

Towards Identification of the Substrates of ATP-Binding Cassette Transporters^{1[OPEN]}

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Most ATP-binding cassette (ABC) proteins function in transmembrane transport, and plant genomes encode a large number of ABC transporters compared with animal or fungal genomes. These transporters have been classified into eight subfamilies according to their topology and phylogenetic relationships. Transgenic plants and mutants with altered ABC transporter expression or function have contributed to deciphering the physiological roles of these proteins, such as in plant development, responses to biotic and abiotic stress, or detoxification activities within the cell. In agreement with the diversity of these functions, a large range of substrates (e.g. hormones and primary and secondary metabolites) have been identified. We review in detail transporters for which substrates have been unambiguously identified. However, some cases are far from clear, because some ABC transporters have the ability to transport several structurally unrelated substrates or because the identification of their substrates was performed indirectly without any flux measurement. Various heterologous or homologous expression systems have been used to better characterize the transport activity and other biochemical properties of ABC transporters, opening the way to addressing new issues such as the particular structural features of plant ABC transporters, the bidirectionality of transport, or the role of posttranslational modifications.

ATP-binding cassette (ABC) proteins are involved in countless cellular processes, mainly in transmembrane transport of a wide range of unrelated molecules. Compared with other organisms, terrestrial plant genomes encode a particularly large number of ABC proteins. For instance, the *Arabidopsis thaliana* genome is predicted to encode 130 ABC proteins and rice (*Oryza sativa*) 133, many more than for human (49) or mouse (53; Hwang et al., 2016). Based on their phylogenetic relationships, ABC proteins were classified originally in several subfamilies named after the functions of their prototypical members (e.g. multiple drug resistance [MDR], heavy metal tolerance [HMT], chloroplast trigalactosyldiacyl glycerol complex, and chloroplast cobalt uptake system). However, in many cases, the prototype function is not conserved in other members of the same subfamily. Therefore, a unified eukaryotic ABC naming system was introduced in 2008; this system classified ABC proteins into eight subfamilies, ABCA to ABCI (Fig. 1),

although plants lack proteins from the ABCH cluster (Verrier et al., 2008). In each subfamily, several proteins have been characterized, and in the case of ABC transporters, their substrates have been identified. However, the vast majority of plant ABC proteins remain uncharacterized.

ABC proteins were first identified in plants as vacuolar xenobiotic transporters involved in cellular detoxification (Martinoia et al., 1993). Since then, plant ABC transporters have been found to be involved in many essential physiological processes such as nutrition,

ADVANCES

- Omics data show that plant genomes contain close to 130 ABC genes, more than twice the number of ABC genes in animal species.
- Knockout and overexpressing transgenic lines have unraveled essential physiological roles such as in plant development and response to (a)biotic stress for many ABC transporters, mainly in *Arabidopsis* and rice.
- Substrates of ABC transporters have often been proposed from physiological data but are less frequently identified from direct transport assays.
- Several expression systems have been used to produce ABC transporters and characterize their biochemical properties.

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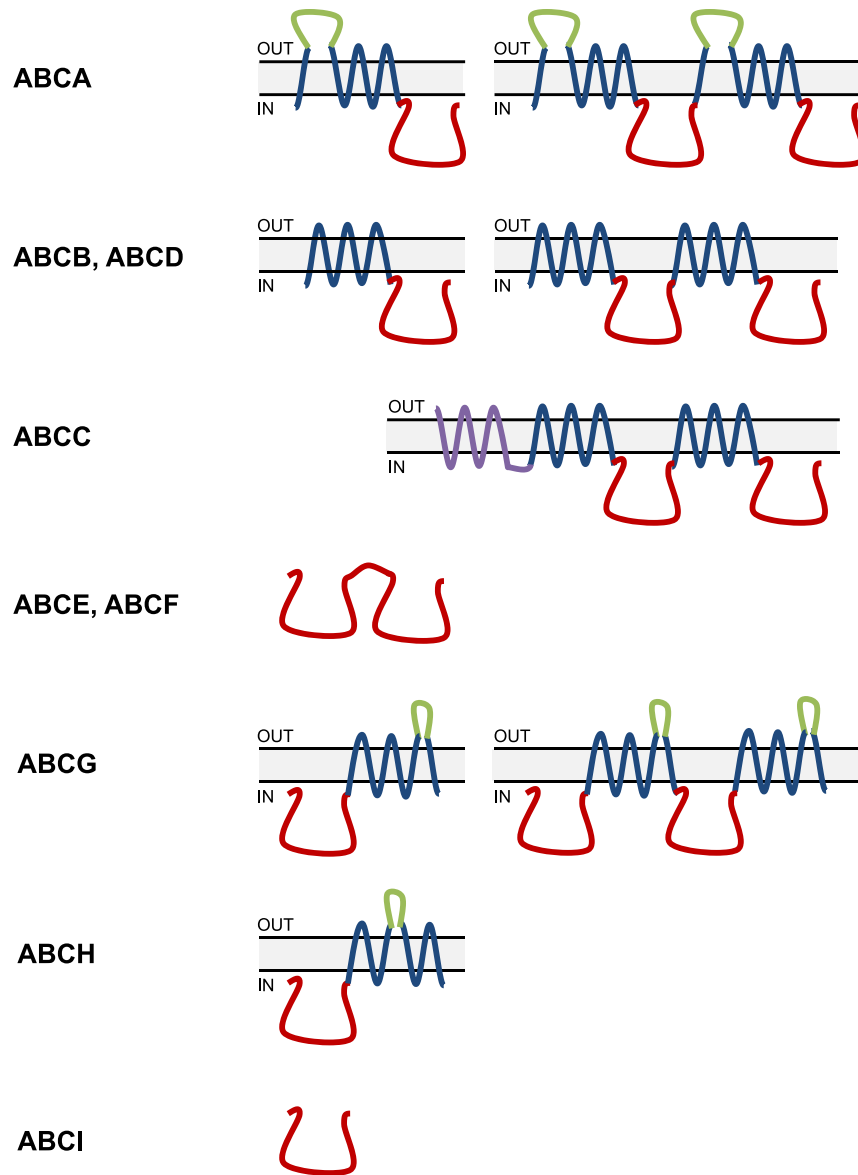


Figure 1. Predicted topologies of ABC subfamilies. Based on their size, orientation, and domain organization, ABC proteins have been classified into eight subfamilies, ABCA to ABCI, but plants lack proteins from the ABCH cluster (Verrier et al., 2008). For each subfamily, the half-size transporter is depicted on the left and the full-size on the right, whenever applicable. The N-terminal side is always on the left and the C-terminal side is on the right side. IN refers to the cytosol and OUT refers to the other side of the membrane. Nucleotide-binding domains (NBDs) are in red, transmembrane domains (TMDs) in blue, and extracytosolic loops in green. The N-terminal extension found in some subfamily C members is depicted in purple.

development, responses to biotic and abiotic stress, and interaction with the environment. The physiological roles of plant ABC transporters have been reviewed (Kretzschmar et al., 2011; Borghi et al., 2015; Hwang et al., 2016; Lane et al., 2016; Park et al., 2017; Do et al., 2018) and will not be discussed further here.

However, the exact substrates transported by most ABC transporters are still debated or unknown. One reason is that some ABC transporters, at least in animals or fungi, are able to transport different structurally unrelated substrates. This is typically the case in the

human (*Homo sapiens*) ABC transporters ABCB1 (also called MDR1 or P-glycoprotein) and ABCG2, which, when overexpressed in some cancer cells, confer resistance to chemotherapy. Thus, identifying one substrate does not necessarily tell the whole story. Another reason is that only a limited number of studies have used direct methods to identify substrates of plant ABC transporters. To address this issue, transport assays involving isolated cells, membrane vesicles, or reconstituted liposomes are essential yet technically challenging (see below). Over the past decades, advances

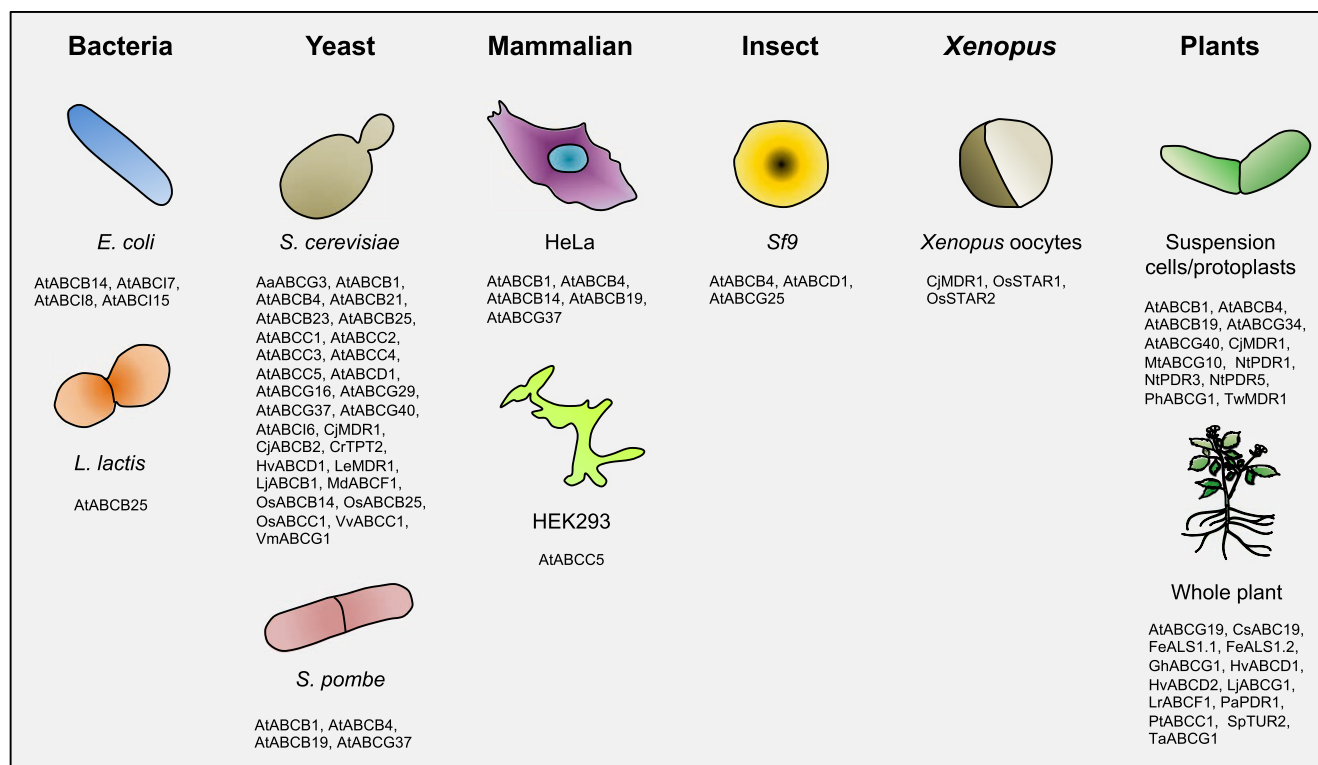


Figure 2. Expression systems used to produce and characterize plant ABC proteins. Abbreviations of plant species are as follows: At, *Arabidopsis thaliana*; Cj, *Coptis japonica*; Cr, *Catharanthus roseus*; Cs, *Cucumis sativus*; Fe, *Fagopyrum esculentum*; Gh, *Gossypium hirsutum*; Hv, *Hordeum vulgare*; Le, *Lithospermum erythrorhizum*; Lj, *Lotus japonicus*; Lr, *Lilium regale*; Md, *Malus domestica*; Mt, *Medicago truncatula*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pa, *Petunia axillaris*; Ph, *Petunia hybrida*; Pt, *Populus trichocarpa*; Sp, *Spirodela polyrrhiza*; Ta, *Triticum aestivum*; Tw, *Tripterygium wilfordii*; Vm, *Vinca minor*; Vv, *Vitis vinifera*.

