Hiring cell gatekeepers
– ABC transporters in plant biotechnology

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Abstract

In order to survive in the environment, a cell has to be able to maintain a constant influx of chemical substances which provide nourishment, it also has to dispose of endogenous or exogenous toxins as well as must perceive and exchange signalling molecules. Such movement needs to be selective and organised. This prerequisite is realised by an array of membrane transporters, channels, carriers and pumps. ATP-binding cassette (ABC) transporters constitute the largest family of membrane transport proteins found in all organisms in every life domain. Researches in recent years have shown recognition of the role that plant ABC transporters play in hormone signalling, heavy metal tolerance, as well as natural product trafficking. In this review article we present plant ABC transporters, their characteristic modular structure and importance in plant physiology. We also point out the recent discoveries that describe their role in drought tolerance by modulation of abscisic acid traffic, and their involvement in subcellular compartmentation of exogenous toxic compounds and/or natural products. Alongside the gradually increasing number of results in fundamental research, plant ABC transporters begin to emerge as novel targets for applied science. We present an outline of the work and ideas that may link genetic engineering of ABC transporters with plant biotechnology. A particular attention is paid to the possibility of alleviating the heavy metal stress and the production of secondary metabolites.

Key words: ABC transporters, plant biotechnology, membrane transport, phytoremediation, secondary metabolites, drought resistance

ABC transporters

ATP-binding cassette (ABC) proteins form a large family that is one of the largest and one of the most ubiquitous on Earth. Its members are found in all living organisms, both prokaryotic and eukaryotic. This characteristic arises from the fact that ABC proteins are absolutely fundamental for cell survival and development. A number of them are responsible for active molecular transport, in which various substrates are translocated across biological membranes at the expense of chemical energy, ATP hydrolysis in this case. This function is vital for maintaining a persistent contact between the cell and its environment (Verrier et al., 2008).

ABC transporters have a well-established position in clinical research because of their role in the emergence of multi-drug resistance in cancer, the resistance to anti-malarial medications or the development of cystic fibro-

sis. Conversely, the role of plant ABC transporters is beginning to be understood despite the fact that these proteins are more numerous and diverse in plants than in other kingdoms.

All ABC proteins contain at least one cytoplasmic domain that possesses catalytic activity directed towards ATP hydrolysis. The catalytic core of this domain is often described as an ATP-binding cassette, hence the name of the entire protein family. The domain itself is called a nucleotide-binding domain (NBD) and is prevalent in all ABC proteins. NBD is highly conserved throughout the course of evolution, with Walker A (GX_4-G-K-[ST]) and Walker B ([RK]-X_3-G-X_3-L-[hydrophobic]$_3$) motifs, as well as ABC signature motif ([LIVMFY]-S-[SG]-G-X_3-[RKA]-[LIVMYA]-X-[LIVMF]-[AG]) being the most representative of this family. ABC transporters also contain a transmembrane domain (TMD), which is respon-

sible for providing transport specificity and substrate recognition (Higgins, 1992). TMD spans the lipid bilayer, and comprises 4-6 alpha-helices. A close cooperation between TMD and NBD is thought to be crucial for the active transport of substrates through the membrane. In this mechanism, conformational changes in NBD triggered by ATP binding and hydrolysis are passed onto TMD, allowing the chemical molecules to be translocated. Regarding the protein architecture, a typical ABC transporter may constitute two NBDs and TMDs, thus being called a full-size ABC transporter, or single NBD and TMD, which are aptly referred to as half-size ABC transporter. Interestingly, a small group of soluble ABC proteins lacking TMDs, therefore most likely not connected with active transport, was identified (Sanchez-Fernandez et al., 2001; Sugiyama et al., 2006). Recently, a new system for the classification of plant ABC transporters has been proposed. The authors based it on a similar system devised previously for human ABC transporters by Human Genome Organisation (HuGO). In this classification, all plant ABC transporters have been divided into eight distinct subfamilies, named alphabetically from ABCA to ABCH (Verrier et al., 2008).

