Secretion of Secondary Metabolites by ATP-Binding Cassette Transporters in Plant Cell Suspension Cultures[1]

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The substrate specificity of the yeast (*Saccharomyces cerevisiae*) pleiotropic drug resistance (PDR)-type ATP-binding cassette (ABC) transporters is extended to plant secondary metabolites of the tropane alkaloid family. Functional analysis of yeast *PDR5* genes in transgenic tobacco (*Nicotiana tabacum* L. cv Bright-Yellow 2 [BY-2]) cell lines suggest that *PDR* genes can be used to stimulate the secretion of secondary metabolites in plant cell suspension cultures.

Higher plants are capable of producing an enormous number of low-molecular weight compounds, often with distinct and very complex structures (Verpoorte, 2000). In addition to their importance for the plant itself, numerous secondary metabolites are also of high value to humans because of their interesting biological activities. Many of these compounds are difficult to produce because they accumulate at extremely low levels in often rare and exotic plant species. The problems related with obtaining useful metabolites from natural resources might potentially be circumvented using cell cultures. Nevertheless, the enormous biosynthetic potential of plant cells is still unexploited, in sharp contrast with that of microorganisms (Hutchinson, 1994; Verpoorte and Memelink, 2002). Increasing the metabolic flux through a biosynthetic pathway to improve the production of certain compounds in plant cells has been the prime target for metabolic engineering in the past. However, low productivity may also be the result of the intrinsic toxicity of plant secondary metabolites, including for the producing plant cell itself. Furthermore, catabolism or intracellular accumulation of plant secondary metabolites may interfere with their production and hamper subsequent purification.

Here, we present evidence that supports the use of genes encoding ABC transporters to circumvent some of the metabolic constraints imposed by plant cells. The ABC protein family corresponds to a large, ubiquitous, and diverse group of proteins, whose molecular structure allows them to transport substrates across biological membranes via the binding and hydrolysis of ATP. The list of substrates that can be transported by ABC transporter proteins is impressive and includes peptides, carbohydrates, lipids, heavy metal chelates, inorganic acids, steroids, and xenobiotics. Particularly interesting is the involvement of ABC transporters in the extrusion of cytotoxic compounds. ABC transporters are associated with the acquisition of multiple drug resistance by pathogenic organisms and with detoxification pathways that deal with either endogenously synthesized or environmental toxic compounds (Higgins, 1992; Rea et al., 1998; Davies and Coleman, 2000; Martinoia et al., 2002).

Plant alkaloids represent one of the largest groups of natural products. A well-studied class of biologically active alkaloids includes the nicotine and tropane alkaloids, synthesized by Solanaceae spp. Both the tropane ring of alkaloids, such as hyoscyamine and scopolamine, and the pyrrolidine ring of nicotine are derived from putrescine via *N*-methyl putrescine (Facchini, 2001). Nicotine production can be elicited in tobacco BY-2 cells (Imanishi et al., 1998), which is a commonly used plant cell model system (Nagata et al., 1992). As a consequence, BY-2 cell cultures serve well to investigate the effect of ABC transporters on the secretion of both exogenously applied and endogenously synthesized secondary metabolites.

The range of functions that the ABC transporter protein family can fulfill in a single organism is clearly illustrated in yeast. The 29 ABC proteins of yeast have been classified into six subfamilies with distinguishable topologies (Decottignies and Goffeau, 1997; Bauer et al., 1999). With respect to secretion of plant secondary metabolites, two of these subfamilies, PDR and multidrug resistance-associated protein, are of particular interest. To identify yeast ABC transporters with substrate specificity for tropane alkaloids, we assessed sensitivity to hyoscyamine and scopolamine of yeast strains deficient for one of the multidrug resistance-associated protein-type transporters *YOR1* and *YCF1*, or one of the PDR-type transporters *SNQ2*, *PDR5*, *PDR10*, and *PDR11* (Fig. 1). The isogenic yeast strains derived from the US50-18C and W303-1A genotypes