have been made in such assays thanks to the efficient expression of proteins in heterologous hosts, such as bacterium, yeast, mammal, insect, or plant cells as well as transgenic plants (Fig. 2).

We have listed 132 plant ABC transporters for which a substrate has been proposed and/or demonstrated even in an indirect way (Supplemental Table S1). In most cases, phenotypic differences between ABC mutant and wild-type plants in response to stresses or data concerning ABC gene expression have allowed putative substrates to be assigned to particular transporters. However, in many cases, direct transport studies are still missing. In this review, we will start by describing the structural determinants of ABC transporters. We will subsequently highlight interesting trends in terms of the substrate profile for each ABC subfamily, focusing on those for which direct transport measurements have been undertaken. We will then assess the methods used to determine substrates and question whether ABC transporters with pleiotropic substrates exist in plants as is the case in animals or fungi. We will further illustrate the point that, even when transporters have been characterized in different expression systems, controversy still may exist regarding the substrates transported. Finally, we will discuss

topics of increasing interest within the field: directionality, multispecificity, posttranslational modifications, and the putative bifunctionality of plant ABC transporters. We hope that this overview will foster further research aimed at determining the substrate profiles of plant ABC transporters.

STRUCTURAL DETERMINANTS OF ABC TRANSPORTERS

Most ABC proteins are predicted to be transporters and usually consist of two similar TMDs and two similar NBDs. They can be full size (two TMDs and two NBDs) or half size (one TMD and one NBD), in which case they homodimerize or heterodimerize to become functional. In addition, the domain order (NBD or TMD first) can vary (Fig. 1). At the primary structural level, the NBDs contain conserved motifs involved in Mg-ATP binding and hydrolysis. Some of these also are found in other families of proteins that bind ATP, but one, the ABC signature, is the hallmark of the ABC family. Both NBDs contribute to the formation of two ATP-binding sites sandwiched at their interface (for review, see Oldham et al., 2008; Rees et al., 2009; Licht and Schneider, 2011).

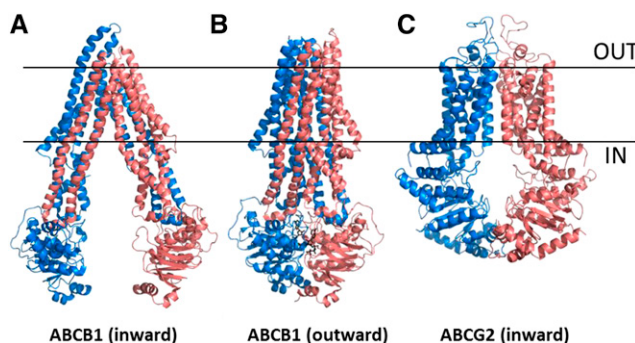


Figure 3. Examples of ABC transporter structures. A, Structure of the mouse ABCB1 transporter (Protein Data Bank [PDB] code 4Q9I) in an inward-facing configuration. The N-terminal half (TMD1 + NBD1) is colored in blue and the C-terminal half (TMD2 + NBD2) is colored in salmon. B, Structure of the human ABCB1 transporter (PDB code 6C0V) in an outward-facing configuration. The N-terminal half (TMD1 + NBD1) is colored in blue and the C-terminal half (TMD2 + NBD2) is colored in salmon. Two ATP molecules (in black) are localized at the interface between the two NBDs. C, Structure of the human ABCG2 transporter (PDB code 5NJ3) in an inward-facing configuration. The two half-size transporters are colored in blue and salmon. The two black lines represent the membrane limits, where IN refers to the cytosol and OUT refers to the other side of the membrane. The structures were drawn with PyMOL.

Generally, the TMDs display higher variation in their primary sequence, yet they typically consist of at least six transmembrane helices per TMD, which span the membrane. Although TMDs are presumed to form the binding site and the pathway through which the substrates are transported, how the cross talk between the NBDs and TMDs occurs at the molecular level remains unknown. In fact, extensive site-targeted mutational studies performed on the ABC transporter from the yeast *Saccharomyces cerevisiae* PDR5 revealed that a single mutation in the NBD abolished the transport of a specific set of substrates but did not affect either the ATPase activity or the efflux of other drugs (Ernst et al., 2008). This suggests that, depending on the substrate, different regions within the TMDs and NBDs might interact to dictate substrate specificity.

The 3D structures of several ABC transporters, mostly from prokaryotes, have been obtained via crystallization and x-ray diffraction. However, cryoelectron microscopy, a rapidly developing technique (for which a Nobel Prize was awarded in 2017), has become a very powerful alternative route toward determining the detailed structure, in particular of membrane proteins, which are notoriously difficult to crystallize. This technique does not require crystallization and, interestingly, allows for the identification of catalytic intermediates (Murata and Wolf, 2018). It was used recently to determine the structures of several ABC transporters. What can we learn from these structures? While the general conformation is conserved among ABC transporters, particularly the organization as two NBDs and two TMDs as well as the conserved NBD motifs, structural variations are observed, for instance between members of different subfamilies (ter Beek et al., 2014). As an example, Figure 3 displays the structures of mammalian ABCB and ABCG transporters.

The major interest of obtaining a 3D structure is to understand at the molecular level how a protein

functions. Interestingly, the comparison of 3D structures obtained under different conditions (e.g. the presence or absence of ATP or substrates; Fig. 3, A and B) led to models of transport mechanisms in which important conformational modifications occur during the catalytic cycle to move the substrate from one side of the membrane to the other (Fig. 4). However, given the ancient origin of the ABC family, a unique mechanism is unlikely and mechanistic diversity can be expected (e.g. regarding substrate binding and how transport is coupled to ATP binding and hydrolysis; Moeller et al., 2015; Locher, 2016).

To date, no detailed 3D structure has been determined for any plant ABC transporter. Taking into account the primary sequence conservation, in particular in the NBDs, there is no reason to believe that the structure of plant ABC transporters is much different from that obtained from other eukaryotes. Particle analysis of two purified ABCG transporters from tobacco (*Nicotiana tabacum*; NtPDR1 and NtPDR5) was obtained by electron microscopy (Pierman et al., 2017; Toussaint et al., 2017) and showed that their size and shape are compatible with the structures of animal ABCG transporters, such as that shown in Figure 3C. Yet, particular features, for instance linked to the binding site of transported substrates or to regulatory domains, are expected and justify the necessity to obtain their detailed 3D structures. A key challenge for crystallography or cryoelectron microscopy is to capture structures with bound allocrites (transported substrates), especially for ABC transporters, which have the capacity to transport several structurally independent substrates, as has been shown for animal and fungal transporters. Structural analysis of mammalian MDR1 has shown the presence of a large cavity that can accommodate various substrates (Aller et al., 2009; Kim and Chen, 2018). It is not clear yet if plant ABC transporters have this capacity of multiple substrates (see below). Until

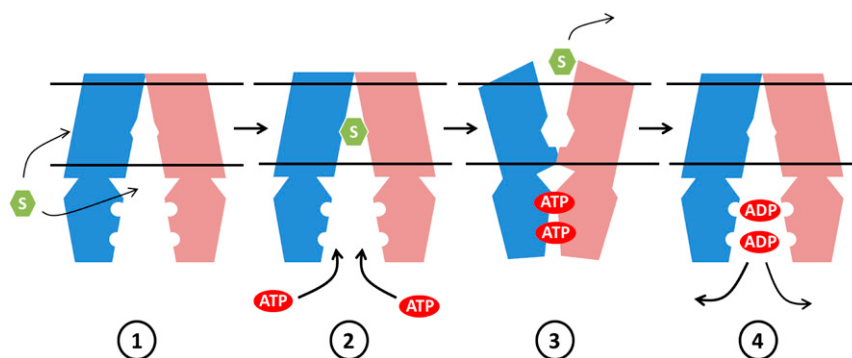


Figure 4. Model of the transport mechanism. 1, The substrate (S) enters the substrate pocket (bottom arrow) localized within the TMDs of the transporter (inward-facing conformation). When hydrophobic, the substrate accumulates within the membrane and laterally accesses the substrate pocket (top arrow). 2, Substrate binding triggers ATP binding. 3, ATP binding induces NBD dimerization and orientates the TMD to the outside (outward-facing conformation), which results in substrate release on the outside. 4, ATP hydrolysis converts the transporter to the starting inward-facing conformation. Note that variations of this model have been proposed. For instance, whether one or two ATPs are hydrolyzed per cycle is still unclear and might depend on the transporter. The two horizontal black lines represent the membrane limits..

detailed 3D structures of plant ABC transporters are experimentally determined, their modeling according to atomic structures of transporters from other kingdoms can be performed. This was the case for the Arabidopsis ABCB transporters involved in auxin transport (Bailly et al., 2012).

SUBSTRATES OF PLANT ABC TRANSPORTERS: AN OVERVIEW

Throughout the following section, each ABC subfamily will be briefly introduced and hallmark examples of substrate identification will be given, highlighting those for which direct transport measurements have been undertaken. Table 1 provides an overview of these data and illustrates the large diversity of substrates of the plant ABC transporters. Exhaustive information is provided for more transporters in Supplemental Table S1.

