

# Identification and characterization of plant transporters using heterologous expression systems

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## Abstract

In recent years major progress has been achieved in the understanding of transport processes in higher plants. The boom in the field of molecular plant physiology led to the identification and characterization of membrane transporters with transport activities for potassium, calcium, sugars, nitrate, ammonium, sulphate, phosphate, amino acids, peptides, and metal ions. Such progress was hardly feasible without heterologous expression of the isolated transport proteins. This review summarizes the different approaches in characterizing plant membrane transporters using heterologous expression systems. By presenting concrete examples, it outlines different cloning strategies, displays the methods used for (i) expression of transport proteins and detection of their function, (ii) biochemical analyses, (iii) explorations of the structure–function relationship through mutational analysis, and concludes with a discussion about the physiological relevance of the analyses in heterologous expression systems.

Key words: Membrane transporters, transport processes, heterologous expression systems, transport proteins, cloning strategies.

## Introduction

Plants are fully autotrophic organisms, with aerial parts fixing CO<sub>2</sub> from the atmosphere and roots taking up mineral ions from the earth's crust. Obviously, together with the photosynthetic machinery, this autotrophic way of life requires controlled expression of a large set of membrane transport systems, responsible for mineral uptake from the soil and translocation towards the shoots, coupled to transport of photosynthetates in the opposite direction. These transporters are therefore integral systems underlying the autotrophic status of plants.

Particularly during the last decade new and detailed insights into the biology of plant transport have been obtained. As is true elsewhere, the field of plant science has been revolutionized by the development and use of molecular biology techniques. Molecular genetic approaches have, for the first time, afforded the opportunity to extract single transporters from the complicated

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network of the plant and have allowed their detailed investigation in isolation. Now seen, with increasing frequency, is the molecular characterization of individual transport proteins that begins with the cloning of the gene or the gene transcript, determination of the nucleotide and amino acid sequences, expression of the gene product and detection of its function followed by biochemical analyses and further elaborated explorations of the structure–function relationship through mutational analysis (for recent reviews see Tanner and Caspari, 1996; Logan *et al.*, 1997; Maurel, 1997; Rentsch *et al.*, 1997; Eng *et al.*, 1998; Fox and Guerinot, 1998). These steps were hardly feasible without the utilization of heterologous expression systems. In this article, the different expression systems used for the characterization of plant transport proteins are described, both the advantages and the risks of heterologous systems are pointed out and the physiological significance of some of the results obtained is discussed. This analysis refers mainly to plasma membrane transporters, with rather special emphasis on K<sup>+</sup> carriers and channels.

### Heterologous expression systems

Several heterologous expression systems have been used for the identification and characterization of plant membrane transporter proteins (Frommer and Ninnemann, 1995).

#### *Escherichia coli*

Although the prokaryote *Escherichia coli* serves as a well-established system for protein production, the heterologous expression of membrane proteins has been successful only in some exceptional cases (e.g. the light-harvesting chlorophyll *a/b* binding protein from pea, Kühlbrandt and Wang, 1991) and even then the protein is not always obtained in a functional form. Although the main problem might be the failure of the expressed protein to fold correctly, many membrane proteins seem to be toxic when expressed in bacteria. Probably because of these limitations, *E. coli* has not become an expression system of choice for plant membrane transporters. Nevertheless, *E. coli* is occasionally used to produce plant transporter proteins for reconstitution, and very recently for direct flux-measurements and complementation assays (Kim *et al.*, 1998; Uozumi *et al.*, 1998).

#### Yeast

Most of the basis of our current knowledge on plant membrane transporters has been obtained by skilful use of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. These organisms have short generation times and can easily be handled. Moreover, the great progress in using yeasts as heterologous systems for the

expression of plant transporters was facilitated by the engineering of yeast strains with deficiencies in specific transport pathways (Riesmeier *et al.*, 1992). By functional complementation, such strains were used for proving the functionality of cloned transporters and for screening cDNA libraries to identify new transporters. In addition, yeast is well suited for flux measurements allowing the determination of substrate specificity of particular transporters. The yeast system has also been used in biochemical approaches, for example, for analysing functional properties of H<sup>+</sup>-ATPases (Palmgren and Christensen, 1994), and to a lesser extent for electrophysiological characterization of ion channels (Bertl *et al.*, 1995).

#### *Xenopus oocytes*

Oocytes of the South-African clawed toad *Xenopus laevis* have been used as a powerful tool to study heterologous gene expression for more than 25 years (Gurdon *et al.*, 1971). The popularity of this expression system is due to several factors. On the one hand, *Xenopus* oocytes are quite robust and easy to handle because of their large size (~1 mm in diameter), and on the other they can efficiently synthesize and assemble a variety of proteins after injection of DNA or RNA either isolated from various tissues or synthesized *in vitro* from cDNA clones. Size and ease of handling have made oocytes well-suited to the measurement of water and solute fluxes, enzymatic activity and, in particular, to electrophysiological and biophysical characterization of electrogenic membrane transporters (for the use of *Xenopus* oocytes as an expression system for plant proteins see Schroeder, 1994; Theodoulou and Miller, 1995).

#### *Insect cells*

An alternative to the expression systems outlined so far is the infection of insect cells by recombinant baculoviruses. The use of this system for producing recombinant proteins has become more widespread due to many factors including potentially high protein expression levels, ease and speed of genetic engineering, and ease of insect cell growth in suspension cultures. Furthermore the recombinant proteins are often biologically active and, for the most part, appear to undergo faithful post-translational processing to produce recombinant products that are very similar to those of the authentic proteins (for reviews see McCarroll and King, 1997; Possee, 1997). Baculovirus-infected cells of the moth *Spodoptera frugiperda* (*Sf9* and *Sf21* cell lines) have been used for biochemical and electrophysiological characterization of plant K<sup>+</sup> channels (Gaymard *et al.*, 1996).

#### *Mammalian cell lines*

Mammalian cell lines have seldom been used for the study of plant transporters. The exceptions mentioned in

this study note the utilization of COS cells, an engineered line of African green monkey kidney cells which can easily be transfected (Gluzman, 1981). Transiently transfected COS cells express the protein at relatively high levels over a short period of time in a burst starting about 24 h post-transfection and lasting for up to 1 week. With regard to the characterization of plant membrane transporters, thus far COS cells have only been used for a few cloning strategies and structure–function relationship studies (Kammerloher *et al.*, 1994; Kristoffersen *et al.*, 1996; Chang and Bush, 1997).