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were kindly provided by Michel Ghislain (Université Catholique de Louvain, Louvain-la-Neuve, Belgium) and Tokichi Miyakawa (Hiroshima University, Higashi, Japan), respectively, whereas yeast strains derived from the BY4741 genotype were obtained from the EUROSCARF collection (Frankfurt). Hyoscyamine and scopolamine levels were tolerated at concentrations of 50 and 100 mM, respectively, in wild-type US50-18C and BY4741 strains and only at concentrations of 20 and 50 mM, respectively, in the wild-type W303-1A strain, which was more sensitive. All isogenic strains showed identical alkaloid sensitivity, except the *pdr5* mutant strain, for which the abovementioned alkaloid concentrations were lethal. Thus, Pdr5p has substrate specificity for tropane alkaloids and is most probably the only ABC transporter involved in transport of these alkaloids in yeast cells. This finding was not surprising because previously other plant secondary metabolites, such as taxol, indole alkaloids, and flavonoids, have been shown also to be substrates for Pdr5p-mediated multidrug transport (Kolaczkowski et al., 1996, 1998). The involvement of Pdr5p in nicotine transport is unclear because deletion of the *PDR5* gene only had a weak effect on nicotine tolerance in the US50-18C genotype (Fig. 1).

We did not further investigate whether these genotype-dependent differences in yeast alkaloid tolerance could be accounted for, e.g. by specific differences in the *PDR* gene network, such as different *PDR5*-coding sequences or different activities of the transcriptional activator *PDR1* (Balzi et al., 1987, 1994). Furthermore, research done with yeast has learned that stress tolerance is not controlled by stress defense genes only, but it also depends on more general metabolic and homeostatic processes (Serrano et al., 1999), in this particular case not necessarily directly related to the drug resistance network, that can exhibit profound variation between distinct yeast genotypes.

To assess the activity of Pdr5p on alkaloid secretion in plants, transgenic BY-2 cell lines expressing the yeast *PDR5* gene were constructed. To this end, the *PDR5*-coding sequence was inserted into the Gateway plant transformation vector pK7WGD2 (Karimi et al., 2002). Because previous reports on heterologous expression of yeast *PDR* genes in plants are contradictory (Grec et al., 2000; Muhitch et al., 2000), we cloned the *PDR5* gene from two different yeast genotypes with distinct alkaloid tolerance, US50-18C and W303-1A. Plant cell transformations were performed by applying the ternary vector system (van der Fits et al., 2000) and following the procedure described by Shaul et al. (1996). Using this transformation procedure, one directly obtains strictly transgenic suspension cultures consisting of a heterogeneous mixture of transformed cells. As a consequence, possible phenotypic effects resulting from overexpression of certain transgenes should be considered as the mean of all effects obtained in multiple independent transformant cell lines. Transgenic lines transformed with either the US50-18C or W303-1A gene construct both correctly expressed the *PDR5* gene, at least at the mRNA level, as verified by reverse transcription PCR (Fig. 2).

Tolerance of the transgenic BY-2 suspension cultures to exogenously applied tropane alkaloids was assessed by measuring cell death after incubation in the presence of increasing concentrations of hyoscyamine. Tobacco BY-2 cells tolerated hyoscyamine perfectly up to 10 mM. Gradual increase of hyoscyamine levels induced an increase in cell death, whereas all suspension-cultured tobacco cells died within 24 h of incubation at concentrations of 50 mM or higher (data not shown). BY-2 cell lines expressing