Plant ABC transporters are more diverse than their homologues in other kingdoms. This fact might be due to the basic limitation of plants, which is their inability to relocate when exposed to unfavourable conditions. Consequently, in order to minimise the effect of adverse external stimuli, plants must have had developed efficient mechanisms to eliminate or considerably reduce any possibility of such influence. Hence the role of ABC transporters in a wide array of processes, such as detoxification, hormone signalling, lipid, ion and secondary metabolite transport. Avoidance of undesirable effects must have a counterpart in advancing those that are beneficial. For ABC transporters, their activity was proposed to have a significant role in mechanisms defining advantageous plant interactions, like bacterial symbiosis and mycorrhiza (Crouzet et al., 2006; Verrier et al., 2008).

Although human ABC transporters are being widely studied, due to their significance in various vital biological processes, as well as serious medical conditions (e.g. multi-drug resistance, cystic fibrosis), their plant counterparts still form a relatively unexplored territory. Later research has demonstrated a gradually increasing number of discoveries within this field of study and some of them possess the potential for developing into commercially exploitable innovations. In this paper, we try to outline the possibilities and concepts that derive from putting various recent findings regarding plant ABC transporters into a wider context of green biotechnology and applied sciences. Our focus is toward the possible involvement of these proteins in the engineering of new drought-resistant plant varieties, efficient production systems for natural products, as well as plants designed to alleviate the negative impact of heavy metals on the environment.

ABC transporters and drought resistance

Fresh water, absolutely essential for survival, still remains a rare commodity in the modern world. Even in the countries referred to as developed, shortages in water supply occur on a regular basis, seriously affecting crop production and yield. This is even more pronounced in poorer regions, contributing severely to the emergence of famine, let alone the dehydration itself persistently posing a grave issue.

Plant response to prolonged periods of drought is a complex mechanism that includes minimising the loss of water that occurs due to transpiration. Reduction in transpirational-water loss is realised by a pair of epidermal guard cells delineating the stomata; the cells reduce the aperture of the stomatal pore, effectively stopping the exchange of water and gasses (Hetherington, 2001). Stomatal opening is induced by light, low CO_2 concentration and high humidity, whereas its closure is prompted by darkness, high CO_2 concentration, low humidity and plant hormone abscisic acid (ABA). It has been shown that during drought plants exhibit a drastic increase in ABA levels, thereby leading later to recognition of its role in drought response (Zeevaart, 1980).

Plant hormone ABA is known to be transported over long distances and distributed via the apoplast (Ackerson, 1982). ABA is a weak acid and, as such, may exist in both protonated (ABAH) and anionic (ABA⁻) form, depending on the pH of the surrounding solution. As an uncharged species, ABAH can readily permeate the plasma membrane, therefore obviating the need for a specific transporter that carries the hormone from the apoplast to the cytoplasm. A similar scheme of chemiosmotic transport was proposed earlier for auxin by Mary Helen Goldsmith in 1981. However, advances in auxin transport and signalling studies showed that this issue is

more complex. An intricate system of auxin carriers has been discovered, including AUX1, LAX3 proteins and several ABC transporters (Vieten et al., 2007). Similarly, most recent studies on *Arabidopsis thaliana* have identified two highly effective and specific ABA carriers (Kang et al., 2010; Kuromori et al., 2010) that belong to the family of ABC transporters: AtABCG40 and AtABCG25 (see Fig. 1). Since drought is known to elevate extracellular pH in apoplast, protonated form of ABA dissociates and becomes negatively charged, thus no longer being able to passively diffuse through the lipid bilayer. Therefore, it was suggested that the existence of an ABA-uptake transporter could potentially mitigate problems associated with chemiosmotic mode of hormone delivery that arise under water stress conditions. (Wilkinson et al., 1997; Kang et al., 2010).

The need for ABA transport system via the cell membrane is illuminated further by the recent characterisation of the soluble intracellular receptors of this hormone. PYR/PYL proteins have been identified as cytosolic ABA receptors that function at the apex of a negative regulatory pathway which controls ABA-signalling (Park et al., 2009). With regard to these latest findings, it has to be noted that initial steps of ABA perception are just beginning to be unravelled and understood, despite the fact that secondary messengers and effectors for ABA are well known and extensively studied (Israelsson et al., 2006).