## Characterization of plant membrane transporters

### Cloning

By the beginning of this decade various membrane transporters were characterized *in planta* (for a contemporary review see Hedrich and Schroeder, 1989) but only two were identified at the molecular level. The plasma membrane H<sup>+</sup>-ATPase from *Arabidopsis thaliana* was cloned with the aid of microsequencing of the purified protein (Boutry *et al.*, 1989; Harper *et al.*, 1989; Pardo and Serrano, 1989) whereas the H<sup>+</sup>-hexose co-transporter from *Chlorella kessleri*, HUP1, was identified by differential screening of a cDNA library taking advantage of the fact that the expression of this transporter in *Chlorella* is hexose-induced (Sauer and Tanner, 1989). The demonstration of HUP1 as a glucose transporter was subsequently confirmed by heterologous expression in yeast (Sauer *et al.*, 1990).

More recently, the identification of most plant transporters has been achieved through the use of heterologous expression systems. The first successes resulted in the cloning of K<sup>+</sup> channels from *Arabidopsis thaliana* (Anderson *et al.*, 1992; Sentenac *et al.*, 1992) by complementation of mutant yeast cells. Yeast mutants deleted for the K<sup>+</sup> transporter genes *TRK1* and *TRK2* (required for high affinity K<sup>+</sup> uptake) are unable to grow on low K<sup>+</sup> media. The single mutant *trk1* and the double mutant *trk1 trk2* require high concentrations of K<sup>+</sup> (*c.* 3 mM and 50 mM, respectively) in the medium to grow at rates achieved by wild-type cells on medium containing micromolar quantities of the ion (Ramos *et al.*, 1985; Ko and Gaber, 1991). The K<sup>+</sup> uptake-defective yeast mutants were transformed with cDNA libraries from *Arabidopsis thaliana* and screened on low K<sup>+</sup> media for rescue of the growth phenotype. A major surprise was that the two inward-rectifying K<sup>+</sup> channels cloned by this approach, KAT1 (Anderson *et al.*, 1992) and AKT1 (Sentenac *et al.*, 1992), are remarkably similar to the animal voltage-dependent outward rectifiers of the *Shaker* type. This underscores another advantage of functional cloning: the ability to identify transporter proteins without implementing a structurally-biased constraint on the search.

The ability to restore K<sup>+</sup> uptake in *S. cerevisiae* mutants has also led to the identification of other classes of plant transport proteins. For example, a K<sup>+</sup> and a cation carrier from wheat roots have been cloned by complementation of yeast *trk* mutants: HKT1, which comprises 10–12 membrane spanning domains (Schachtman and Schroeder, 1994), and LCT1, which contains 8 to 10 transmembrane segments (Schachtman *et al.*, 1997). Interestingly, HKT1 and LCT1 exhibit no significant sequence similarities with other transport proteins.

The strategy of cloning by functional complementation has not only proven successful for the isolation of K<sup>+</sup> transporters but also, using yeast strains with appropriate deficiencies in other transport activities, plant carriers have been cloned that have transport activities for sucrose (Riesmeier *et al.*, 1992), amino acids (Frommer *et al.*, 1993; Hsu *et al.*, 1993; Kwart *et al.*, 1993), peptides (Steiner *et al.*, 1994), ammonium (Ninnemann *et al.*, 1994), copper (Kampfenkel *et al.*, 1995), sulphate (Smith *et al.*, 1995), calcium (Hirschi *et al.*, 1996), iron (Eide *et al.*, 1996), and zinc (Grotz *et al.*, 1998).

In theory, functional cloning by expression in *Xenopus* oocytes should provide an alternative means of identifying plant transporters. In this approach, cRNA synthesized from a subdivided plant cDNA library is injected into *Xenopus* oocytes and followed by assessment of individual oocytes for the detection of transport activity and subdivision of the positive cDNA pools, ultimately to identify the transporter cDNA. This type of expression cloning has been shown to be very powerful for the isolation of animal membrane receptors and ion channels (Jentsch *et al.*, 1990). However, although initial reports were promising (Cao *et al.*, 1992) this procedure has yet to be used successfully for the isolation of a plant membrane transporter.

A different and elegant method for identifying plant membrane proteins resulted in the cloning of plant plasma membrane water channels. COS cells were transfected with an *Arabidopsis thaliana* root cDNA library and screened with an antiserum raised against purified integral plasma membrane proteins from *A. thaliana* roots. The immunoselection led to the identification of five genes which could be shown to code for proteins with water transport-facilitating activities when expressed in *Xenopus* oocytes (Kammerloher *et al.*, 1994). Further improvements of this immunoscreening method have been developed using either *E. coli* or COS cells for the systematic rapid cloning of plant cDNAs encoding proteins with membrane spanning domains (Shi *et al.*, 1995; Kristoffersen *et al.*, 1996). The function of the newly detected proteins, however, remains to be identified.

### Functional characterization

Despite noteworthy exceptions (Lesage *et al.*, 1994), cloning by functional complementation of a yeast mutant

defective for the transport of a specific solute simultaneously demonstrates that the cloned transporter is indeed endowed with transport activity for the corresponding solute. In other cloning strategies, the activity of the expressed protein must be assessed separately. To date, in all cases where the substrate specificity of a plant transporter has been unambiguously determined, the approaches have relied on the use of heterologous expression systems. While yeasts have proven to be the systems of choice for many studies (Sauer *et al.*, 1990; Hechenberger *et al.*, 1996; Muchhal *et al.*, 1996) other systems including *Xenopus* oocytes (Müller-Röber *et al.*, 1995; Ketchum and Slayman, 1996; Gaymard *et al.*, 1998), the insect cell system (Gaymard *et al.*, 1996; Czempinski *et al.*, 1997; Zimmermann *et al.*, 1998), and *E. coli* (Kim *et al.*, 1998; Uozumi *et al.*, 1998) are also being exploited. Indeed, the first example of such a strategy has been provided by the cloning and characterization of the HUP1 glucose carrier from *Chlorella* (Sauer and Tanner, 1989; Sauer *et al.*, 1990; see above). Recently, K<sup>+</sup> transporters identified among EST clones from the *Arabidopsis* genome sequencing project, or through a PCR strategy based on sequence alignments and identification of highly conserved regions, have been shown to be endowed with K<sup>+</sup> transport activity by complementing *S. cerevisiae* *trk* mutants (Quintero and Blatt, 1997; Santa-Maria *et al.*, 1997; Fu and Luan, 1998) or *E. coli* (Kim *et al.*, 1998). However, in this context it should be noted that there are still several putative plant transport systems (Köhler and Neuhaus, 1998; Schuurink *et al.*, 1998) and ionotropic receptors (Lam *et al.*, 1998) which have to be assigned a function.

