 and 2.5 ± 1.8 (n = 15) for the wild type and the RNAi line, respectively. The lack of a visible phenotype for abcb21 RNAi lines may be a result of the putative functional redundancy of other uncharacterized ABCB transporters expressed in these tissues, or phenotype differences may only be observed under special stress conditions.

**Discussion**

Thus far, membrane transporters of auxin have been studied intensively from the viewpoint of the polar movement of the IAA molecule along the vertical axis of the plant body, to which PIN proteins provide a dominant contribution. Similarly, both ABCB1 and ABCB19, as well as auxin importers of the AUX1/LAX family are also relevant for the downward movement of auxin in parenchyma cells. Another ABC protein of the B family, ABCB4, is expressed specifically at the root epidermis, which mediates the upward movement of the IAA molecule. In this study, we have demonstrated that a new member of the ABCB subfamily, ABCB21, is expressed in the abaxial side of cotyledons, in junctions of lateral organs in the aerial part and in pericycle cells adjacent to the protoxylem poles of Arabidopsis root, and further this ABC transporter recognized IAA as a transport substrate. Also, this transporter protein binds NPA, resulting in inhibition of IAA transport.

The initiation of the LR formation process is triggered by the entrance of the IAA molecule from the xylem parenchyma into pericycle cells (Laskowski et al. 1995, Himanen et al. 2002). In the microarray analysis of the abcb21 RNAi lines, the expression of LAX3, PIN5 and SHY2 involved in LR development was significantly down-regulated (Bouchard et al. 2006, Blakeslee et al. 2007) (Supplementary Table S2). We then observed how ABCB21 expression is altered during the LR initiation process with ABCB21 promoter–GUS transformants (Supplementary Fig. S6). At the dividing pericycle cell, ABCB21 expression disappeared when LR initiation occurs, which is phase II to III according to the literature (Vanneste et al. 2005). This suggests that ABCB21 may be relevant for LR initiation, but once the initiation occurs the role of this transporter can be terminated. We thus expected that knock-down lines would show phenotype alterations in root development, but no clear difference was observed (Supplementary Fig. S5). This does, however, occur relatively frequently in the knock-down lines, or even in knock-out mutants of ABC transporter genes, because there is a high redundancy of this gene family in plants. If an uncharacterized transporter of functional redundancy is identified, the double knock-out mutant might provide a more informative phenotype as reported for ABCB1 and ABCB19 (Noh et al. 2001).

The abcb21 loss-of-function line (Wisconsin No. CS849895) showed a very low germination rate, and germinated plants did not show a clear auxin-related phenotype either, including LR...
development. This suggests that the main phenotype of ABCB21 loss of function is the failure of seed development, and the minority of seeds that could somehow compensate the ABCB21 function, by as yet unidentified IAA transporters, can germinate. Thus, we analyzed the biochemical function of ABCB21 in the root using RNAi lines. The possibility cannot be eliminated that the RNAi construct for suppression of ABCB21 might influence the expression of other auxin transporters, but at least a microarray analysis of RNAi lines revealed that the expression of other ABC transporters was not strongly influenced. While we could not find phenotype alterations in our experiments, our data strongly support that ABCB21 is a transporter regulating auxin movement in various tissues.

Conflicting reports on the transport directionality of ABCB4 have been reported (Terasaka et al. 2005, Cho et al. 2007, Lewis et al. 2007). Likewise, the closest ABCB4 homolog, ABCB21, seems to export IAA from mesophyll protoplast but, based on yeast assays, functions as an auxin importer (Figs. 5, 7). One possible explanation for this behavior might be the molecular environment of the assay systems where different

**Fig. 7** Transport assay of ABCB21 in yeast. (a) Import assay (high external IAA): IAA and the diffusion control, BA, were used, and the retention was measured in yeast cells (strain JK93da) expressing ABCB21, and ABCB1 and ABCB4 as controls. Bars represent the same as (b). (b) Cold IAA (10 µM) was pre-loaded and the import assay was conducted. Control 1 (pNEV) and control 2 (pDR196). (c) Export assay (low external IAA): these yeast transformants were pre-treated with IAA on ice and then export activity was measured (see the Materials and Methods for details). As diffusion control, BA is used. (d) Specific binding of NPA to microsomes prepared from yeast expressing ABCB21, ABCB1 or ABCB4. (e) NPA inhibition of IAA retention (import assays) in yeast expressing ABCB21 and its closest homolog, ABCB4. Data shown are mean activities ± SEs, 3–5 individual measurements with four replicates each. Significant differences from the vector control (P < 0.05) are indicated by asterisks.
endogenous proteins may influence the transport function. For instance, the immunophilin-like protein TWISTED DWARF1 (TWD1) can modulate the function of ABCBs (Geisler et al. 2003, Bouchard et al. 2006), i.e. when co-expressed with ABCB1, TWD1 showed an opposite effect on ABCB1-mediated auxin efflux activity in yeast and HeLa cells (Bouchard et al. 2006). As another example, coordinated auxin transport by ABCB–PIN interactions has also been reported (Noh et al. 2003, Blakeslee et al. 2007), i.e. ABCB4 showed different auxin transport directionalities depending on its PIN partner (Cho et al. 2007). Obviously, these interacting partners are absent in the yeast expression system. In this study carefully comparing ABCB1, B4 and B21 transport activity in yeast, we suggest another mechanism in that the cytoplasmic IAA concentration is a key factor determining the directionality of IAA transport (Figs. 5, 7): when cytoplasmic auxin concentrations are low (approximately nM order) such as in the yeast loading assays that were conducted in the absence of non-labeled IAA, both ABCB21 and B4, unlike ABCB1, import auxin (Fig. 7). However, when cytoplasmic auxin concentrations are kept relatively high (approximately mM order), either by addition of non-labeled IAA to the protoplast uptake buffer at time point 0 (Fig. 5b) or by yeast loading prior to removal of the extracellular supernatant during the export assays (Fig. 7c), both ABCB21 and B4 catalyze in the export direction like ABCB1. This proposed mechanism is supported by IAA pre-loading experiments that blocked ABCB21- and ABCB4-mediated IAA import (Fig. 7b). In that respect, ABCB21 resembles ABCB4 as a root-localized auxin efflux transporter with auxin uptake activity at low auxin concentrations (Kubeš et al. 2012).