An interesting initial observation was that AtABCG25 protein is present mainly in vascular parenchyma cells, where enzymes that biosynthesize ABA are also expressed. Overexpression of AtABCG25 resulted in conferring resistance to exogenously applied ABA, as well as reduced transpiration, causing diminished water loss in comparison with the wild-type plants. Similarly, loss-of-function *atabcg25* mutants were hypersensitive to hormone treatment. These data are consistent with the idea that AtABCG25 is an ABA exporter responsible for the delivery of the hormone to the extracellular environment of the guard cells, which in consequence performs a stomatal closure, therefore reducing water loss (Kuromori et al., 2010).

At the same time, AtABCG40 was described as a plasma-membrane protein responsible for ABA delivery into guard cells. Knockout AtABCG40 mutants exhibited a decreased ABA-uptake and delayed ABA response, as well as reduced drought tolerance. This observation directly conjoined the protein with its role as an ABA importer significant for rapid responses to environmental stress (Kang et al., 2010).

Characterization of AtABCG25 and AtABCG40 ABA transporters has a potentially important role in manipulating plant adjustment to drought stress, which is a prerequisite for plant survival in scarcely irrigated areas. Alongside conferring pest resistance and improving nutritional value, augmenting crop performance under drought conditions appears to be one of the major challenges for green biotechnology applied in agricultural development. Numerous approaches and methods have been proposed throughout the years for dealing with these issues, including traditional selection, molecular breeding, genetic engineering and various combinations thereof. Engineering plants with enhanced drought resistance through genetic manipulation was proposed to be related, in a number of cases, with altering ABA metabolism, transport and response (Manavalan et al., 2009). Two ABC transporters from ABCG family presented in this section pave another path for green biotechnology, focused on providing new drought-tolerant varieties.

ABC transporters and production of secondary metabolites

Plants are known to produce a multitude of diverse chemical substances. Variety of low-molecular-weight compounds, called secondary metabolites, are engaged in plant response to biotic and abiotic stresses, and also play an important role in the life cycle of the plants and mediate their interaction with the environment. This versatile repertoire of chemical species makes plants a natural source for active substances of industrial or biological use, such as: food additives, insecticides, pharmaceuticals and fragrances (Harborne, 2001; Dixon, 2001). The large and still growing commercial demand for natural products, coupled with huge productive potential of plants, which is powered by photosynthesis, have prompted scientists to devise a promising alternative for more conventional systems that are applied in the production of low-molecular-weight compounds and recombinant proteins.