Beyond providing a means for revealing the transport activity of a given system, the heterologous expression of cloned transporters can provide additional information about transport kinetics ( $K_m$  values for various substrates), transport mechanisms (energetic coupling to the transmembrane proton electrochemical gradient), and pharmacological properties (Riesmeier *et al.*, 1993, for sucrose carriers). It should be noted, however, that the transport properties and bioenergetics of the sucrose and amino acid symporters were initially defined using purified plasma membrane vesicles and imposed proton electrochemical differences (Bush, 1993a).

With regard to K<sup>+</sup> transport, flux studies in *S. cerevisiae* using K<sup>+</sup> and Rb<sup>+</sup> allowed the characterization of high-affinity K<sup>+</sup> uptake systems (Schachtman and Schroeder, 1994; Fu and Luan, 1998), and the identification of the transporter mediating the high-affinity K<sup>+</sup> uptake of barley roots (Santa-Maria *et al.*, 1997). Using <sup>42</sup>K<sup>+</sup> and <sup>22</sup>Na<sup>+</sup> the transport mechanism of the wheat K<sup>+</sup> carrier HKT1 was revealed to be a Na<sup>+</sup>-coupled co-transport with a stoichiometry of K<sup>+</sup>:Na<sup>+</sup> ≈ 2 (Rubio *et al.*, 1995). To elaborate the transport characteristics of the transporter LCT1, radiotracer measurements were

combined with a negative yeast growth assay. Flux studies showed that LCT1 mediates <sup>45</sup>Ca<sup>2+</sup> as well as <sup>109</sup>Cd<sup>2+</sup> uptake. A prolonged exposure of LCT1-expressing cells to these cations led to toxic accumulations of Ca<sup>2+</sup> or Cd<sup>2+</sup>, which manifested itself in inhibited growth (Clemens *et al.*, 1998).

Flux measurements have also been performed with *Xenopus* oocytes. Swelling experiments to show the function of water channels is but one example (Maurel *et al.*, 1993). By far the greatest use of this expression system has been to analyse the electrical features of expressed transporters (Boorer *et al.*, 1992; Aoshima *et al.*, 1993; Tsay *et al.*, 1993; Schachtman and Schroeder, 1994). The combination of electrical and radiotracer flux measurements revealed the dependence of the transport activity on both extracellular pH and membrane voltage for several carriers (hexose, nitrate, amino acids, and sucrose). Based on such observations, the transport mechanism of these transporters has been deduced to be an electrogenic proton co-transport with stoichiometries of H<sup>+</sup>:sucrose=1, H<sup>+</sup>:amino acid=1, and H<sup>+</sup>:NO<sub>3</sub><sup>-</sup>>1 (Boorer *et al.*, 1992, 1996a, b; Tsay *et al.*, 1993; Boorer and Fischer, 1997). Furthermore, for the amino acid transporter, freeze-fracture electron microscopy allowed an estimate of the number of transporters expressed in the oocyte plasma membrane. With this information, the transport rate of a single carrier molecule could be calculated to be 350–800 transport cycles per second (Boorer *et al.*, 1996a). In this context it should be noted that, due to such relatively low transport rates, the electrophysiological analyses of carriers are difficult and could lead to distorted conclusions. Thus, since the membrane current is proportional to the cell surface × the transporter density × the turnover rate of the transporter (Tyerman and Schachtman, 1992), low rates of transport are more likely to be detected with large cells such as *Xenopus* oocytes.

Ion channels have transport rates (in the range of 10<sup>7</sup> ions per second or even higher) that are much higher than those of carriers (10–1000 transport events per second), and electrophysiological analysis of their transport activity is therefore easier. To date, several plant ion channels have been cloned (including chloride channels, Hechenberger *et al.*, 1996; Lurin *et al.*, 1996) but thus far, only plant potassium channels have been investigated in detail. All but one of the K<sup>+</sup> channels share a common structure of six transmembrane spanning helices. The exception, KCO1 from *Arabidopsis*, comprises only four transmembrane domains (Czempinski *et al.*, 1997). Using the oocyte expression system with the two-electrode voltage-clamp technique, the pharmacological and biophysical features of some of these channels were determined (Schachtman *et al.*, 1992; Kochian *et al.*, 1993; Véry *et al.*, 1994, 1995; Cao *et al.*, 1995b; Hedrich *et al.*, 1995; Hoshi, 1995; Müller-Röber *et al.*,

1995; Ketchum and Slayman, 1996; Gaymard *et al.*, 1998; Lacombe and Thibaud, 1998; Moroni *et al.*, 1998).

Several channels which were not functional in oocytes have been characterized in insect cells applying the patch-clamp technique (Gaymard *et al.*, 1996; Czempinski *et al.*, 1997; Zimmermann *et al.*, 1998). Additional information could be acquired from patch-clamped yeast cells expressing KAT1 or AKT1 (Bertl *et al.*, 1994, 1995, 1997). Besides the two-electrode voltage-clamp technique, the patch-clamp technique has been adapted to the oocyte system to explore the activity of a single transport molecule (Schachtman *et al.*, 1992; Cao *et al.*, 1995b; Hedrich *et al.*, 1995; Hoshi, 1995; Müller-Röber *et al.*, 1995; Zei and Aldrich, 1998). From these observations transport rates in the range of millions of ions per second could be estimated.

The data obtained so far indicate that plant K<sup>+</sup> channels are not only potassium-selective pores in the membrane, but can be directly regulated by the membrane voltage, intracellular and extracellular protons, and cytoplasmic nucleotides. Furthermore, based on the transport direction under physiological conditions, plant potassium channels have been segregated into two groups: inwardly rectifying channels (KAT1, AKT1, AKT2/3 from *Arabidopsis*; KST1, SKT1 from potato) and outwardly rectifying channels (KCO1, SKOR from *Arabidopsis*; for references see Table 1).