Subfamily A

Subfamily A consists of forward-oriented (TMD-NBD) transporters. One full-size and 11 half-size transporters have been identified in Arabidopsis. Only one half-size transporter, AtABCA9 (*Arabidopsis thaliana* ABC2 homolog11) has been characterized. It is involved in lipid metabolism by mediating the transport of fatty acids to the endoplasmic reticulum (Kim et al., 2013).

Subfamily B

Subfamily B also includes forward-oriented full- and half-size transporters. P-glycoprotein (PGP)/MDR are full-size proteins, whereas HMT/ABC transporters of the mitochondrion (ATM), transporters associated with antigen processing (TAP), and prokaryotic lipid

A-like exporters, putative, are half-size. The Arabidopsis genome codes for an impressive total of 29 ABCB proteins, the second most furnished subfamily after the ABCGs.

PGP/MDR

PGP/MDR transporters are the most studied ABCB transporters. In Arabidopsis, several of them (AtABCB1/AtPGP1/AtMDR1, AtABCB4/AtPGP4/AtMDR4, AtABCB19/AtPGP19/AtMDR11, and AtABCB21/AtPGP21) were shown to transport auxins (Supplemental Table S1). The spatiotemporal distribution of these phytohormones directs plant growth, and the asymmetrical distribution of auxin is governed by transporters. According to the transporter, different types of auxins are transported. However, varying data also were obtained depending on the expressing host. Although most of these proteins have been studied thoroughly for their ability to mediate auxin transport, some members also were found to mediate the translocation of other metabolites such as xenobiotics, malate, benzoic acid (BA), or alkaloids in both homologous and heterologous expression systems.

AtABCB1 expression in yeast and mammalian cells suggested a possible lack of specificity of the transporter, as BA also was transported (Geisler et al., 2005; Blakeslee et al., 2007), although this weak activity generally is used as a negative control for auxin transport in plant assays. Later, both AtABCB1 and AtABCB19 were expressed in *Schizosaccharomyces pombe* (Yang and Murphy, 2009; Kim et al., 2010; Bailly et al., 2014) and shown to mediate the efflux of radiolabeled indole-3-acetic acid (IAA), the main auxin, in this system as well. However, in this case, no clear export of radiolabeled BA was observed. Another example of conflicting results comes from AtABCB14, which is expressed in guard cells. Analysis of knockout plants seemed to

Table 1. Diversity of substrates of plant ABC transporters

Only transporters for which the substrates have been identified by direct transport (flow across a membrane) are listed. See text for details and references.

ABC Subfamily	Transporter Name	Substrate(s)
ABCA	AtABCA9	Fatty acids/acyl-CoA
ABCB	AtABCB1	Auxins
	AtABCB4	Auxins
	AtABCB14	Malate/auxins
	AtABCB15	Auxins
	AtABCB19	Auxins
	AtABCB21	Auxins
	AtABCB25	Glutathione polysulfides
	CjMDR1	Alkaloids
	CjABCB2	Alkaloids
	LeMDR1	Alkaloids
	LjABCB1	Auxins
	OsABCB14	Auxins
	TwMDR1	Alkaloids
	ZmABCB1	Auxins
ABCC	AtABCC1	Xenobiotics
	AtABCC2	Xenobiotics
	AtABCC3	Xenobiotics
	AtABCC4	Xenobiotics
	AtABCC5	Organic anions/phytate
ABCF	VvABCC1	Anthocyanins
	MdABCF1	S-RNase
ABCG	AaABCG3	Sesquiterpenes
	AtABCG14	Cytokinins
	AtABCG16	Jasmonic acid
	AtABCG25	ABA
	AtABCG29	Monolignols
	AtABCG30	ABA
	AtABCG34	Alkaloids
	AtABCG36	Auxins/cadmium
	AtABCG37	Auxins
	AtABCG39	Xenobiotics
	AtABCG40	ABA
	CrTPT2	Alkaloids
	MtABCG10	Isoflavonoids
	NbABCG1	Sesquiterpenes
	NbABCG2	Sesquiterpenes
	NtABCG1	Diterpenes
	NtABCG3	Coumarins
ABCI	NpABCG1	Diterpenes
	PaPDR1	Strigolactones
	PhABCG1	Volatiles
	VmABCG1	Alkaloids
	OsSTAR1/OsS-TAR2	UDP-Glc

indicate a possible link with malate transport. Direct import of malate by AtABCB14 was indeed demonstrated in both *Escherichia coli* and HeLa cells (Lee et al., 2008). However, this transporter was found later to be also expressed in vascular tissues, where it seems to act in the polar transport of auxin along with AtABCB15 (Kaneda et al., 2011). More comprehensive transport assays need to be performed to demonstrate the ability of these two transporters to transport auxins, as the defects in polar auxin transport might as well be due to altered xylem development. Later, auxin transporters

also were identified in rice, maize (*Zea mays*), and *Lotus japonicus* (Supplemental Table S1).

CjABCB1 (CjMDR1) was found to be highly expressed in the rhizome of *Coptis japonica*, and substrates were identified using three different expression systems. In *Xenopus laevis* (*Xenopus*) oocytes, CjABCB1 was found to mediate the import of the alkaloid berberine (Shitan et al., 2003). The specificity of the transporter was then assessed by performing drug sensitivity assays in *S. cerevisiae*. CjABCB1 transformants were more sensitive not only to berberine but also to 4-nitroquinoline *N*-oxide. Finally, CjABCB1 was expressed in *Catharantus roseus* suspension cells, and surprisingly, no transport of berberine was observed. However, the endogenous alkaloids ajmalicine and tetrahydroalstonine accumulated significantly more in cells expressing CjABCB1 than in control lines (Pomahacová et al., 2009). Later, another transporter from *C. japonica*, CjABCB2 (CjMDR2), also was found to be expressed in the rhizome, and expression in yeast demonstrated its ability to act as a berberine importer (Shitan et al., 2013).

HMT/ATM

The Arabidopsis genome encodes three HMT/ATM half-size ABCB transporters localized to the mitochondria and involved in the biogenesis of cytosolic iron-sulfur proteins (Kushnir et al., 2001; Chen et al., 2007; Bernard et al., 2009; Luo et al., 2012). However, direct identification of the actual substrates was only performed for AtABCB25 (AtATM3/AtSTA1). Indeed, upon its expression in *Lactococcus lactis*, a transportomics approach using membrane vesicles revealed that glutathione (GSH) polysulfides are likely to be the substrates serving as precursors for iron-sulfur cluster assembly (Schäedler et al., 2014). AtABCB25 also was suggested to be involved in cadmium (Cd) and lead tolerance, but no direct transport assays were performed with these substrates (Kim et al., 2006). Recently, an HMT/ATM transporter from rice, OsABCB23, also was found to be involved in the transport of iron-sulfur precursors (Zuo et al., 2017).

TAP

Out of the three TAP transporters present in Arabidopsis, only one, AtABCB27 (AtALS1/AtTAP2), has been characterized until now. The transporter was localized to the tonoplast and suggested to be involved in aluminum (Al) metabolism (Larsen et al., 2007). A tonoplast-localized TAP transporter from rice, OsABCB25 (OsALS1), was characterized and seems to be responsible for Al import into the vacuole (Huang et al., 2012). In *S. cerevisiae*, OsABCB25-expressing cells were more sensitive to Al toxicity, but no difference in internal Al contents was observed. However, this could be explained by the fact that the protein was not properly localized to the tonoplast but rather in other internal membranes in yeast cells. No Al transport was

observed when the protein was expressed in *Xenopus* oocytes (Huang et al., 2012).

Subfamily C

Subfamily C (15 members in *Arabidopsis*), previously known as MRP (multidrug resistance-associated proteins), is composed of forward-oriented (TMD-NBD) full-size ABC transporters, many of which contain an additional hydrophobic N-terminal extension. They were first identified after the observation that glutathionated compounds are actively transported into the vacuole and energized by ATP but independently of the proton motive force (Martinoia et al., 1993; Lu et al., 1997, 1998; Tommasini et al., 1998; Liu et al., 2001). The ability to transport GSH conjugates was demonstrated for many ABCC transporters expressed in yeast. The first reports demonstrated the transport of GSH S-conjugates in microsomal vesicles by AtABCC1 (AtMRP1) and AtABCC2 (AtMRP2; Lu et al., 1997, 1998). Later, Raichaudhuri et al. (2009) showed that AtABCC1 mediates the uptake of radiolabeled folates and antifolates, whereas AtABCC2 was found to transport glucuronate conjugates (Liu et al., 2001). In addition, both transporters also mediated Cd, mercury, and arsenic (As) tolerance by transporting phytochelatin (PC), a GSH oligomer, into the vacuole (Song et al., 2010, 2014a; Park et al., 2012). Growth tests revealed that cells expressing either AtABCC1 or AtABCC2 were more resistant to As than controls, but only in the presence of TaPCS1, a PC synthase from wheat (*Triticum aestivum*). In contrast to plants, yeast does not synthesize PCs. Direct transport performed with *S. cerevisiae* microsomal vesicles confirmed the ability of AtABCC1 or AtABCC2 to transport apoPC and PC₂-As (Song et al., 2010) as well as PC₂-Cd (Park et al., 2012; Song et al., 2014a) complexes. In addition, AtABCC2, and to a lesser extent AtABCC1, also transported ABA as its glucosyl ester ABA-GE (Burla et al., 2013). Finally, chlorophyll catabolites, herbicides, and organic anions also were proposed as putative substrates (Lu et al., 1998; Frelet-Barrand et al., 2008), adding to the long list of conjugated metabolites thought to be sequestered to the vacuole by these transporters.

In vitro assays using yeast microsomal vesicles showed that AtABCC3 (AtMRP3) transports GSH-conjugated chlorodinitrobenzene and the chlorophyll catabolite Bn-NCC-1 (Tommasini et al., 1998). More recently, AtABCC3 also was shown to confer resistance to high Cd, probably by mediating the vacuolar sequestration of phytochelatin-Cd complexes (Bovet et al., 2003; Brunetti et al., 2015). Transport of the antifolate methotrexate by AtABCC4 was demonstrated using yeast microsomes (Klein et al., 2004). Microsomes isolated from *S. cerevisiae* cells expressing AtABCC5 displayed ATP-dependent transport of estradiol-17-(β -D-glucuronide) (Gaedeke et al., 2001). Expression in HEK293 mammalian cells led to an increase in the specific binding of radiolabeled glibenclamide, a sulfonylurea known to induce stomatal

opening in plants (Lee et al., 2004). Later, Nagy et al. (2009) showed that AtABCC5 mutant seeds displayed a particularly low phytate (inositol hexakisphosphate) content. By performing transport experiments using yeast microsomal vesicles, the transport of phytate by AtABCC5 was confirmed (Nagy et al., 2009).