When compared with chemical synthesis, as well as microbial and mammalian cell culture systems, plant-based platforms demonstrate many advantages. Plant cells possess enzymes required for post-translational modification of proteins, which are lacking in bacteria.
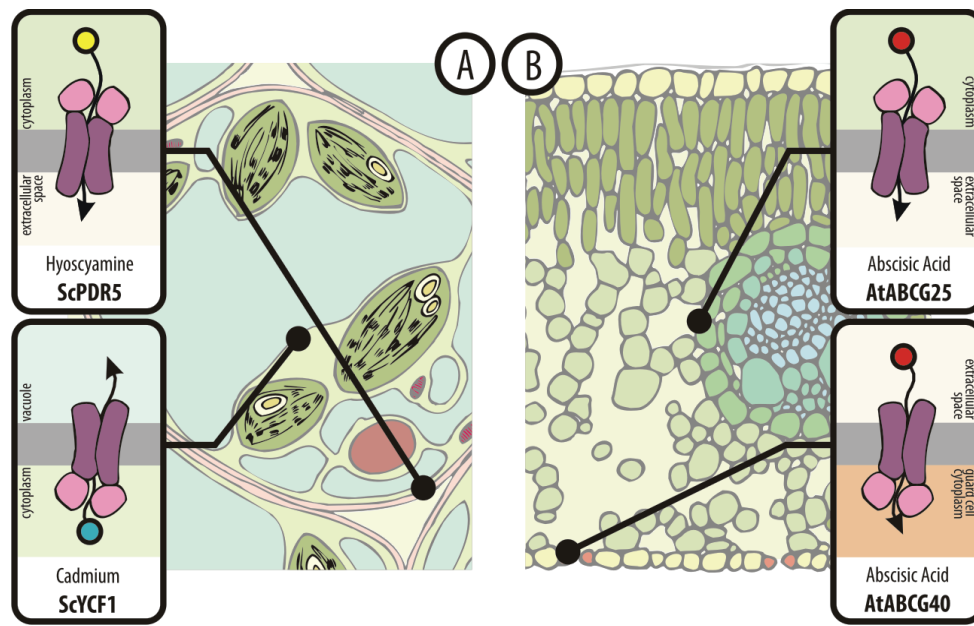
Fig. 1. Example of ABC transporters considered for various plant biotechnological projects. (A) Heterologous expression of *Saccharomyces cerevisiae* ABC transporters within a plant cell. Tonoplast (ScYCF1) or plasma membrane (ScPDR5) localised proteins may be potentially applied in processes such as phytoremediation and/or molecular farming. (B) Two plant ABCG transporters and their putative localisation shown on a leaf section. Influencing the endogenous level of ABA opens up possibilities to modulate plant response to drought stress. Note: Transmembrane domain (TMD) and nucleotide-binding domain (NBD) are represented in violet and pink colours, respectively. Colour dots represent potential molecules translocated across the membranes as indicated in the inset caption.

Plant-derived products are free from bacterial toxins, animal pathogens, viruses and potentially dangerous substances used normally in chemical synthesis. Additionally, some of the low-molecular-weight compounds cannot be readily synthesised by any other organism. Indeed several methods for obtaining valuable secondary metabolites have been proposed and applied, resulting in introduction of commercially successful products, e.g. ginseng saponins, shikonin and berberine (Verpoorte and Mamalink, 2002; Smith, 2005; Zhao and Verpoorte, 2007). Beside the fact that our understanding of biosynthetic pathways in plants is still limited, the production of biologically active chemicals in plants poses several limitations. Often the product may be toxic for the plant in question, significantly decreasing the biomass growth and causing cell death. Moreover, troublesome isolation methods are required for the extraction of the compounds from the contents of the cell. Finally, the molecule of interest can be subjected *in vivo* to degradation by the native enzymes of its producer. All these issues contribute to unnecessary complications in the design of the process and negatively influence its effi-

ciency, rendering it overly expensive and making it less feasible. However, these drawbacks can be easily overcome if the desired compound is removed from the plant cell. This can be achieved, for instance, if the cells are grown in suspension or hairy roots cultures, enabling the metabolites to be secreted. Thus, by the constant exchange of the spent medium, cells are left in a working volume, free from co-products and the product itself. This strategy, called perfusion cultivation, also facilitates isolation of the product, because soluble compounds can be purified directly from the medium, avoiding energy-consuming process of cell homogenisation (Hellwing et al., 2004; Weathers et al., 2010).

It is known that ABC transporters are involved in export of various low- and high-molecular-weight compounds from cells. Proteins that belong to this family are responsible for multi-drug resistance phenomenon in pathogens and cancers, actively removing toxic pharmaceuticals from the cells. ABC transporters show substrate specificity for various secondary metabolites, e.g. steroids, terpenoids, flavonoids and alkaloids – which include compounds used in medicine, such as scopolamine

and morphine. In plants, synthesis of these substances is often associated with plant-environment interactions, formation of root exudates, response to abiotic and biotic stresses, and interaction with insects or symbiotic nitrogen fixing bacteria (Higgins, 1992; Rea et al., 1998; Martinoia et al., 2002).