#### Biochemical characterization

In addition to the investigation of the function of plant transporters, other methods have been used to gain

further information. Biochemical approaches have been developed for the characterization of phosphorylation and glycosylation status of the transporter proteins and also the assembly of the components of several transport systems. Such analyses require the isolation of large quantities of protein in a controlled environment. Since, as mentioned above, expression in *E. coli* does not seem to offer particular advantages, most success in this field has up to now relied on expression in yeast or in insect cells. However, even in these eukaryotic systems, problems regarding correct targeting of the heterologously expressed protein to the cell membrane can occur. Most, or even all, of the synthesized polypeptides may be trapped in internal membranes (Villalba *et al.*, 1992; Regenberg *et al.*, 1995; Gaymard *et al.*, 1996; Erhardt *et al.*, 1997). Yet problems in the targeting of heterologously expressed proteins do not exclude the development of biochemical approaches and the purification of the protein in a functional state. Absence of targeting to the cell membrane can facilitate purification protocols (Villalba *et al.*, 1992). By the same token, the use of yeast strains defective in the secretion pathway (sec mutants) has yielded insights in the phosphorylation processes occurring during the movement of newly synthesized ATPases from the ER to the cell surface (Chang and Slayman, 1991).

Illustrative approaches for the purification of plant membrane transport proteins have been developed for K<sup>+</sup> channels and plasma membrane H<sup>+</sup>-ATPases. Direct purification of K<sup>+</sup> channels from plant tissue is highly challenging work (Zeilinger, 1994) due to the very low

**Table 1.** Functionality of potassium transporters in different heterologous expression systems

Transporter	Expression system				Reference
	Yeast	<i>Xenopus</i> oocytes	<i>Sf9</i> cells	<i>E. coli</i>	
AKT1	+	— <sup>a</sup>	+		Sentenac <i>et al.</i> , 1992 Gaymard <i>et al.</i> , 1996
SKT1	—	— <sup>a</sup>	+		Zimmermann <i>et al.</i> , 1998
KAT1	+	+	+	+	Anderson <i>et al.</i> , 1992 Schachtman <i>et al.</i> , 1992 Gaymard <i>et al.</i> , 1996 Marten <i>et al.</i> , 1996 Uozumi <i>et al.</i> , 1998
KST1		+	+		Müller-Röber <i>et al.</i> , 1995 Zimmermann <i>et al.</i> , 1998
AKT2 <sup>b</sup>	—	—	—	+	Cao <i>et al.</i> , 1995b Uozumi <i>et al.</i> , 1998
AKT3 <sup>b</sup>		+			Ketchum and Slayman, 1996
KCO1		— <sup>c</sup>	+		Czempinski <i>et al.</i> , 1997
AtKUP1	+ <sup>d/-e</sup>	—		+	Fu and Luan, 1998 <sup>d</sup> Kim <i>et al.</i> , 1998 <sup>e</sup>

<sup>a</sup> AKT1 and SKT1 build functional channels when co-expressed in *Xenopus* oocytes (Dreyer *et al.*, 1997). Meanwhile it could also be shown that SKT1 alone is functional in oocytes (S Zimmermann and B Müller-Röber, unpublished results).

<sup>b</sup> AKT2 and AKT3 are encoded by the same gene showing two start-codons in the same open reading frame. AKT2 is the protein synthesized if the first start codon is used, and AKT3 the protein if the second codon is used.

<sup>c</sup> Injection of KCO1-encoding cRNA into *Xenopus* oocytes seems to be poisonous for the cells (S Zimmermann and B Müller-Röber, unpublished results).

abundance of these proteins in the membrane. In contrast, the heterologous expression of K<sup>+</sup> channels in the baculovirus/insect cell system and their subsequent purification has proven to be much more effective. The *Arabidopsis* K<sup>+</sup> channel AKT1 has been obtained with high purity (a single band on a silver stained gel) in sufficiently large amounts (c. 1 mg protein from a 1.0 l cell culture) to allow the demonstration that, like its *Shaker* family counterparts in animal cells, AKT1 takes on a tetrameric structure (Daram *et al.*, 1997).

Although by comparison to K<sup>+</sup> channels, the plasma membrane H<sup>+</sup>-ATPase is much more abundant, its functional characterization after purification from plant tissues remains problematic due to the presence and the similarity of multiple isoforms in many tissues. The first comparison of the functional properties of isoforms of the plant plasma membrane H<sup>+</sup>-ATPase was obtained by expression in *S. cerevisiae* (Palmgren and Christensen, 1994). Using inducible promoters, which allowed independent control for the expression of the endogenous yeast H<sup>+</sup>-ATPase and that of the heterologous pump, three isoforms of the *Arabidopsis* pump, AHA1, AHA2 and AHA3, were produced individually and their biochemical properties characterized. Each of the isoforms displayed qualitatively similar enzymatic properties, but quantitative differences were found in turnover rates for ATP hydrolysis, the apparent affinities for ATP or sensitivity to pH. This first demonstration of functional differences between members of the same gene family led to the suggestion that such diversity is central to solute transport integration at the whole plant level, allowing each tissue to express the set of transport systems suited to the role that this tissue plays in the plant (Sussman, 1994).

#### Analyses of structure–function relationships

Determination of the amino acid sequence of a transporter through the sequence of its cloned cDNA can provide an initial glimpse of its putative structure. Based on the inferred hydrophilic and lipophilic character of different parts of the protein, transmembrane domains and sometimes extramembraneous regions can be postulated. However, as such analyses can lead to strongly distorted models, the need for direct experimental approaches, such as the investigations aimed at elucidating the structure of the *Drosophila* Shaker K<sup>+</sup> channel (compare the initially proposed topology, Kamb *et al.*, 1987, with the latest model, Durell *et al.*, 1998) and the *Arabidopsis* NAT2/AAP1 amino acid carrier (Chang and Bush, 1997), is inescapable.

To elucidate the topology of NAT2, NAT2, c-myc epitopes that are readily detectable by immunolabelling were fused to the C-terminus and the N-terminus of the protein. Expression in yeast revealed that this engineering

did not disrupt the function of the protein. After heterologous expression in COS cells, only the C-terminal epitope was detected on the cell surface indicating the C-terminus is oriented on the outside and the N-terminus on the inside. In addition, the epitope-tagged proteins were expressed in a cell free translation system, incorporated into microsome membranes, and digested with a protease. From the resultant proteolytic fragments it could be concluded that NAT2 contains 11 transmembrane domains, a cytosolic N-terminus and an extracellular C-terminus (Chang and Bush, 1997). In contrast, hydropathy analysis of the NAT2 sequence had suggested 12 membrane-spanning segments (Frommer *et al.*, 1993; Hsu *et al.*, 1993).

The topology of the *Arabidopsis* K<sup>+</sup> channel KAT1 has been investigated by a different technique. Varying lengths of the N-terminus of the protein were fused with alkaline phosphatase (PhoA), and the resulting chimaeric proteins were expressed in *E. coli*. The enzyme activity of the PhoA moiety indicated the localization of the phosphatase, since PhoA is active when it is exported into the periplasm, whereas it remains inactive in the cytoplasm (Manoil and Beckwith, 1986). Based on this screen, the postulated topology of the channel, with six transmembrane domains (Anderson *et al.*, 1992), was confirmed (Uozumi *et al.*, 1998).