VvABCC1 was found to act as a vacuolar transporter of anthocyanins in ripening grape berry (*Vitis vinifera*; Francisco et al., 2013). Direct transport of malvidin 3-O-glucoside was demonstrated using microsomes isolated from ABCC1-expressing *S. cerevisiae* cells. Interestingly, transport was greatly enhanced in the presence of GSH, which seems to be cotransported with the anthocyanins, but without the formation of an anthocyanin-GSH conjugate.

Rice OsABCC1, which localizes to the tonoplast, was studied for its involvement in As accumulation in the grain (Song et al., 2014b). Knockout plants were more sensitive to As and accumulated significantly more As in their grain. The correlation of metal concentrations with the expression of OsABCC1 suggests that the transporter restricts the distribution of As to the grain by sequestering it into the vacuoles of vascular bundle companion cells connected directly to the grain. Toxicity assays performed with OsABCC1-expressing yeast cells revealed that PC is most likely necessary for the vacuolar sequestration of As, similar to what was observed for AtABCC1 in *Arabidopsis*.

Subfamily D

Subfamily D contains forward-oriented (TMD-NBD) half-size and full-size proteins. The full-size peroxisomal AtABCD1/PXA1/PED3/PMP2/CTS was first described for its involvement in the peroxisomal import of substrates for β -oxidation and the glyoxylate cycle (Zolman et al., 2001; Footitt et al., 2002, 2007; Hayashi et al., 2002; Hooks et al., 2007; Kunz et al., 2009; Park et al., 2013). However, AtABCD1 also has been suggested to play a role in the biosynthesis of jasmonates, which are lipid-derived signaling molecules, by importing a jasmonic acid precursor and/or its acyl-CoA derivative into the peroxisome (Theodoulou et al., 2005). AtABCD1 was expressed in *S. cerevisiae* and found to localize to peroxisomes as it is the case in plants. ATPase assays on a purified peroxisomal fraction revealed stimulation by fatty acyl-CoAs and sensitivity to aluminum fluoride. Moreover, the *S. cerevisiae* *pxa1 pxa2 Δ* mutant, lacking endogenous peroxisomal transporters and unable to grow on oleic acid, was complemented by AtABCD1 (Nyathi et al., 2010). Later, De Marcos Lousa et al. (2013) used the same yeast strain to show that the transport of oleic acid by AtABCD1 required peroxisomal acyl-CoA synthetases and to demonstrate a functional and physical interaction between the transporter and acyl-CoA synthetases. Other reports also indirectly linked AtABCD1 to the synthesis of multiple secondary metabolites, namely BA, probably by importing cinnamic acid/cinnamoyl-CoA into the peroxisome (Bussell et al., 2014); flavonoids,

by mediating fatty acid breakdown, ultimately leading to the induction of flavonoid biosynthetic enzymes (Carrera et al., 2007); ubiquinone, likely by importing *p*-coumaric acid/coumaroyl-CoA (Block et al., 2014); acetate (Hooks et al., 2007); and indole 3-butyric acid (IBA)/IBA-CoA (Park et al., 2013).

Two peroxisomal transporters from barley (*Hordeum vulgare*), HvABCD1 and HvABCD2, also were found to be involved in fatty acid β -oxidation (Mendiondo et al., 2014). Indeed, the expression of both proteins in *Arabidopsis* complemented an *atabcd1* mutant, and the expression of HvABCD1 in yeast partially complemented *pxa1 pxa2Δ* for fatty acid β -oxidation.

Subfamilies E and F

Subfamilies E and F are composed of soluble ABC proteins that possess two NBDs but no TMD and, therefore, probably are not involved per se in transport processes unless associated with other membrane partners. MdABCF1 (MdABCF) from apple (*Malus domestica*) is involved in the uptake of *S*-RNase in pollen tubes (Meng et al., 2014). Bimolecular fluorescence complementation and yeast two-hybrid assays showed a direct interaction between the protein and *S*-RNase, and down-regulation of the protein in pollen tubes reduced their *S*-RNase uptake capacity.

Subfamily G

In contrast with other subfamilies, ABCG transporters are characterized by a reverse orientation (NDB-TMD). Subfamily G is composed of both half-size proteins, known as white-brown complex (WBC) transporters, and full-size proteins, known as pleiotropic drug resistance (PDR) transporters. This subfamily is by far the largest in plants (43 members in *Arabidopsis*), and its outstanding diversification is thought to be correlated with the adaptation of plants to the land environment (Hwang et al., 2016). Indeed, in land plants, these transporters have been linked with cuticle formation, defense mechanisms, hormone transport, and seed germination, all of which are essential traits for survival on land. Subfamily G is the most represented according to the number of publications dealing with substrate identification (Supplemental Table S1).

WBC

The first plant WBC transporter to be characterized was GhABCG1 (GhWBC1) from cotton (*Gossypium hirsutum*; Zhu et al., 2003). As for all other WBC transporters described until now, GhABCG1 was found to localize to the plasma membrane. By analogy to its human homologs, it was proposed to transport lipids and was found to be necessary for the proper development and elongation of cotton fibers. Since the initial report, many other half-size ABCG transporters were found to be involved in the translocation of hydrophobic compounds such as lipids or sterols (Supplemental Table S1). Several cases have been studied in more detail.

Several WBC transporters were suggested to be involved in the translocation of cutin precursors across membranes for the deposition of the plant cuticle (Pighin et al., 2004; Luo et al., 2007; Panikashvili et al., 2007, 2010, 2011; Ukitsu et al., 2007; Le Hir et al., 2013; Li et al., 2013; Qin et al., 2013; Zhu et al., 2013b; Wu et al., 2014). Multiple WBC transporters in *Arabidopsis* and rice also were described as being involved in exine biosynthesis in pollen grains, probably by mediating the transport of sporopollenin precursors (Quilichini et al., 2010, 2014; Choi et al., 2011, 2014; Le Hir et al., 2013; Qin et al., 2013; Wu et al., 2014; Yadav et al., 2014; Zhao et al., 2015; Chang et al., 2016; Yim et al., 2016). Similarly, several transporters were found to be involved in the translocation of suberin precursors and Casparian strip biogenesis (Landgraf et al., 2014; Shiono et al., 2014; Yadav et al., 2014; Yamauchi et al., 2015; Fedi et al., 2017). However, until now, no direct assay was able to demonstrate the transport of lipidic molecules by any WBC transporter; thus, substrates have been proposed solely by analyzing mutant and/or overexpressing phenotypes (Supplemental Table S1). The main obstacle to performing direct transport, of course, is the hydrophobic nature of these metabolites, which makes such assays very difficult to carry out using conventional methods.

In addition to the lipidic substrates described above, half-size ABCG transporters also were shown to be involved in the translocation of very diverse metabolites. Direct transport experiments were performed for a few of these.

AtABCG16/AtJAT1 was selected in *S. cerevisiae* for its capacity to confer resistance to toxic concentrations of jasmonic acid (Li et al., 2017). Moreover, the *AtABCG16*-expressing yeast cells acquired the capacity to export jasmonic acid. In the plant, *AtABCG16* localized to the plasma membrane as well as to the nuclear envelope. Interestingly, the transported substrate seemed to differ according to the localization: jasmonic acid at the plasma membrane and jasmonoyl-Ile, a biologically active analog, at the nuclear envelope. Since *AtABCG16* is a half-size transporter, the authors suggest that the different subcellular localization and substrates might be linked to the formation of different homodimers/heterodimers (Li et al., 2017).

AtABCG19 (*AtWBC19*) was reported to confer specific resistance to kanamycin in *Arabidopsis* (Mentewab and Stewart, 2005). It was later found that, when expressed in *Populus tremuloides*, this gene also conferred tolerance to three other aminoglycoside antibiotics (Kang et al., 2010a). Furthermore, although no transport assays were performed, the same transporter was later linked to metal homeostasis, as mutant seedlings showed altered transcriptome and reduced iron uptake upon exposure to kanamycin, in addition to a decreased zinc/copper content (Mentewab et al., 2014).

Three WBC transporters from *Arabidopsis* and one from rice were proposed recently to act in the cellular translocation of ABA. This phytohormone is of crucial

agronomical importance, as it induces the expression of stress-related genes leading, among others, to stomatal closure when plants are exposed to drought. AtABCG25 (AtWBC26) was isolated by genetic screening for ABA sensitivity, and mutants were found to display several ABA-related phenotypes, such as impaired stomatal movement and germination (Kuromori et al., 2010; Kang et al., 2015). Interestingly, this gene was expressed in *Sf9* insect cells, and it was the first report of the use of this expression system to analyze the transport activity of plant ABC transporters. The ATP-dependent uptake of ABA in vesicles expressing AtABCG25 was observed (Kuromori et al., 2010) and later confirmed by performing cellular transport assays with the same expression system (Kuromori et al., 2014). AtABCG22 also was found to regulate stomatal aperture (Kuromori et al., 2011), whereas AtABCG16 (AtWBC16) was linked with ABA tolerance upon pathogen infection (Ji et al., 2014). Finally, OsABCG5 (OsRCN1) from rice also was proposed recently to act in ABA translocation in guard cells, in addition to its function in root suberization (Matsuda et al., 2016), again based merely on physiological observations.

In two concomitant reports, AtABCG14 was found to be involved in the root-to-shoot translocation of another essential class of phytohormones, cytokinins (Ko et al., 2014; Zhang et al., 2014). These growth-promoting signaling molecules facilitate communication between belowground and aboveground structures. The transporter was localized at the plasma membrane of the root pericycle, phloem, and procambial cells, in which cytokinin biosynthesis genes are expressed. The *atabcg14* mutant exhibited severe growth retardation, and levels of trans-zeatin-type cytokinins were reduced severely in shoots and increased in roots of the mutant. Translocation assays using endogenous radiolabeled trans-zeatin confirmed the importance of AtABCG14 in the xylem loading of the hormone for long-distance transport.

Finally, one half-size ABCG transporter, PhABCG1, was found to be involved in the active export of volatile organic compounds from petunia (*Petunia hybrida*) flowers. Heterologous expression in tobacco Bright Yellow-2 (BY-2) suspension cells was achieved, and direct transport of volatile benzenoids was demonstrated (Widhalm et al., 2015; Adebessin et al., 2017).