Goossens and co-workers (2003) have suggested that this feature of ABC transporters can be utilised to acquire secretion of tropane alkaloids into the growth medium. As a subject of investigation, they chose ScPDR5 from *Saccharomyces cerevisiae*, which is known to mediate transport of taxol, flavonoids, as well as indole and tropane alkaloids (Kolaczkowski et al., 1996). Furthermore, *pdr5* yeast mutant is more sensitive to hyoscyamine, an alkaloid which is sufficient to arrest growth and cause cell death even in low molar concentrations. They have shown that heterologous expression of ScPDR5 in *Nicotiana tabacum* BY-2 cells confers higher plant tolerance to hyoscyamine, most probably by secretion of this toxic compound (see Fig. 1). These results suggest that ABC transporters may be used to acquire secretion of tropane alkaloids, among other low-molecular-weight compounds, thus facilitating production of biologically active substances in plant cell cultures.

ABC transporters also play an important role in biosynthetic pathways of secondary metabolites. They transport intermediates through biological membranes, a condition that is often necessary for completing a synthesis process, because some stages of the pathway take place in different cellular compartments. Therefore, it was suggested that plant ABC transporters may become important targets in engineering strategies aimed at the synthesis of the compounds that plants do not normally produce (Kunze et al., 2002). It is noteworthy that the expression of several ABCG transporters is highly induced upon treatment with various elicitors. Such chemicals, e.g. copper chloride in legumes, not only modulate the expression of ABC transporters but also induce the production of phenolic compounds (Tebayashi et al., 2001).

ABC transporters and phytoremediation

Contamination of soil and water in heavily industrialised countries poses a major threat for the environment and human health. Among the most widespread and hazardous chemical substances found in urban and industrial areas are heavy metals and their compounds; mainly lead (Pb) and cadmium (Cd) (Salt et al., 1998). In long-term exposure, Pb and Cd cause adverse health effects, affecting central nervous system, blood cells and kidneys. Cd and its compounds are found primarily in soils containing waste materials deposited by zinc mines, in sludge-amended soils and soils treated with cadmium-rich phosphate fertilisers (Raskin and Ensley, 2000). High toxicity of Cd and Pb is due to their ability to bind to sulfhydryl groups of amino acids that causes inactivation or denaturation of proteins. Cd and Pb ions displace inorganic co-factors, such as Mn^{2+}, Mg^{2+} and Cu^{+}, from crucial transcriptional factors and metalloproteins, as well as generate reactive oxygen species, causing further cell damage (Goyer, 1997; Schützendübel et al., 2001).

Methods employed for chemical removal of heavy metal contamination are cumbersome and prohibitively expensive. Bearing this in mind, researchers began to investigate phytoremediation – more cost-effective approach aimed at dealing with this issue, which takes advantage of plants' remarkable ability to concentrate, metabolise and deposit heavy metal compounds in their tissues (Salt et al., 1998). Alongside, classic selection techniques, genetic engineering has been proposed for the development of plants suitable for environmental clean-up of contaminated soil. In order to mitigate the harmful effect of heavy metals, plants have developed detoxification mechanisms that exert chelating compounds such as metallothioneins and phytochelatins (PC). The former are small, cystein-rich cytosolic proteins, found additionally in animals, where they serve fairly similar purpose, while the latter are glutathione polymers also present in fungi, nematodes and algae. Once conjugated with glutathione, heavy metals may be removed from the cytosol and safely sequestered in the vacuole (Clemens et al., 1999; Clemens, 2001). Recently two *Arabidopsis* ABC transporters from so-called ABCC subfamily, namely AtABCC1 and AtABCC2, have been identified as vacuolar PC transporters, underpinning the important role of such transport systems in heavy metal resistance (Song et al., 2010).

These observations suggest that efficient alleviation of heavy metal stress requires effective modes of transport to be employed by the organism. Sequestration of Pb and Cd and their conjugates in the vacuole, as well as their removal from the cytoplasm are energy-consuming processes, as the compounds need to be translocated across the membranes against their chemiosmotic gradients. ABC transporters are naturally among the choice