Obviously, to gain an understanding of the molecular basis of the catalysis of transmembrane transport, detailed structural information beyond the elucidation of topology of the transporter proteins is required. With notable exceptions, including the recently solved structure of the *Streptomyces lividans* K<sup>+</sup> channel KcsA (Doyle *et al.*, 1998), there is a paucity of direct structural data on transport proteins. Most of our knowledge in this field originates from the manipulation of transport proteins using recombinant DNA techniques and subsequent assays in heterologous systems for effects on function. In accordance with this, three different approaches have been used: random mutagenesis, site-directed mutagenesis, and creation of chimaeric proteins.

In the case of random mutagenesis, a preparation of randomly mutated transporter cDNA is generated and screened upon expression in a heterologous host, such as *S. cerevisiae*. Clues regarding structure–function relationships are subsequently gleaned from the sequence and detailed characterization of the mutants exhibiting altered transport function. In many cases, this approach can be the most efficient way of investigating the structure of the protein. However, the usefulness of random mutagenesis can be limited unless the output of the system can be designed to distinguish between mutations, such as non-sense or frameshift mutations (Ros *et al.*, 1999), that confer trivial loss-of-function phenotypes and those that alter sites specifically required for transporter function.

Site-directed mutagenesis presupposes a concrete

suspicion that a specific part(s) of the protein determines a distinct feature. This suspicion can arise from sequence comparison with homologous proteins, from random mutagenesis, or from the pharmacological attributes of the transporter. The chemical properties of inhibitors, as well as their molecular interactions with the transporter, often provide information about amino acid residues that are involved in substrate binding and translocation (Crestfield *et al.*, 1963; Westhead, 1965). Accordingly, single amino acids are selectively changed or parts of the protein are truncated and the functional features of the mutant system investigated. One limitation of this approach is the intrinsic bias in selecting specific regions for mutagenesis while another is the inefficiency of site-directed mutagenesis compared to random mutagenesis when the targeted region is large.

The third approach, creation and analysis of chimaeric proteins, is applicable to homologous proteins strongly differing in only a few functional characteristics. Creation of chimaeric proteins between both transporters can help to localize the sites in the proteins being responsible for the difference.

Structure–function studies have been performed for different plant transporters, among them several water channels, the Na<sup>+</sup>-coupled K<sup>+</sup> carrier HKT1, the hexose-H<sup>+</sup> symporters HUP1 and HUP2, the sucrose-H<sup>+</sup> symporter AtSUC1, as well as the potassium channels KAT1 and KST1. Results of studies on these transporters are summarized below.

Plant water channels (aquaporins) belong to the major intrinsic proteins (MIP family) with six putative transmembrane segments (for a review, see Maurel, 1997). The water flux through many aquaporins can be blocked by extracellular mercury. Using site-directed mutagenesis, the mercury-sensitive site of  $\gamma$ -TIP and  $\delta$ -TIP (aquaporins expressed on the tonoplast) was shown to be a cysteine residue in the third transmembrane domain (Daniels *et al.*, 1996). In contrast, the plasma membrane water channel RD28 is not sensitive to Hg<sup>2+</sup>. Engineering of a cysteine residue in the fifth transmembrane helix created a mercury sensitivity (Daniels *et al.*, 1994) indicating that both the third and the fifth transmembrane segment are involved in the formation of the aqueous pore. In a further study, it was shown that the activity of  $\alpha$ -TIP is regulated by phosphorylation (Maurel *et al.*, 1995). Mutating putative targets for a phosphorylation, three serines were identified which each can independently modulate water transport by the  $\alpha$ -TIP protein (Maurel *et al.*, 1995). The control of permeability by channel phosphorylation has also been investigated for the plasma membrane channel PM28A. Mutational analysis revealed two serines which were phosphorylated when the protein was expressed in *Xenopus* oocytes. However, biochemical investigations led to the conclusion that only one of these

two sites is used to modulate the channel activity *in planta* (Johansson *et al.*, 1998).

A model system for structure–function studies on plant transporters belonging to the major facilitator superfamily is the *Chlorella* glucose-H<sup>+</sup> symporter HUP1 (for review see Tanner and Caspari, 1996). Transporters in this family are believed to contain 12 transmembrane segments. To determine structural elements of HUP1 involved in sugar binding and transport, randomly produced mutants of the *HUP1* gene were screened in *Schizosaccharomyces pombe* for an altered affinity toward the toxic sugar analogue 2-deoxyglucose. Whereas yeast cells transformed with the wild-type HUP1 were highly sensitive to 2-deoxyglucose, mutations that altered the protein in either the 5th, 7th or 11th transmembrane domains conferred decreased sensitivity (Will *et al.*, 1994, 1998). In addition, site-directed mutagenesis revealed that a residue at the end of the first transmembrane domain (D44) is essential for the activity of the transporter (Caspari *et al.*, 1994). In subsequent studies this region of the protein was shown to be crucial for substrate specificity as well.

HUP2, another monosaccharide-H<sup>+</sup> symporter in *Chlorella*, shares 74% homology to HUP1, but differs significantly from HUP1 in the former's higher specificity for D-galactose. The expression of chimaeric proteins in yeast led to the conclusion that this difference involves the amino-terminal part of the first extracellular loop, and site-directed mutagenesis pinpointed the distinction to a single amino acid (N45; Will and Tanner, 1996; Will *et al.*, 1998). As a whole, the data support the proposal that the 1st, 5th, 7th, and 11th transmembrane segments line the sugar translocation path and determine its specificity. Additionally, the role of histidine residues in the transport of hexoses has been investigated. The substitution of all of the histidine residues in HUP1 to arginine has no effect on the transport activity in *S. pombe* indicating that the extracellular histidines are not required for substrate binding nor for coupling of transport of the solute with co-transported protons (Caspari *et al.*, 1994).

In comparison, a histidine highly conserved among plant sucrose-H<sup>+</sup> transporters has been identified to be part of the sucrose binding pocket of the sucrose carrier AtSUC1. Previous biochemical investigations showed that, when the classical histidine denaturing agent diethyl pyrocarbonate (DEPC) was applied from the outside face of the plasma membrane, the sucrose carrier was inhibited. Significantly, DEPC inhibition was blocked when sucrose was present in the reaction mixture, suggesting the DEPC-sensitive residue was at, or conformationally linked to, the sucrose binding site (Bush, 1993b). Site-directed mutagenesis subsequently inferred that the corresponding residue was located in the loop between the first and second transmembrane segment (Lu and Bush, 1998), supporting the hypothesis that this region of sugar transporters determines the substrate specificity.