PDR

In contrast to WBC proteins, which are found in all phyla, PDR transporters are present only in plants and fungi. They were first characterized in the yeast *S. cerevisiae* (Servos et al., 1993; Balzi et al., 1994; Bissinger and Kuchler, 1994), the genome of which encodes nine PDR proteins (Decottignies and Goffeau, 1997). Some yeast PDR transporters have been shown to be involved in the resistance to a large set of functionally and structurally unrelated compounds, such as fungicides, herbicides, pesticides, antibiotics, and detergents (Rogers et al., 2001). In plants, PDR transporters are involved in

the response to biotic and abiotic stress, and different members transport a wide diversity of substrates.

The first indication that PDR transporters could be involved in the secretion of defense-related metabolites was obtained by Jasiński et al. (2001). The authors identified NpABCG1 (NpPDR1) from *Nicotiana plumbaginifolia* and provided evidence that this plasma membrane PDR transports sclareol, an antifungal diterpene secreted by trichomes (leaf hairs). Many other plant PDR transporters then were found to be involved in the response to biotic stress. In tobacco, *NtABCG1* (*NtPDR1*) expression was found to be induced by sclareol and cembrene, and the transport of both diterpenes by *NtABCG1* was demonstrated in tobacco BY-2 cells (Crouzet et al., 2013). In addition, stimulation of the ATPase activity of purified *NtABCG1* by these substrates as well as by the sesquiterpene capsidiol was demonstrated recently (Pierman et al., 2017). PhPDR2 from petunia was reported recently to act as a putative exporter of toxic steroids in trichomes. Mutant plants were found to be more susceptible to *Spodoptera littoralis* infection, and mutant trichomes displayed reduced petuniasterone and petuniolide contents (Sasse et al., 2016). Two functionally redundant transporters from *Nicotiana benthamiana*, NbABCG1 (NbPDR1) and NbABCG2 (NbPDR2), were found to be essential for resistance against *Phytophthora infestans*, and the double mutant was found to reduce the secretion of the sesquiterpene capsidiol, the major *Nicotiana* spp. phytoalexin involved in postinvasion defense (Shibata et al., 2016; Rin et al., 2017). As both transporters also were found to be involved in preinvasion defense, the authors also suggest that they could additionally transport diterpenes, but this remains to be demonstrated.

CrABCG1 (CrTPT2) from *Catharanthus roseus* and VmABCG1 (VmTPT2) from *Vinca minor* were found to be involved in the secretion of monoterpenoid indole alkaloids at the leaf surface, thereby also suggesting a role in herbivore defense. Both transporters were expressed in *S. cerevisiae* and found to mediate the efflux of catharanthine and vincamine, respectively (Yu and De Luca, 2013; Demessie et al., 2017). In *Medicago truncatula*, MtABCG10 was found to act as a transporter of isoflavonoid precursors, thereby also regulating plant-microbe interactions. Indeed, mutant plants displayed reduced levels of medicarpin and were more sensitive to pathogen infection. Moreover, direct transport of the phenylpropanoid precursors liquiritigenin and 4-coumarate could be demonstrated by expressing the protein in tobacco BY-2 cells (Banasiak et al., 2013; Biala et al., 2017). Recently, AaABCG3 (AaPDR3) expressed in *Artemisia annua* T-shaped trichomes was proposed to mediate the transport of the sesquiterpene β -caryophyllene. Indeed, the level of β -caryophyllene was decreased in loss-of-function plants and increased when the transporter was overexpressed. In addition, yeast cells expressing AaABCG3 specifically accumulated more caryophyllene than control cells (Fu et al., 2017).

AtABCG34 (AtPDR6) was first suggested to be involved in the root exudation of organic acids (Badri

et al., 2008). However, a recent report showed its involvement in the defense against necrotrophic fungi by secreting camalexin, a major Arabidopsis phytoalexin, at the leaf surface (Khare et al., 2017). Mutant plants displayed increased sensitivity to *Alternaria brassicicola* and *Botrytis cinerea* and secreted less camalexin than the wild type. The expression of AtABCG34 in tobacco BY-2 cells conferred resistance to camalexin, thereby further confirming the in planta data. AtABCG36 (AtPDR8/AtPEN3) is one of the best described examples of a PDR transporter for which multiple substrates have been proposed. Among these are compounds involved in plant defense against pathogens. AtABCG36 was found to contribute to nonhost resistance against fungal pathogens (Kobae et al., 2006; Stein et al., 2006; Campe et al., 2016). It is localized at the plasma membrane, recruited to the infection site (Underwood and Somerville, 2013), and involved in glucosinolate-dependent pathogen defense (Clay et al., 2009). The identity of the chemicals secreted by AtABCG36 is still unknown, but indole-type metabolites, precursors of 4-O- β -D-glucosyl-indo-3-yl formamide, were proposed as substrates (Lu et al., 2015).

The knockout mutants of two Arabidopsis PDR transporters, AtABCG36 and AtABCG37 (AtPDR9/AtPIS1), are hypersensitive to auxinic compounds (Ito and Gray, 2006; Strader and Bartel, 2009). Using various heterologous expression systems, AtABCG37 was reported to transport a range of auxinic compounds, including the IAA precursor IBA, but not free IAA. As both transporters localize to the outer side of root cells, they were suggested to export IBA to the rhizosphere (Strader and Bartel, 2009; Ruzicka et al., 2010). However, as described above, auxins are not the only putative substrates of AtABCG36 and AtABCG37, and the physiological relevance of IBA transport by these transporters remains to be elucidated.

Strigolactones were first identified as growth stimulants for the parasitic weed *Striga*, but they are better known for their ability to induce the hyphal branching of mycorrhizal fungi, thereby greatly facilitating mycorrhization. Based on the observation that PDR transporters could secrete diterpenes, Kretschmar et al. (2012) postulated that sesquiterpene lactones also could be excreted by a transporter of this family. They indeed observed that PhPDR1 expression from petunia was induced by strigolactones. Furthermore, PhPDR1 mutant plants displayed reduced strigolactone secretion and strongly delayed mycorrhization. In addition, heterologous expression of PaPDR1, the homolog of PhPDR1 from *Petunia axillaris*, in Arabidopsis conferred increased resistance to synthetic strigolactones (Kretschmar et al., 2012). In a more recent report (Sasse et al., 2015), PaPDR1 was found to display a dual polar localization within roots and, therefore, also presumably mediates the root-to-shoot transport of strigolactones. All these results strongly suggest that PhPDR1/PaPDR1 act as strigolactone transporters. The tobacco strigolactone transporter, NtABCG6 (NtPDR6), also has been identified and was found to be involved in shoot branching (Xie et al., 2015).

Monolignols are phenolic compounds (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) acting as monomeric precursors for the synthesis of lignin, the defining constituent of wood. They are synthesized in the cytosol and, therefore, must be transported across cellular membranes to reach the cell wall, where they are oxidized and polymerized. Miao and Liu (2010) performed extensive in vitro assays using vacuolar and plasma membrane vesicles to demonstrate that monolignol transport across the plasma membrane and their sequestration into vacuoles are likely to involve ABC transporters. However, they could not identify the transporter responsible for their observation. Two years later, AtABCG29 was reported to be the plasma membrane monolignol transporter in Arabidopsis. Indeed, AtABCG29 loss-of-function mutants displayed lignification defects and were more sensitive to exogenous *p*-coumaryl alcohol supply. Moreover, the direct transport of *p*-coumaryl alcohol by AtABCG29 could be demonstrated in yeast cells and microsomes (Alejandro et al., 2012).

In addition to the half-size AtABCG25 transporter described above, three PDR transporters were found to be involved in ABA transport in Arabidopsis. Whereas AtABCG25 mediates ABA export (Kuromori et al., 2014), AtABCG40 acts as an ABA importer. Indeed, overexpression of AtABCG40 leads to an increased ABA accumulation in tobacco BY-2 cells as well as in yeast (Kang et al., 2010b). More recently, two additional PDR transporters, AtABCG30 and AtABCG31, were found to act as ABA importer and exporter, respectively. Both transporters work in a coordinated manner in the endosperm (AtABCG31) and the embryo (AtABCG30) of Arabidopsis seeds to suppress seed germination until conditions are favorable (Kang et al., 2015). This work also confirmed the previously described functions of AtABCG25 (Kuromori et al., 2010) and AtABCG40 (Kang et al., 2010b) in seed germination.

Other PDR-type transporters (AtABCG36, OsABCG36, CsABCG36, and CsABCG40; Supplemental Table S1) were found to be induced by multiple abiotic stresses, but the direct contribution of ABA still remains to be demonstrated.

Subfamily I

Plant ABCI proteins bear similarities with bacterial multimeric-type ABC transporters, and several members of this family were predicted to be targeted to the chloroplast or mitochondria. These protein systems are presumed to assemble with other subunits in planta to form functional ABC transport systems. This is the case of the mitochondrial cytochrome *c* maturation complex, the chloroplast iron-sulfur center biogenesis complex, the chloroplast trigalactosyldiacyl glycerol complex, and the chloroplast cobalt uptake system (Supplemental Table S1).

Plant ABCI proteins that do not relate to existing families in other organisms also were characterized. In Arabidopsis, this is the case for AtABCI16 (AtALS3/

AtSTAR1), a membrane domain that was proposed to be involved in UDP-Glc transport. Indeed, mutant plants were found to be more sensitive to Al toxicity and phosphate starvation than the wild type, and this second phenotype could be rescued by exogenous application of UDP-Glc (Larsen et al., 2005; Gabrielson et al., 2006; Huang et al., 2010; Dong et al., 2017). The rice homolog, OsSTAR2, also was found to be responsible for Al tolerance in rice mediated by UDP-Glc. OsSTAR2 is a TMD that interacts with OsSTAR1, an NBD. The interaction between both moieties was shown by bimolecular fluorescence complementation in onion (*Allium cepa*) epidermal cells (Huang et al., 2009). Here again, exogenous application of UDP-Glc could rescue the growth defect of mutants exposed to Al toxicity. Direct transport was then demonstrated by coinjecting *Xenopus* oocytes with RNA coding for OsSTAR1 and OsSTAR2 and monitoring the efflux of radiolabeled UDP-Glc (Huang et al., 2009).

HOW TO IDENTIFY SUBSTRATES OF ABC TRANSPORTERS

In many cases, substrates are inferred from phenotypes of plants that no longer express, or that over-express, an ABC transporter. Metabolite profiling of tissue extracts and/or of the exudate for those transporters that are localized to the plasma membrane might suggest possible substrates. However, caution is required because artifactual results might occur. For instance, preventing the expression of an ABC transporter involved in the secretion of a hormone might have consequences on various metabolic pathways.