of candidates suitable for such action, and indeed their involvement in heavy metal resistance and transport has been shown in plants and fungi (Kim et al., 2007). Two ABC transporters from yeast are known to be responsible for Cd deposition in the vacuole. ScYCF1 (see Fig. 1) from budding yeast *S. cerevisiae* is responsible for pumping glutathione-conjugated Cd through the vacuolar membrane (Li et al., 1997). Similarly, SpHMT1 found in fission yeast *Schizosaccharomyces pombe* transports phytochelatin-Cd complexes into the vacuole (Ortiz et al., 1995). In plants, several ABC transporters from *A. thaliana* have been shown to be implicated in heavy metal tolerance and indirectly in their transport (Kim et al., 2007). Among them, AtMRP3 partially restores Cd resistance if heterologously expressed in yeast *ycf1* mutant with inactive Cd-conjugate transporter (Tommasini et al., 1998). A mitochondial transporter AtATM3, which is a close homologue of SpHMT1, confers Pb and Cd resistance. It has been suggested that overexpression of AtATM3 enhances translocation of Cd to the shoots, although the mechanism behind this effect is not yet fully understood (Kim et al., 2006). Lastly, it was proposed that AtPDR8 from ABCG family of ABC transporters has a role in Cd resistance, extruding Cd from root epidermal cells (Kim et al., 2007).

Preparation of plants suitable for phytoremediation of heavy metal polluted soil is a complex and multi-levelled issue. Firstly, a right cultivar has to been chosen, providing maximum possible biomass growth in a reasonably short time, with regard to the climatic conditions expected at the site of their future application. Secondly, plants must be able to efficiently uptake heavy metals and solubilise their compounds bound tightly to the soil particles. Thirdly, one must ensure they possess a mechanism that transfers heavy metals acropetally to the shoots, which would allow for the mechanised harvesting of plant over-ground biomass for further processing. Finally, toxic compounds have to be deposited safely in a secluded cell compartment where they would not impede cellular functions (Song et al., 2010; Salt et al., 1998). In spite of the findings described earlier in the article, it has been shown that the last of the issues, sequestration of heavy metal compounds in the vacuole might be improved with the aid of ABC transporters. In heterologous expression experiment, ScYCF1 from yeast was introduced into *A. thaliana* cultured cells and seedlings. Analogously to its role in *S. cerevisiae*, this trans-

porter provided enhanced Pb and Cd resistance. When grown on medium rich in Pb and Cd, transgenic plants produced higher biomass and, in comparison with the wild type, exhibited elevated Cd and Pb content. Concordantly, the vacuolar concentration of glutathione-conjugated cadmium was four times higher in *YCF1* mutants, confirming subcellular sequestration of this pollutant. The authors also state that they are developing *YCF1* yellow poplar trees to gain high biomass productivity needed for phytoremediation (Song et al., 2003).

A concluding word

ABC transporters have been already recognised in many crucial biological processes associated with the translocation of molecules through biological membranes. The contribution of such transporters to cell physiology is undisputed and permanently intriguing for basic research. The diversity of roles of ABC transporters in non-plant taxa suggests that they might have many varied functions in plants as well. Identifying new homologues, as well as deciphering their potential physiological role is crucial for the understanding of many basic processes *in planta*. One of the remarkable observations of plant genome inventories in *Arabidopsis*, rice and recently *Lotus japonicus* was the identification of a large number (>120) of putative ABC transporter encoding genes (Sanchez Fernandez et al., 2001, Jasiński et al., 2003, Sugiyama et al., 2006), in comparison to other multicelullar organisms (~50). This might suggest that plant ABC transporters are not only more diverse than their homologues in other kingdoms but possibly more specialized as well. For instance, high gene redundancy observed in legume plants within the so-called ABCC and ABCG subfamilies of ABC transporters (Sugiyama et al., 2006; Jasiński et al., 2009) may indicate that gene duplication of some full-size ABCs occurred as a result of the adaptation to a new species-specific function. Recognition of such specialisation might be advantageous, for instance, while considering the engineering of production/transport of secondary metabolites. Those substances are often species-specific, and so are their biosynthetic pathways and translocation mechanisms. The issue of specificity should be taken into consideration when developing new strategies, alongside the conclusion that plant ABC transporters offer a solid platform for the construction of efficient transport systems, being a promising biotechnological tool in the near future.

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