In contrast to aquaporins or sugar carriers which both belong to large protein families sharing structural and functional similarities, nothing was known regarding the structure of the sodium-coupled potassium carrier HKT1. To identify regions of this protein involved in the binding and transport of Na<sup>+</sup> and K<sup>+</sup>, two approaches involving both random and site-directed mutagenesis have been developed. Analysis of the mutant proteins expressed in *S. cerevisiae* indicate that HKT1 harbours distinct and separate binding sites for Na<sup>+</sup> and K<sup>+</sup>. The loop between the putative 9th and 10th transmembrane segments appears to be part of the Na<sup>+</sup> binding site, whereas the 6th transmembrane segment is evidently involved in the binding of K<sup>+</sup> (Rubio *et al.*, 1995; Diatloff *et al.*, 1998).

At the present time, the most detailed information on structure–function relationships of a plant transporter family is available for plant K<sup>+</sup> channels. In this connection, KAT1 (*Arabidopsis thaliana*) and KST1 (*Solanum tuberosum*), which are both expressed in guard cells, have evolved into model systems for these studies. These plant potassium channels belong to the large family of voltage-gated potassium channels showing a topology of six transmembrane domains (see above) and a pore region with the typical signature sequence TxxTxGYGD (Heginbotham *et al.*, 1994) between the 5th and 6th segment. Extensive use of site-directed mutagenesis and the biophysical characterization of the resulting mutants expressed in oocytes identified this region as the permeation pathway for potassium ions. Accordingly, altered pharmacology and selectivity of the channel mutants could be correlated to an altered growth phenotype of complemented yeast cells (Anderson *et al.*, 1994; Uozumi *et al.*, 1995; Becker *et al.*, 1996; Ichida and Schroeder, 1996; Nakamura *et al.*, 1997; Dreyer *et al.*, 1998).

By exploiting both the microbial aspects and the genetic capabilities of the yeast system, elements of the GYG motif within the pore signature sequence of the K<sup>+</sup> channel KAT1 have been shown to play a key role in recognition of potassium and the ability of the channel to discriminate between ions (Nakamura *et al.*, 1997). It was possible to perform a combinatorial analysis in which all possible amino acid substitutions at the YG sites within this signature sequence were tested for function and for ion selectivity by expression of the mutant channels in yeast (and subsequently supported by independent analysis in *Xenopus* oocytes). The results of this study established specific roles for these sites and revealed structural constraints that could not have been discovered by other (non-combinatorial) strategies. For example, it was shown that only the wild-type YG and the conservative mutant FG channels are completely selective for K<sup>+</sup> over other ions. It was also learned that essentially any amino acid substitution at the site normally occupied by glycine abolishes channel function yet glycine substitution mutants can function if accompanied by compensatory

mutations that decrease the size of the side chain at the adjacent site, normally occupied by tyrosine. Thus, the absolute requirement for glycine in an otherwise wild-type channel was concluded to arise from an intrinsic physical restraint on the channel pore. Evidently, the tyrosine side chain interferes with channel function if the amino acid beside it (normally G) contains a side chain of any size. Importantly, the basic structure–function features of the KAT1 pore gleaned from numerous studies involving heterologous expression in yeast (Anderson *et al.*, 1994; Uozumi *et al.*, 1995; Becker *et al.*, 1996; Nakamura *et al.*, 1997) are generally supported by the recently solved structure of the K<sup>+</sup> channel KcsA from *Streptomyces lividans* (Doyle *et al.*, 1998), underscoring the relevance, in this case, of data generated from a heterologous system.

Besides altering the ionic selectivity and pharmacological properties of the K<sup>+</sup> channel, mutations in the pore region can also affect the voltage-dependent activity (Becker *et al.*, 1996; G Lemaillot, unpublished data), indicating that this region is involved in the conformational changes during opening and closing. Plant K<sup>+</sup> uptake channels like KAT1 and KST1 open upon hyperpolarization, and close when the membrane is depolarized. This voltage-dependent activity (gating) is likely to be triggered by movement of the 4th transmembrane domain which displays a high density of positively charged amino acids (Cao *et al.*, 1995a; Dreyer *et al.*, 1997; Hoth *et al.*, 1997a; Marten and Hoshi, 1997, 1998; Zei and Aldrich, 1998). The gating machinery of plant K<sup>+</sup> channels is further modulated by both intracellular and extracellular protons (Hedrich *et al.*, 1995; Hoshi, 1995; Müller-Röber *et al.*, 1995). For the K<sup>+</sup> channel KST1, it has been shown that extracellular histidines are involved in a pH-sensing mechanism (Hoth *et al.*, 1997b). The deletion of all extracellular histidine residues removed the pH-effect on the steady-state activity of KST1. In contrast, a similar approach carried out on the closely related channel KAT1 did not influence its pH-sensitivity at all (Ichida and Schroeder, 1996), indicating that other amino acids besides histidine are involved in the pH-sensing mechanism.

Finally, further approaches, aimed at elucidating the structure–function relationships of plant K<sup>+</sup> channels, based on expression of green fluorescent protein (GFP)-tagged channels in the insect cell system and/or in the two-hybrid system, have revealed domains involved in channel clustering (Erhardt *et al.*, 1997) and/or channel tetramerization (Daram *et al.*, 1997). In the former approach, a yeast two-hybrid screen (Fields and Song, 1989; for a recent review see Niethammer and Sheng, 1998) using a C-terminal fragment of the potato guard cell K<sup>+</sup> channel KST1 as bait led to the identification of the two novel K<sup>+</sup> channel proteins SKT2 and SKT3. Interactions between the C-terminal fragment of KST1