The activity of a transporter is vectorial: a substrate is moved from one compartment to another one. Testing a transporter thus requires the possibility to follow the transfer of a substrate from one compartment to another one. The more direct approach consists of expressing the transporter of interest in a heterologous cellular system, such as yeast or plant suspension cells. As the substrate is normally not synthesized by the host, it has to be added in the incubation medium. For a plasma membrane-localized importer, the internal concentration of the substrate is expected to increase upon expression of the transporter. For a plasma membrane exporter or for a transporter localized to internal membranes, there is a prerequisite that the substrate diffuses to some extent within the cells before being transported back to the external medium or within organelles if the transporter is expressed. Since many of the substrates at a certain concentration are toxic to the cell, an alternative route consists of comparing the cell growth of wild-type and ABC-expressing cells in the presence of increasing concentrations of the putative substrate.

A more direct way to follow transport consists of purifying sealed membrane vesicles from the expressing host. There are tools to obtain inverted vesicles with the ATP-binding site on the outer face (Sze, 1985;

Larsson et al., 1994; Johansson et al., 1995). In this case, an exporter is expected to transport within the vesicle. ATP and the substrate are added to the incubation medium, and the accumulation of the substrate into the vesicle can be readily measured. If there is any doubt that the measured transport does not occur through the transporter that is expressed, this transporter can be solubilized, purified, and eventually reconstituted in artificial lipid vesicles and transport can be measured as for native vesicles. An important issue here can be substrate presentation, especially for hydrophobic compounds. This approach, however, is laborious and time consuming. Many, but not all, ABC transporters have their ATP hydrolysis activity stimulated in the presence of the transported substrates (Decottignies et al., 1994; Herget et al., 2009; Kaur et al., 2016). This property can be used to identify substrates indirectly. This method, which avoids the necessity of a vectorial system, was applied recently to characterize the substrate specificity of AtABCD1 (Nyathi et al., 2010), AtABCB25 (Schädler et al., 2014), and, more recently, NtABCG1 (Pierman et al., 2017). However, a negative result cannot easily be interpreted, since there are ABC transporters whose ATP hydrolysis activity is not stimulated by their substrates. A positive result also needs caution, because this method does not distinguish a molecule that is actually transported from a molecule that might stimulate the ATPase activity without being transported.

When there is no clue concerning the substrate or when the possibility of several structurally distinct substrates (i.e. pleiotropy) has to be evaluated, at least two approaches can be undertaken (Fig. 5). One relies again on the possible activation of ATPase activity by the substrates. As activity can be followed by colorimetric assays compatible with automation, membrane vesicles or purified ABC transporters can be used for the high-throughput screening of large collections of chemicals. Another approach is referred to as transportomics and consists of mixing sealed membrane vesicles containing the ABC transporter with all the metabolites extracted from tissues in which the protein is expressed (Krumpalova et al., 2012). After incubation with ATP, the internal content of the vesicles is analyzed by gas chromatography or HPLC-based metabolomics. This technique has been used successfully to characterize AtABCB25 (Schädler et al., 2014). The interesting point of this method is that it focuses on physiologically relevant substrates, which can putatively be in contact with the transporter. It also measures transport in conditions near the *in vivo* situation, where competition between many compounds occurs. Now, it should be stressed that the transport of hydrophobic molecules (this concerns many secondary metabolites) might be more challenging, because these compounds diffuse freely through the membrane and interfere with the monitoring of the transport activity catalyzed by the ABC transporters. As a matter of fact, one role of some ABC transporters might be to expel from membranes hydrophobic compounds whose

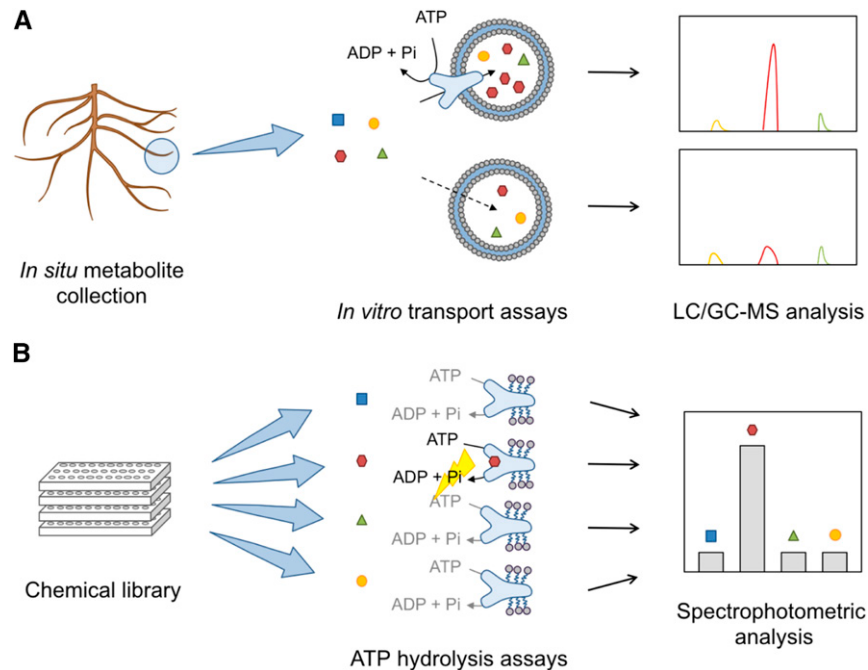


Figure 5. Schematic workflow of two complementary approaches to assess the putative pleiotropy of ABC transporters. A, Transportomics consists of monitoring the transport of physiologically relevant substrates under conditions allowing competition to occur. As a first step, metabolites from wild-type and mutant organs are extracted under conditions in which the transporter of interest is expressed. These extracts are then incubated in the presence of ATP and membrane vesicles with and without the transporter. Vesicles are purified subsequently, and metabolites accumulated in the vesicles are determined by liquid chromatography/gas chromatography-mass spectrometry (LC/GC-MS)-based metabolomics. Substrates actively transported should accumulate at higher levels in vesicles that contain the transporter than in those that do not. B, Activity assays consist of monitoring the ATPase activity of a candidate transporter in the presence of putative substrates. In this case, individual substrates are brought in contact with the purified transporter. Most, but not all, ABC transporters have their ATPase activity stimulated in the presence of transported substrates. ATPase activity can be monitored easily by spectrophotometric assays compatible with automation.

local concentration might threaten the membrane integrity Box 1.

WHICH EXPRESSION SYSTEM TO USE TO IDENTIFY SUBSTRATES OF ABC TRANSPORTERS

Several different expression systems have been used to produce plant ABC transporters and identify their substrates (Fig. 2; Supplemental Table S1). Each system has its own advantages and disadvantages, yet yeast remains the most used model for heterologous expression. This brief survey of the systems used to characterize plant ABC transporters illustrates that guidelines, but no general rules, can be drawn.

Although *E. coli* often is used to obtain large amounts of recombinant proteins for functional and structural studies, only one full-size ABC transporter has been partially characterized using this system (Lee et al., 2008). This illustrates the fact that bacterial hosts often are not convenient for expressing plant membrane proteins. Nevertheless, *Lactococcus lactis* was used to express, purify, and assess the transport activity of AtABCB25, which makes it a valuable alternative ex-

pression system (Schaedler et al., 2014), especially given its relative ease to obtain inside-out vesicles.

The yeast *S. cerevisiae* appears to be a useful expression system to identify substrates of plant ABCB- and ABCC-type transporters at the cellular level. Moreover, interaction studies between plant ABCI proteins or one ABC transporter with other proteins (i.e. PIN transporters) could be investigated efficiently using the yeast two-hybrid or split-ubiquitin system (Supplemental Table S1). However, the expression of transporters of the ABCG subfamily in *S. cerevisiae* appears to be more problematic. Indeed, SpABCG1, AtABCG37, and NpPDR5 did not localize properly to the plasma membrane in this host. Therefore, phenotypic characterization examined through growth or transport assays was difficult to interpret (van den Brûle et al., 2002; Ito and Gray, 2006; Ruzicka et al., 2010; Tous-saint et al., 2017). Yang and Murphy (2009) shifted to the yeast *S. pombe* and optimized strains and vectors to functionally characterize auxin transporters. Since that report, heterologous expression of AtABCG37 in *S. pombe* was used successfully to demonstrate the transport of the auxin IBA (Ruzicka et al., 2010).

BOX 1. Why is a Transporter Needed?

When discussing the role of a transporter, two aspects need to be considered: kinetics and thermodynamics. Kinetics deals with the rate of transport. Free diffusion of a molecule through a membrane depends on its hydrophobicity and its size. A small and hydrophobic metabolite readily diffuses through a membrane while a hydrophilic compound, especially of large size, does not cross a membrane unless diffusion is facilitated by a transporter. Thermodynamics is related to energy and, in particular, to the concentration gradient of a molecule that can be built across a membrane. If no source of energy is provided, the transport remains passive, following Fick's law. In this case, the concentration in the receiving compartment can never be higher than that in the donor compartment. There are circumstances where this can be problematic: for instance, when a compound has to be evacuated from a compartment because of its toxicity. This is particularly the case of hydrophobic molecules,

which can concentrate within membranes and disrupt their integrity. An example of this is diterpenes, which are massively synthesized in, and secreted from, trichome cells of tobacco. These metabolites are highly hydrophobic and thus diffuse across the plasma membrane, yet they require ABC transport to maintain their internal concentration as low as possible, thereby avoiding cytotoxicity (Crouzet et al. 2013). As another example, emission of volatiles was thought to occur solely by diffusion. Modeling of their transport in the petal or leaf epidermis concluded that simple diffusion of volatiles through the plasma membrane would result in a too high residual concentration in the plasma membrane and, as a consequence, in cell intoxication (Widhalm et al. 2015). This was experimentally demonstrated upon the identification of an ABC transporter involved in the secretion of benzenoid volatiles in petunia (Adebesin et al. 2017).

The vaccinia virus/T7 RNA polymerase expression system in human HeLa cells has become a standard for assaying mammalian ABCB proteins. Therefore, this system was used predominantly for transport measurements at the cellular level of plant P-glycoprotein-like transporters (Geisler et al., 2005) and some other full-size ABC transporters (Lee et al., 2004; Kang et al., 2010b; Ruzicka et al., 2010).