This model is in line with the identification of putative cytoplasmic regulatory, structural elements in ABCB4 apparently absent in constitutive exporters, ABCB1 and B19 (Yang and Murphy 2009, Kubeš et al. 2012). Taken together, an ABCB protein may show such plasticity for the transport function depending on its subcellular expression altering cytoplasmic auxin concentrations. However, at this time, we cannot exclude the possibility that additional regulatory components, such as protein kinases or protein–protein interactions, further tune ABCB21 directionality (Cho et al. 2007, Yang and Murphy 2009).

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana plants (ecotype Columbia) were grown on soil in growth chambers with 100 or 120 μmol m⁻² s⁻¹ light in a 16 h light/8 h dark cycle at 21°C. The sterile growth condition was produced as described elsewhere (Terasaka et al. 2005). To analyze ABCB21 expression under various growth regulators, seeds were grown on a nylon mesh (20 μm pore) over Murashige and Skoog (MS) medium for 14 d under the same light cycle described above. Roots were subjected to various treatments by gentle transfer of the mesh to the new medium. Treatments were stopped by immediate freezing of seedlings in liquid N₂. Sequence data for the genes in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: PGP/ABCB21 (At3g62150), PGP/ ABCB4 (At2g47000) and β-tubulin (At5g12250).

Cloning of ABCB21 cDNA

The full-length coding sequence of ABCB21 cDNA (At3g62150) with 12 and 17 bp 5’- and 3’-untranslated regions, respectively, was isolated by RT–PCR with two primers, ABCB21-Full-Fw and ABCB21-Full-Rv (Supplementary Table S3). The PCR product was subcloned in the pENTR1A vector (Invitrogen).

Expression analysis

Total RNA was isolated with the RNAeasy Plant Mini-Kit (Qiagen). Reverse transcription was done with SuperScript III reverse transcriptase (Invitrogen), followed by incubation with RNase H (Invitrogen).

Real-time PCR was performed with the Rotor-Gene 3000A (Corbett Research), using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to the manufacturer’s instructions. Briefly, the PCR mixture consisted of 10 ng of cDNA template, 5 pmol primers, 1 μl of fluorescent probe provided with the above kit, and 12.5 μl of Platinum Quantitative PCR SuperMix-UDG in a total volume of 25 μl. The standard reaction condition was as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 50°C for 30 s, 72°C for 30 s. The primers (ABCB21-Fw and ABCB21-Rv) are listed in Supplementary Table S3. Agarose gel electrophoresis, RNA transfers onto Hybond-N+ membranes (GE Healthcare) and hybridization with the 1.1 kb ABCB21 fragment (positions +2,001 to +3,063) were performed using standard procedures. The primers (ABCB21-northern-Fw and ABCB21-northern-Rv) are listed in Supplementary Table S3.

Promoter:GUS construct and tissue-specific expression

To create the ABCB21 promoter:reporter construct (ProABCB21:GUS), two PCR primers flanking the regions from −753 to +15 bp were made (ABCB21Pro-Fw and ABCB21Pro-Rv) (Supplementary Table S3). PCR was performed using genomic DNA as a template and KOD-Plus polymerase (TOYOBO). The PCR product was subcloned into the entry vector pDONR221 to transfer the promoter region of ABCB21 into the binary vector pGWBB203 by the Gateway system (Invitrogen). BP and LR reactions were performed according to standard procedures. Agrobacterium tumefaciens GV3101 (pMP90) was used for transformation via the floral dip method (Clough and Bent 1998). The T2 generation were histochemically stained to detect GUS activity as described elsewhere (Terasaka et al. 2005). Tissue sections (10 μm thick) embedded in Technovit 7100 (Heraeus Kulzer) were prepared by Leica microscope (RM2155) and observed with a Zeiss microscope (Axioskop2).
Sucrose density gradient fractionation of plant membranes