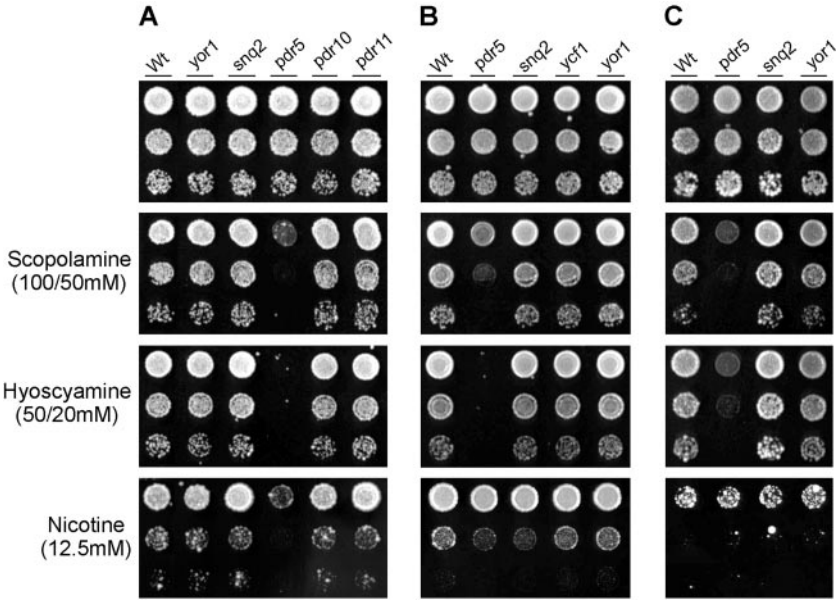

Figure 1. Yeast tolerance to tropane and nicotine alkaloids. A, Strain US50-18C (Wt) and its derivatives AD1 (*yor1*), AD2 (*snq2*), AD3 (*pdr5*), AD4 (*pdr10*), and AD5 (*pdr11*). B, Strain BY4741 (Wt) and its derivatives Y02409 (*pdr5*), Y03951 (*snq2*), Y04069 (*ycf1*), and Y05933 (*yor1*). C, Strain W303-1A (Wt) and its derivatives DHR5-4c (*pdr5*), DHR5-2c (*snq2*), and MLC26-1a (*yor1*). Strains were grown in liquid yeast extract/peptone/dextrose medium to saturation. Serial dilutions were dropped on solid yeast extract/peptone/dextrose medium containing scopolamine, hyoscyamine, or nicotine, as indicated. The two concentrations represent the tolerance levels of wild-type US50-18C and BY4741 strains (left value) and of wild-type W303-1A strain (right value), respectively. Growth was recorded after 2 d, except in the presence of nicotine (4 d).

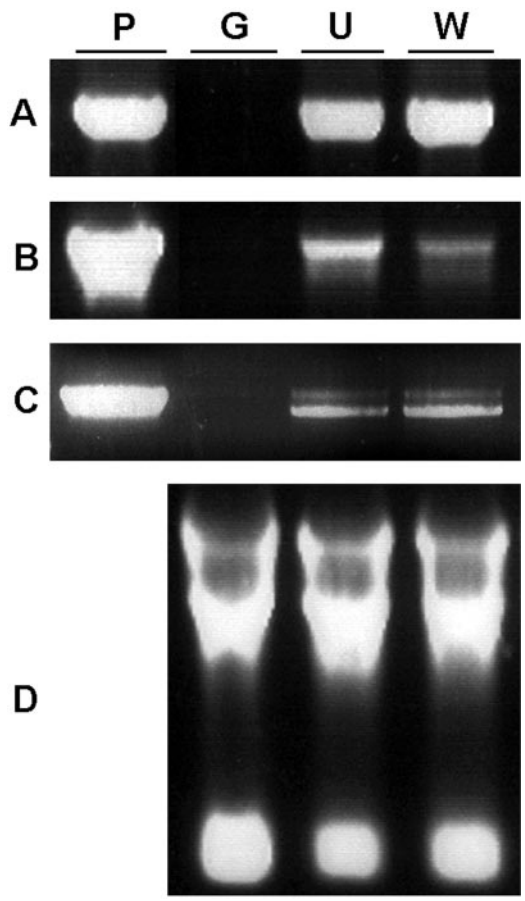

Figure 2. *PDR5* expression analysis in transformed BY-2 cells. *PDR5* expression in transformed BY-2 cells was verified by RT-PCR analysis. Three primer pairs were used, respectively spanning nucleotides 1 to 1,043 (A), 907 to 2,828 (B), and 2,670 to 4,536 (C) of the 4,536-nucleotide-long *PDR5* open reading frame. Lanes G, U, and W represent BY-2 cell lines transformed with pK7WGD2-GUS, pK7WGD2-ScPDR5-US50, and pK7WGD2-ScPDR5-W303, respectively. The PCR reaction performed on plasmid pK7WGD2-ScPDR5-W303 was used as a size control (lane P). Ethidium bromide-stained rRNA was used as a loading control (D).

the different yeast *PDR5* genes displayed to varying extent an increased tolerance to hyoscyamine when compared with the control lines transformed with the β-glucuronidase (*GUS*) gene. Lines expressing the *PDR5* genes from the yeast genotype W303-1A were the most tolerant. Fold increase in cell death, after incubation in the presence of 30 mM hyoscyamine for 24 h, decreased approximately 35% in the *W303-1A-PDR5*-expressing lines (Fig. 3A), whereas *US50-18C-PDR5*-expressing lines displayed a 15% decrease in hyoscyamine-induced cell death (data not shown). Fluorescence microscopy analysis of BY-2 cells illustrated in vivo the induction of cell death and the positive effect of the W303-1A Pdr5p transporter on cell viability in the presence of hyoscyamine (Fig. 3B). Growth performance of transgenic tobacco cells with the W303-1A Pdr5p transporter on solid hyoscyamine-containing medium also clearly improved when compared with the control cell lines (Fig. 3C).