Expression in insect cells and *Xenopus* oocytes appears to be unusual for plant ABC transporters. Indeed, few transporters were expressed successfully and characterized using each system. In *Xenopus* oocytes, two techniques were assayed for transport assays: the uptake of a substrate quantified by its fluorescence and by HPLC analysis (Shitan et al., 2003; Xu et al., 2016) and the depletion of a radiolabeled substrate confirmed by an electrophysiological technique (Huang et al., 2009). In two other cases, however (AtABCG37 and OsABCB25), transport assays in *Xenopus* oocytes failed (Ito and Gray, 2006; Huang et al., 2012), possibly because the proteins did not localize properly to the plasma membrane. It is worth mentioning here that, despite their limited use in expressing plant ABC transporters, insect cells are a popular expression system for mammalian ABC, especially when purification and reconstitution are intended.

Finally, heterologous expression in other plant species also was used to efficiently characterize ABC transporters. Transport assays at the cellular level also were achieved with plant material, working on isolated protoplasts or suspension cells (Kang et al., 2010b; Miao

and Liu, 2010; Kubeš et al., 2012; Adebesin et al., 2017; Biala et al., 2017; Khare et al., 2017; Miao et al., 2017; Pierman et al., 2017). In the latter case, tobacco BY-2 cells offer several advantages: they grow rapidly in a simple medium and can be genetically transformed by *Agrobacterium tumefaciens* or biolistics (Nagata et al., 1992).

MONOSPECIFICITY OR POLYSPECIFICITY?

Several ABC transporters exhibit poor substrate specificity and are able to transport functionally and structurally unrelated compounds. This feature was at the origin of alternative ABC subfamily names such as MDR in animals and PDR in yeast. For instance, PDR5 from *S. cerevisiae* and the human ABCB1 (MDR1/P-glycoprotein) are able to export hundreds of chemically unrelated compounds. From a structural perspective, P-glycoprotein was found to exhibit a large 6,000 Å³ internal cavity with 30 Å separating the two NBDs and is predicted to contain at least seven different drug-binding sites (Safa, 2004; Aller et al., 2009). Yet, because transporter-drug interactions are believed to be very dynamic, the mechanism of multidrug recognition and selection is still unknown. Concerning PDR5, multiple binding regions were identified, and it seems that molecular volume is the key determinant for substrate selection: efficient substrates have surface volumes of 200 to 300 Å³, while substrates that are smaller than 90 Å³ are transported with low efficiency (Golin et al.,

2000, 2003). Other ABC transporters seem to be much more specific regarding their substrate. As depicted in Supplemental Table S1, there is no clear example so far of a plant ABC transporter for which a very large substrate profile has been identified unambiguously. Yet, there are cases for which at least two different families of compounds with very different structures have been proposed. For instance, in addition to the auxinic compounds IBA and 2,4-dichlorophenoxyacetic acid (Strader and Bartel, 2009), AtABCG36 also was suggested to be involved in the transport of toxins (Kobae et al., 2006; Stein et al., 2006; Campe et al., 2016), callose precursors (Clay et al., 2009), Cd (Kim et al., 2007), or thymidine (Ziegler et al., 2017). Yet, no direct transport experiment was performed at the cellular or vesicular level in any case. In contrast, the transport of both 2,4-dichlorophenoxyacetic acid and IBA was demonstrated at the cellular level for AtABCG37 (Ruzicka et al., 2010). However, this transporter also was suggested to be involved in the secretion of coumarins in response to iron deficiency (Rodríguez-Celma et al., 2013; Fourcroy et al., 2014; Ziegler et al., 2017). This was demonstrated by transport assays carried out with NtABCG3/NtPDR3, its tobacco homolog, expressed in BY-2 cells. In addition, these results suggested that glycosylated coumarins, rather than free coumarins, are transported out of the cell (Lefèvre et al., 2018). Whether AtABCG37 is involved directly in the export of both coumarins and auxinic compounds still remains to be determined.

A third example of ABC transporter suggested to be involved in the translocation of structurally unrelated compounds is that of the peroxisomal protein AtABCD1. Characterization of mutant plants suggested fatty acyl-CoA (Footitt et al., 2002; De Marcos Lousa et al., 2013), IBA/IBA-CoA (Park et al., 2013), jasmonate precursors (Theodoulou et al., 2005), cinnamic acid/cinnamoyl-CoA (Bussell et al., 2014), and *p*-coumaric acid/coumaroyl-CoA (Block et al., 2014) as putative substrates. However, only its involvement in fatty acid translocation was investigated at the biochemical level by performing both influx and ATPase stimulation experiments (Nyathi et al., 2010; De Marcos Lousa et al., 2013). Nevertheless, it is tempting to speculate that the transporter recognizes the CoA esterified moiety and that all these diverse substrates in fact share a common structural feature. A last example of the putative pleiotropy of plant ABC transporters is that of CjMDR1 from *C. japonica*. Yeast sensitivity assays found that the transporter could translocate the alkaloid berberine, reticuline and its precursor berberine, as well as 4-nitroquinoline *N*-oxide, a quinolone derivative. However, CjMDR1 still demonstrated some specificity, as other chemicals such as quinine or cycloheximide did not seem to be recognized by the transporter (Shitan et al., 2003). A later report even made the model more complex, as CjMDR1 heterologously expressed in *C. roseus* suspension cells did not mediate berberine transport but did translocate endogenous alkaloids (Pomaháčová et al., 2009).

The examples described above raise the problem that very few plant ABC transporters have been tested for their specificity. Meaning that at least two putative substrates, close or distant analogs, have been tested by indirect and/or direct assays.

In contrast, some plant ABCs have shown quite high substrate specificity. This is the case of the Arabidopsis transporter AtABCB1, which can only transport a specific subset of auxins and is able to discriminate between the closely related synthetic auxins 1-naphthalene acetic acid (1-NAA) and 2-NAA (Geisler et al., 2005). Other examples are those of the ABA transporters AtABCG25 and AtAABCG40, which can discriminate between the *S*- and *R*-enantiomers of this hormone (Kang et al., 2010b; Kuromori et al., 2010). The example of AtABCG40 is quite typical of the caution that needs to be taken when assessing substrates to ABC transporters based on physiological data. Indeed, AtABCG40 was first thought to behave in a pleiotropic way, with putative substrates ranging from diterpenes (Campbell et al., 2003; Seo et al., 2012) to lead-related metabolites (Lee et al., 2005; Zhu et al., 2013a), until it was later found to be involved in ABA metabolism in a forward genetic screen (Kang et al., 2010b). Heterologous expression in yeast and tobacco BY-2 cells and direct transport experiments confirmed the ability of AtABCG40 to transport ABA. Interestingly, competition experiments between lead and ABA were performed, and the transporter displayed very high substrate specificity, suggesting that the phenotypes related to pathogen defense and lead were most probably caused by impaired ABA signaling.

The ABC transporter family is not the only one for which substrate monospecificity or polyspecificity is discussed. Indeed, there are secondary transporters (e.g. belonging to the major facilitator superfamily and multidrug and toxic compound extrusion families) that were shown to transport substrates with different structures (Remy and Duque, 2014). This raises interesting questions Box 2 concerning kinetic and thermodynamic aspects of these secondary transporters compared with ABC transporters, which are primary pumps (Maathius and Sanders, 1993; Zhang et al., 2015, 2016; Wagner et al., 2017).

DIRECTIONALITY OF TRANSPORT

Whatever the subcellular localization of an ABC transporter, its ATP-binding domain most likely is localized within the cytosol and the direction of transport (import/export) is defined in relation to the cytosol. Whereas in prokaryotes ABC transport systems involved in either import or export have been described extensively, it has long been accepted that eukaryotic ABC transporters act exclusively as exporters. In fact, prokaryotic uptake transporters are structurally and mechanistically distinct from their exporter counterparts, as they usually require highly specific periplasmic or membrane-anchored substrate-binding proteins

BOX 2. ABC, MSF, and MATE: Which Is the Winner?

Besides ABC transporters, other transporter families, in particular the major facilitator superfamily (MFS) and multidrug and toxic compound extrusions (MATEs), are also involved in energy-dependent transport of a large variety of structurally unrelated substrates. However, they do not directly depend on ATP but use, as a driving force, the proton electrochemical gradient generated by a proton pump ATPase. MATEs are usually proton-substrate symporters while MFS transporters generally, but not always, function as proton-substrate antiporters (Remy and Duque 2014). From a kinetic point of view, ATP-driven pumps like ABC transporters usually have a low turnover number compared to other transporters that do not need to couple two functions: ATP hydrolysis and transport of a substrate. Regarding thermodynamic considerations, the gradient of substrate concentration that can be obtained on both sides of a membrane is substantially higher for an ABC transporter. Indeed, for a secondary transporter, the proton electrochemical potential is defined as $F\Delta\psi - 2.3RT\Delta pH$, which, for a plant plasma membrane, gives a value of approximately 18 kJ/mol, taking into account a pH difference of 2 and a transmembrane potential of -150 mV (Maathius and Sanders 1993). For an uncharged molecule, which is transported with a proton, this theoretically allows for a concentration gradient of approximately 40,000-fold on both sides of the membrane (for more detail, see Zhang et al., 2015).

Of course, these values and thus, the performance of the transporters, might fluctuate according to the cell type and the physiological conditions. For instance, the potential will drop in the presence of K^+ ions which are transported within the cell. On the other hand, in healthy cells, the free energy of ATP hydrolysis into ADP and P_i corresponds to more than 50 kJ/mol, thus higher than the value obtained for a proton electrochemical gradient across the membrane. Moreover, an ABC transporter has two binding sites for ATP and it is not clear yet whether one or two ATP are hydrolyzed by cycle. In fact, some ABC transporters possess a degenerated nucleotide binding site in which several key residues necessary for hydrolyzing ATP within the Walker A, Walker B, and H-loop, as well as the ABC signature motif, are replaced by non-canonical amino acids. However, very few studies have determined the stoichiometry of transport and ATP hydrolysis, and the role and prevalence of this degenerated NBD remains a matter of debate (for review, see Wagner et al., 2017). In the case where both NBDs would be active, however, the free energy per cycle would be doubled, resulting in a deeper concentration gradient across the membrane (for more details, see Zhang et al., 2016). Thus, although secondary transporters like MATE and MFS work more rapidly than ABC transporters, the latter can build steeper concentration gradients across a membrane.

to display import activity (for review, see Lewinson and Livnat-Levanon, 2017). Except for the peculiar ABCI subfamily, such substrate-binding proteins are not found in plants. Yet, recent data suggest that plant ABC transporters from the B and G subfamilies still might display import activities and even conditional reversibility between export and import.