and C-terminal fragments of SKT2 and SKT3 were independently confirmed by Western blot-related assays, utilizing K<sup>+</sup> channel C-termini fused to green fluorescent proteins. Analysis of fluorescence microscopy images of insect cells expressing GFP or a fusion of GFP to the complete KST1 polypeptide revealed formation of channel clusters within the cell membrane. In contrast, a fusion protein between GFP and KST1 deleted for the C-terminus region, the so-called K<sub>HA</sub> domain (corresponding to about the last 80 amino acids) which is highly conserved among plant K<sup>+</sup> channels, clearly displayed an even, i.e. non-clustered, distribution of fluorescence along the plasma membrane. Additional electrophysiological studies showed that both fusion proteins displayed functional features similar to those of the wild-type channel. Taken as a whole, these data support the hypothesis that domain K<sub>HA</sub> mediates channel protein interactions, leading to channel clustering, but is not essential to channel subunit tetramerization (see section on Biochemical characterization) and channel activity (Erhardt *et al.*, 1997). In the latter approach, the entire cytoplasmic C terminus (the 564 amino acids downstream of the 6th transmembrane segment) of *Arabidopsis* AKT1 channel was expressed in the baculovirus system and shown to form highly stable tetrameric structures, suggesting a role for this region in channel tetramerization (Chérel *et al.*, 1996; Daram *et al.*, 1997). Expressing various domains from this region as bait and prey in yeast two-hybrid tests revealed subsets of interactions, that could be involved in the tetramerization process as suggested by Daram *et al.* (1997) or in channel-protein interactions or clustering. It might be likely that interactions between C-termini of different channel subunits as revealed by Ehrhardt *et al.* (1997) between KST1 and SKT2 and SKT3 have a physiological significance. Co-expression of different wild type as well as mutant K<sup>+</sup> channel proteins has unveiled that plant potassium channels have the potential to form heteromultimeric structures composed of different of  $\alpha$ -subunits (Dreyer *et al.*, 1997). Such phenomena, also reported for animal K<sup>+</sup> channels (for a review see Jan and Jan, 1997), could allow the cell to increase and control the functional diversity of the channels expressed on its membrane.

### Problems with expression systems

The previous sections have demonstrated the powerful application of heterologous expression systems for the cloning and functional characterization of plant membrane transporters. Nevertheless, there are problems and risks involved in the utilization of the particular heterologous systems and these are now outlined. However, it is emphasized that the utility of an expression system should not be judged by the quantity of the statements below. It is obvious that the more a system is used and the more

sophisticated the measurements are, the more is known about the limitations. *Xenopus* oocytes, for instance, represent the most documented system in this regard.

A simple example might point out one of the uncertainties in the use of the oocyte expression system. The determination of the single channel conductance of the plant K<sup>+</sup> channel KAT1 expressed in *Xenopus* oocytes led to partially inconsistent results (compare Schachtman *et al.*, 1992, with Cao *et al.*, 1995b; Hedrich *et al.*, 1995; Hoshi, 1995; Zei and Aldrich, 1998), raising the discussion as to whether the observed currents are caused by the heterologously expressed gene or originate from oocyte-intrinsic transporters (Methfessel *et al.*, 1986). Oocytes possess endogenous transport systems that influence their growth, maturation and fertilization. Thus it is important to be aware of endogenous currents that may already be present in the native oocyte, although the amplitude of most of the endogenous ionic conductances is highly variable among oocytes from different batches and frogs and it is not uncommon for several of the native currents to be absent altogether or to be present at only very low levels. A frequently used strategy to estimate the errors by intrinsic currents is to compare the electrical features of RNA-injected oocytes with those of oocytes from the same batch being untreated or injected with water or antisense RNA. However, this strategy does not provide absolute controls since it has been shown that expression of heterologous membrane proteins in *Xenopus* oocytes can induce endogenous currents (Tzounopoulos *et al.*, 1995). Thus it is possible that even non-functional transporters expressed in oocytes might induce significant currents which are not present in control cells. Therefore, not only the presence of a current but also, when possible, additional pharmacological characterizations may be required to prove unequivocally the functional expression of the heterologous protein.

The occurrence of intrinsic currents set another limit to the use of the oocyte system for the investigation of plant membrane transporters. On the one hand, the plant plasma membrane can be strongly polarized ( $< -200$  mV) and its transporters can require strongly negative potentials for their activation or energetic coupling, while on the other hand, even in oocytes with low background currents, hyperpolarizing the membrane beyond  $-170$  mV can result in activation of large endogenous negative currents possibly exceeding the magnitude of the current of the heterologously expressed transporter. Hence, the most trustworthy voltage range of an oocyte is limited from  $-170$  mV to  $+90$  mV. However, in special cases this limitation could partially be overcome by using appropriate voltage-pulses (Dreyer *et al.*, 1998). Finally, it must be noted that in several experiments aimed at characterizing new channels and carriers, functional expression was not observed. For example, the injection of certain channel cRNAs into

oocytes did not result in significant changes in the membrane conductance (Table 1; Cao *et al.*, 1995b, Gaymard *et al.*, 1996; Dreyer *et al.*, 1997; Kim *et al.*, 1998; Zimmermann *et al.*, 1998). The baculovirus–insect cell system has provided an alternative system for characterizing those plant K<sup>+</sup> channels, e.g. AKT1, whose expression in *Xenopus* oocytes fails to produce electrical signals (Gaymard *et al.*, 1996). The reasons why a channel or transporter is or is not expressed in a functional state according to the expression system used remain unknown.

Beyond providing an alternative for functional expression, the baculovirus insect cell system has another advantage compared to *Xenopus* oocytes. *Sf9* or *Sf21* cells are more directly amenable to patch-clamp studies, because they are not surrounded by a vitelline membrane that must be removed before electrodes can be attached. However, the insect cell system has its own inconveniences. The expression vectors, i.e. the recombinant baculovirus encoding for the gene of interest, are by comparison rather difficult to construct and to purify routinely. In addition, the usefulness of these expression vectors is essentially limited to *Sf9* or *Sf21* cells whereas vectors that function for heterologous expression in oocytes can also be used in cell types such as COS and Chinese hamster ovary cells (CHO cells) and thus offer complementary opportunities of investigation on the channel of interest. Furthermore, *Sf9* cells die within only 1–3 d after infection, allowing fewer experimental possibilities compared to transfected oocytes, which can be used up to 1 week after injection of cRNA or DNA vectors. Besides these technical inconveniences the use of infected *Sf9* or *Sf21* cells for electrophysiological purposes is limited as well. Endogenous currents, which are less documented in the literature than those of oocytes but which occasionally resemble those of the transporters under investigation, can disturb the measurements (Cao *et al.* 1995b; Gaymard *et al.*, 1996; Marten *et al.*, 1996; Czempinski *et al.*, 1997; S Zimmermann, unpublished data). In addition, the cell membrane withstands poorly the large hyperpolarizations that may be required to study plant channels.