Remarkably, nicotine caused complete growth arrest and cell death at levels of 10 mM only, indicating that the metabolites that plants produce inside the cells can be toxic for themselves and that this toxicity can retard growth of secondary metabolite-producing plant cells. Growth performance on nicotine-containing medium did not improve in any of the *PDR5*-expressing BY-2 cell lines (data not shown). This finding was not unexpected because Pdr5p substrate specificity for nicotine in yeast was not as obvious as in the case of tropane alkaloids (Fig. 1). Nevertheless, preliminary experiments indicated that endogenously synthesized nicotine alkaloids could,

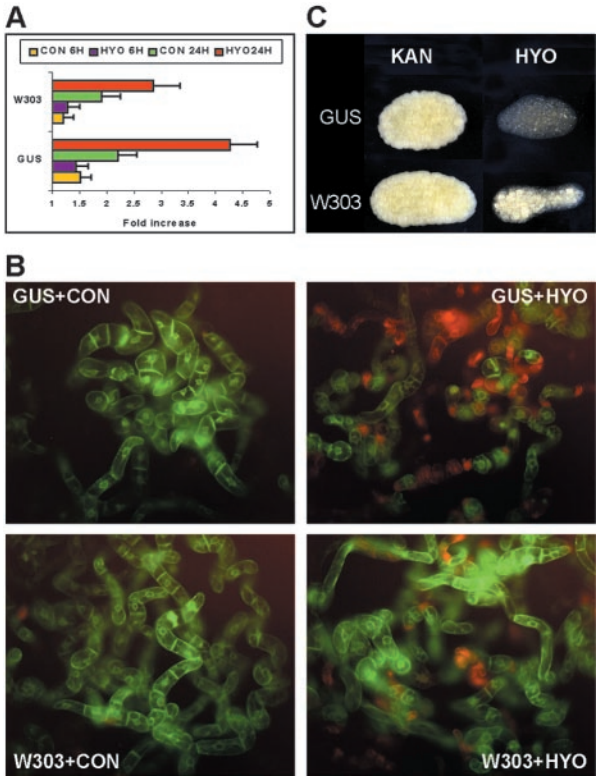

Figure 3. Tolerance of transformed BY-2 cells to hyoscyamine. A, Hyoscyamine-induced cell death in transformed BY-2 cells. Three-day-old transformed BY-2 cell cultures were incubated in the absence (CON) or presence (HYO) of 30 mM hyoscyamine for 24 h. Cell death was assayed at two time points (6 and 24 h) by Evans blue staining (Turner and Novacky, 1974) and is indicated as the fold increase in optical density at OD_{600} relative to the value at the start of the experiment. Values are the mean of three independent experiments. B, Fluorescence microscopy analysis of transformed BY-2 cells. Samples were incubated for 48 h in the absence (CON) or presence (HYO) of 30 mM hyoscyamine as described above. Living cells display green-fluorescent proteins, whereas dead cells have turned red. C, Growth performance of transformed BY-2 cells on hyoscyamine. Three-day-old BY-2 cultures (300-μL volumes) were dropped on solid medium with either 50 μg mL^{-1} kanamycin (KAN) or 70 mM hyoscyamine (HYO), as indicated. Growth was recorded after 15 d. GUS and W303 represent BY-2 cell lines transformed with pK7WGD2-GUS and pK7WGD2-ScPDR5-W303, respectively.

to some extent, be exported to the medium (data not shown).

In conclusion, the data presented here provide evidence that ABC transporters can be used to stimulate the secretion of secondary metabolites and might eventually be useful to enhance secondary metabolite production in plant cell cultures. The diverse functions and substrate specificities that are assigned to ABC transporters and the rapidly increasing number of plant ABC transporter gene sequences that are being identified definitely create an extremely rich gene pit, which awaits to be further explored.

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