Sucrose gradient fractionation was performed basically according to the method described by van den Brule et al. (2002) with the following modifications: Arabidopsis roots (10 g) were homogenized in 3 vols. of 50 mM HEPES-KOH, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol (DTT), 250 mM sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF) using a mortar and pestle. The homogenate was centrifuged for 15 min to remove the debris, and the microsomal membrane fraction was collected by ultracentrifugation at 100,000 g for 40 min. The pellet was resuspended in 3 ml of gradient buffer (10 mM Tris–MES, pH 7.0, 1 mM DTT, 250 mM sucrose and 1 mM PMSF) and centrifuged at 100,000 g for 3 h on a non-continuous sucrose gradient from 20% to 45% (w/v) in the same buffer using a Beckman SW41Ti rotor. Gradient fractions were collected from the interface between different sucrose concentrations, and used for immunoblot analysis.

Western blotting

A keyhole limpet hemocyanin conjugate of an oligopeptide of ABCB21, at position 1 (n-MDSVIESEEGLKVDSPNRAD-c), was injected into rabbits according to a standard protocol (Hayashikasei Co. Ltd.). After the third boost, the antiserum was recovered and used for immunoblot analysis. The immunoblot procedure is as described previously (Yazaki et al. 2006). For each membrane marker, antibodies against plasma membrane H+–ATPase (AHA), vacuolar H+-pyrophosphatase (AVP1) and endoplasmic reticulum luminal BiP were used. For the Western blot of Fig. 5a, we used young whole plants.

Transport experiments with protoplast

Intact Arabidopsis mesophyll protoplasts were prepared from rosette leaves of plants grown on soil under white light (100 μmol m^-2 s^-1, 8 h light/16 h dark, 22°C), and auxin import and export experiments were performed as described (Geisler et al. 2005). Briefly, intact protoplasts were isolated and loaded by incubation with 1 μM IAA for 10 min in SD media (pH 4.5), followed by two washing steps. Finally, export was started by 10 min incubation in SD media (pH 5.5) at 30°C. Aliquots of 1 ml were collected, filtered twice with cold water, and the retained radioactivity was quantified by scintillation counting. Some import assays were conducted with a pre-loading step with 10 μM IAA for 10 min in SD media (pH 4.5) or in the presence of 10 μM NPA in the loading media. NPA binding experiments were carried out as described in Kim et al. (2010).

ABCB21 RNAi transformants

To prepare the RNAi construct, a gene-specific 240 bp fragment from ABCB21 cDNA (corresponding to nucleotide positions +1,908 to +2,148) was subcloned into the destination vector pGWB80 via the donor vector pDONR221 (Invitrogen). The PCR primers (ABCB21ir-Fw and ABCB21ir-Rv) used are listed in Supplementary Table S3. The resulting plasmid, pGWB80 via the donor vector pDONR221. This vector, pDR196GW-ABCB21, was used to transform the yeast gef1 (Gaxiola et al. 1998) or JK93da (Santelia et al. 2005) strains by the lithium acetate method (Ito et al. 1983). For IAA loading experiments, JFK93da transformants were grown to OD600 = 1, washed and incubated at 30°C with 1 μM 5-[^3H]IAA (specific activity 7.4 × 10^11 Bq mmol^-1), American Radiolabeled Chemicals) and 5[^3H]BA (9.3 × 10^11 Bq mmol^-1), American Radiolabeled Chemicals) in SD media (pH 5.5). For IAA efflux experiments, yeast cells were loaded at 0°C for 15 min with 1 μM 5-[^3H]IAA (specific activity 7.4 × 10^11 Bq mmol^-1), American Radiolabeled Chemicals) and 5[^3H]BA (9.3 × 10^11 Bq mmol^-1), American Radiolabeled Chemicals) in SD media (pH 4.5), followed by two washing steps. Finally, export was started by 10 min incubation in SD media (pH 5.5) at 30°C. Aliquots of 1 ml were collected, filtered twice with cold water, and the retained radioactivity was quantified by scintillation counting.

Supplementary data

Supplementary data are available at PCP online.

Acknowledgments

We would like to thank Dr. W. Frommer (Carnegie Institution, Stanford, CA, USA) for pDR196, Dr. Tsuyoshi Nakagawa (Shimane University) for pGWB vectors, Dr. R.A. Gaxiola (University of Connecticut) for the yeast strain gef1, Dr. M. Boutry (Univérité Catholique de Louvain, Belgium) for anti-H+-ATPase antibodies, Dr. N. Koizumi (Osaka Prefecture
University, Japan) for anti-BiP antibodies, Dr. M.H. Sato (Kyoto Prefectural University, Japan) for anti-V-PPase antibodies, Dr. Hiroo Fukuda and Dr. Kuninori Iwamoto (The University of Tokyo, Japan) for ABCB21 Gene Chip technical support, and V. Vincenzetti (University of Zürich) and L. Charrier (University of Fribourg) for excellent technical support. DNA sequencing was conducted with the Life Research Support Center in Akita Prefectural University.

References