The first plant ABC for which such uptake activity was reported is CjMDR1 (Shitan et al., 2003). Both *C. japonica* suspension cells and *Xenopus* oocytes expressing CjMDR1 were found to display ATP-dependent uptake of the alkaloid berberine from the extracellular medium. Later, the Arabidopsis auxin transporter AtABCB4 also was suggested to display such uptake activity (Santelia et al., 2005; Terasaka et al., 2005). Expression of the transporter in *S. cerevisiae* resulted in

hypersensitivity to IAA (Santelia et al., 2005), whereas expression in HeLa cells resulted in net auxin accumulation (Terasaka et al., 2005). Interestingly, treatment with the inhibitor of polar auxin transport *N*-1-naphthylphthalamic acid seemed to revert auxin flux toward efflux. However, a later report from Cho et al. (2007) demonstrated AtABCB4-mediated efflux activity of the artificial auxin NAA in tobacco BY-2 cells while also demonstrating that the primary role of the transporter in roots is auxin efflux. In an attempt to rule out these discrepancies, Yang and Murphy (2009) conducted a thorough characterization of the transport properties of AtABCB4 expressed in *S. pombe*. These experiments further confirmed the substrate concentration-dependent switch from auxin influx to efflux described previously by Terasaka et al. (2005). Such findings were

later corroborated by similar experiments performed in *S. pombe* as well as BY-2 and HeLa cells (Kubeš et al., 2012). Two other members of the ABCB subfamily, AtABCB14 and AtABCB21, also were suggested to display import activity. The expression of AtABCB14 in *S. cerevisiae* and HeLa cells resulted in active uptake of the organic acid malate (Lee et al., 2008), whereas the expression of AtABCB21 in the same system displayed reversible IAA transport, as did AtABCB4 (Kamimoto et al., 2012).

Although the data described above seem to demonstrate that some plant ABCB transporters mediate substrate uptake, a close analysis of these findings reminds us of the complexity of performing and analyzing transport data, especially considering amphipathic substrates. Indeed, as highlighted by Jenness and Murphy (2014), the drawback of performing *in vivo* transport assays using unicellular systems lies in the fact that apparent uptake is actually measured as total cellular accumulation. This combines endogenous and transporter-mediated uptake as well as membrane- and transporter-bound substrate. Another interpretation of the apparent reversible uptake phenotype displayed by AtABCB4 and AtABCB21, therefore, would be related to transporter-assisted diffusion: in their outward-facing conformation, the transporters would bind their substrate, which then could accumulate within cellular membranes via lipophilic diffusion without any actual active substrate uptake occurring (Jenness and Murphy, 2014). This example further demonstrates the importance of performing in-depth *in vitro* biochemical characterization of ABC transporters in order to fully understand their actual mechanism of action (Box 3).

In addition to ABCB transporters, two members of the G subfamily, AtABCG30 and AtABCG40, also have been proposed to act as importers, thereby regulating ABA homeostasis in Arabidopsis seeds. The first evidence of such a phenotype arose from the ability of AtABCG40 to mediate ABA accumulation in BY-2 cells as well as in *S. cerevisiae* (Kang et al., 2010b). While one could argue that these experiments suffer from the same drawbacks as those performed with B subfamily transporters, in planta flux experiments as well as phenotypic analysis of single and double mutants strongly support the idea that multiple importers and exporters act in a coordinated manner to control seed germination until conditions are favorable (Kang et al., 2015).

As a last point, we would like to mention the chance of an artifactual import phenotype due to a possible mislocalization of ABC transporters in heterologous hosts. For instance, as mentioned above, plant ABCG exporters expressed in yeast usually are retained in internal membranes. In this case, when the putative substrate added to the external medium diffuses through the plasma membrane, it can be actively exported by the ABC transporter into internal vesicles, leading to a cellular accumulation, as would be the case for a plasma membrane-localized importer. Hence, the importance

of demonstrating the subcellular localization of a transporter expressed in a heterologous host.

DO POSTTRANSLATIONAL MODIFICATIONS OF PLANT ABC TRANSPORTERS AFFECT THEIR SUBSTRATE SPECIFICITY?

Once synthesized, or even during its synthesis, a protein can undergo many posttranslational modifications. Some, like glycosylation, are stable and essentially irreversible; others, like phosphorylation, are reversible and often participate in the enzyme's regulation. So far, there are few data available concerning posttranslational modifications of plant ABC transporters and even fewer concerning their role.

Glycosylation takes place in the secretory pathway and thus concerns the region facing the lumen of the secretory pathway (i.e. the extracellular medium for those transporters that are targeted to the plasma membrane). External loops of ABC transporters generally are limited in size and thus offer little room to the glycosylation machinery. Although the virtual irreversibility of glycosylation does not support a regulatory role, glycosylation might take part in folding, trafficking, or even substrate specificity. For instance, the mammalian MRP4 mediates the cellular efflux of a wide variety of structurally diverse endogenous and xenobiotic molecules. Mutation of the two glycosylation sites (Asn-746 and Asn-754) did not modify the plasma membrane targeting, yet vesicular transport assays showed that preventing glycosylation affected the transport of prostaglandin E2 but not of estradiol glucuronide, indicating that glycosylation participates in defining the substrate specificity (Miah et al., 2016). Although many secreted proteins also are glycosylated in plants (Strasser, 2016), to our knowledge, a single ABC transporter, NtPDR1 from tobacco, so far has been shown to be glycosylated on the third external loop at position Asn-738 (Pierman et al., 2017).

Phosphorylation is an important posttranslational modification for many proteins and for animal and plant ABC transporters in particular (Aryal et al., 2015). Several reports have shown that the phosphorylation of plant ABC transporters from different subfamilies modifies their transport activity. Indeed, the auxin transporter ABCB19 is inhibited by the blue light photoreceptor kinase PHOTOTROPIN1 (Christie et al., 2011). Another auxin transporter, AtABCB1, has its transport activity stimulated by the PINOID protein kinase, most likely phosphorylating Ser-634. However, negative regulation of AtABCB1 takes place in the presence of a third partner, TWISTED DWARF1 (Henrichs et al., 2012). Phosphorylation of AtABCC1 Ser-846 is important for conferring As resistance when expressed in the yeast *S. cerevisiae* (Zhang et al., 2017). As a last example, AtABCG36/PEN3/PDR8 is phosphorylated on several residues. Ala mutagenesis of Ser-40 or Ser-45 prevented penetration resistance against the powdery

BOX 3. Bifunctional ABC Transporters: A New Paradigm?

Although ABC transporters seem to exhibit a remarkable mechanistic diversity (for review, see Locher 2016), all current models provide the same general framework involving a switch between an inward- and an outward-facing conformation driven by both ATP binding and hydrolysis (Figure 4). However, the recent biochemical characterization of Arabidopsis ABCD1 suggests that at least some ABC transporters might possess additional mechanistic complexity. AtABCD1, also known as PXA1 (Zolman et al. 2001), PED3 (Hayashi et al. 2002), PMP2, or COMATOSE/CTS (Footitt et al. 2007), was first described for its involvement in the peroxisomal import of fatty acids for β -oxidation and the glyoxylate cycle (2007; Footitt et al. 2002; Hooks et al. 2007; Kunz et al. 2009; Park et al. 2013). However, AtABCD1 has also been suggested to play a role in the biosynthesis of jasmonates, which are lipid-derived signaling molecules, by importing a jasmonic acid precursor and/or its acyl-CoA derivative into the peroxisome (Theodoulou et al. 2005). For biochemical characterization of the transporter, AtABCD1 was expressed in *S. cerevisiae* and found to be localized in peroxisomes as in planta. ATPase assays on a purified peroxisomal fraction revealed stimulation by fatty acyl-CoAs and sensitivity to aluminum fluoride.

Moreover, the yeast *pxa1pxa2* Δ mutant, lacking endogenous peroxisomal transporters and unable to grow on oleic acid, was complemented by the expression of AtABCD1 (Nyathi et al. 2010). Later, De Marcos Lousa et al. (2013) used the same yeast strain to show that transport of oleic acid by AtABCD1 required peroxisomal acyl-CoA synthetases and were able to demonstrate a functional and physical interaction between the transporter and peroxisomal acyl-CoA synthetase in planta. They then expressed the protein in *Sf9* insect cells and showed that transfected cells acquired fatty acyl-CoA thioesterase activity. By analyzing a point mutant of AtABCD1 defective for this thioesterase activity but retaining ATPase activity, they suggest that the cleavage of CoA occurs during the transport cycle and is biologically relevant. This is, to our knowledge, the only indication of a plant bifunctional ABC transporter with an additional intrinsic enzymatic function possibly regulating the transport cycle of the protein. It is worth mentioning here that such a polytopic behavior has also recently been shown for the bacterial ABC transporter MsbA, which is able to couple ATP hydrolysis to an adenylate kinase activity as part of its catalytic cycle (Kaur et al. 2016).

mildew pathogen *Blumeria graminis* f. sp. *hordei* (Underwood and Somerville, 2017).

In summary, there are few reports concerning the posttranslational regulation of plant ABC transporters and, in particular, how it might affect substrate specificity. However, considering the wealth of data regarding other proteins, it might be expected that at least some ABC transporters are highly regulated at the functional level. This possibility has to be considered when choosing an expression system. A phylogenetically distant host is less prone to perform the functional modifications that take place in the original host.

CONCLUSION

Recent years have seen remarkable progress in the characterization of ABC transporters in plants. The availability of mutants in model plants such as Arabidopsis and rice has enabled the discovery of the physiological functions of many of these proteins that allow plants to interact efficiently with their environment. However, as different groups worked on identical

transporters, contradictions arose regarding the actual substrates translocated. One possible reason for these discrepancies is that, as in their animal or fungal counterparts, plant ABC transporters may translocate multiple structurally unrelated substrates. A second possibility, however, could be that the different phenotypes arose from a single substrate (e.g. a hormone), which impacts multiple different metabolic processes. While recent data seem to favor the latter hypothesis, in many cases, direct transport data are still missing. In addition, other specific features of plant ABC transporters, which might modulate their activity or specificity, have been poorly investigated (see Outstanding Questions). We are convinced that the combination of classical cellular and genetic approaches with thorough biochemical characterization of plant ABC transporters will be necessary to tackle these complex issues.

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. Comprehensive overview of all functionally characterized plant ABC transporters.

OUTSTANDING QUESTIONS

- Could some plant ABC transporters be pleiotropic, i.e., have multiple structurally-unrelated substrates?
- From a biochemical point of view, how can some plant ABC transporters act as both importers and exporters?
- Could posttranslational modifications modulate the substrate profile of plant ABC transporters?
- Do plant ABC transporters have particular structural and enzymatic features compared to animal and fungal transporters?

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