Problems regarding the use of mutant yeast strains for the identification and characterization of plant K<sup>+</sup> transporters have been stressed recently (Madrid *et al.*, 1998). The strains of *S. cerevisiae* used for these approaches contain null alleles of one or both of the high-affinity K<sup>+</sup> transporter genes, *TRK1* and *TRK2*. These mutations cause hyperpolarization of the membrane potential. In the case of the *trk1 trk2* double mutant, membrane hyperpolarization is extreme and can lead to artefacts in the estimation of the  $K_m$  values of K<sup>+</sup> carriers complementing the yeast mutant. Potentially more serious than this, *trk1 trk2* mutants show an ectopic low-affinity potassium uptake in which K<sup>+</sup> is accumulated through many proteins whose normal role is not that of K<sup>+</sup> transport.

If the hyperpolarized membrane potential of these mutants can transform their own non-K<sup>+</sup> transporters into K<sup>+</sup> transporters, it is evident that the same may occur with some plant transporters. One example in this context might be the cloning of the low affinity cation transporter LCT1 (Schachtman *et al.*, 1997).

### Physiological relevance of analyses in heterologous systems and perspectives

A reliable characterization of the functional and pharmacological features of a given transporter, leading to an unequivocal fingerprint, is a prerequisite towards the elucidation of its role *in planta*. Unfortunately, together with the problem mentioned regarding ectopic K<sup>+</sup> uptake in yeast *trk1 trk2* mutants, a wide set of observations advise that caution should be taken with the data obtained from heterologous systems. By way of illustration, several studies on potassium channels show that the results acquired from heterologous expression systems are ambiguous. Véry *et al.* (1994) and Cao *et al.* (1995b), for example, reported that changes in the expression level of the heterologous channel within the same expression system (*Xenopus* oocytes in these studies) can induce changes in both functional and pharmacological features of the channel. Moreover, comparisons of the features of the same K<sup>+</sup> channel expressed in various systems, yeast, insect cells or oocytes, unveiled further inconsistencies (Table 2). For instance, KAT1 showed a high permeability for NH<sub>4</sub><sup>+</sup> ions when expressed in oocytes, but was rather impermeant to this ion when expressed in yeast or insect cells (Schachtman *et al.*, 1992; Uozumi *et al.*, 1995; Bertl *et al.*, 1995; Marten *et al.*, 1996). By the same token, KST1 was 15-fold more sensitive to extracellular Cs<sup>+</sup> in insect cells than it was in oocytes (Zimmermann *et al.*, 1998).

Differences between features of transporters expressed in oocytes and those derived *in planta* for the native protein have also been reported. The two channels KAT1 and KST1 are believed to be the guard cell K<sup>+</sup> uptake channels of *Arabidopsis thaliana* and *Solanum tuberosum*, respectively (Müller-Röber *et al.*, 1995; Nakamura *et al.*, 1995). However, when expressed in *Xenopus* oocytes, these channels are less sensitive to Ca<sup>2+</sup>, Cs<sup>+</sup>, and H<sup>+</sup> than are the channels of the guard cells (Table 2; Brüggemann *et al.*, 1999). Similar findings were reported for the hexose carrier HUP1. The isolated protein in oocytes differs from the native transporters in *Chlorella* at least in its pH and voltage dependence (Tanner and Caspari, 1996). Such differences clearly indicate that (i) the functional features and the activity of a transporter not only depend on the right ligation of the amino acid chain but also on its correct folding and further transcriptional processes, and (ii) the expressed sequences do not form single proteins, isolated in a virtual membrane, but

**Table 2.** Electrophysiological properties of the plant potassium channels KAT1 and KST1 in different expression systems

Channel feature	Expression system			
	<i>Xenopus</i> oocytes	Sf9 cells	Yeast	<i>in situ</i> (guard cells)
Voltage-dependence, activation threshold	~ -80 mV	~ -60 mV	~ -120 mV	~ -100 mV
pH-dependence, maximum shift in $V_{\frac{1}{2}}$	~ 20 mV			~ 100 mV
Sensitivity to blocking ions				
TEA <sup>+</sup> , Inhibition (10 mM)	~ 70%		~ 70%	
Cs <sup>+</sup> , $K_i$ at -130 mV	~ 1100 $\mu$ M	~ 90 $\mu$ M		
Cs <sup>+</sup> , $V_{Block\frac{1}{2}}$ (1 mM Cs <sup>+</sup> )	~ -155 mV			~ -90 mV
Ca <sup>2+</sup> , $V_{Block\frac{1}{2}}$ (20 mM Ca <sup>2+</sup> )	< -250 mV			~ -190 mV
Permeability for NH <sub>4</sub> <sup>+</sup>	+	-	-	+

Data were obtained from KAT1, KST1, and potato and *Arabidopsis* guard cell K<sup>+</sup> uptake channels (Schachtman *et al.*, 1992; Bertl *et al.*, 1995; Müller-Röber *et al.*, 1995; Uozumi *et al.*, 1995; Véry *et al.*, 1995; Marten *et al.*, 1996; Dreyer *et al.*, 1997; Dietrich *et al.*, 1998; Zimmermann *et al.*, 1998; Brüggemann *et al.*, 1999).

rather pieces of a three-dimensional puzzle, interacting with other surrounding proteins present within the membrane, in the cytoskeleton or in the cytosol, in the heterologous host cell as well as in the original cell. One must be aware that such interactions within a heterologous host cell may result in distorted features. In addition, reconstitution in the heterologous host cell of the variety of interactions that normally occur in the original cell, by co-expression experiments, is probably beyond of our reach at the present time. Several reports regarding plant K<sup>+</sup> channels have highlighted the variety of interactions that could occur *in planta*, by providing evidence for the existence of mechanisms such as interactions between various  $\alpha$ -subunits leading to heteromultimeric channels (Dreyer *et al.*, 1997), interactions with  $\beta$ -subunits (Tang *et al.*, 1996; Fang *et al.*, 1998), channel clustering (Ehrhardt *et al.*, 1997), and channel activity probably tuned by phosphorylation and dephosphorylation events (Li *et al.*, 1998). From what is known for animal K<sup>+</sup> channels, regulatory mechanisms involving, for example, interactions with intracellular compounds that reduce or oxidize cysteine and methionine residues (Ruppersberg *et al.*, 1991; Ciorba *et al.*, 1997), phospholipids (Honoré *et al.*, 1994; Baukowitz *et al.*, 1998; Shyng and Nichols, 1998) or *N*-glycosylation events (Schwalbe *et al.*, 1995) are also likely.

To circumvent misunderstandings that might arise from the investigation of plant transporters only within a heterologous expression system, recent studies have allowed cloned transporters to journey back to their roots (and leaves). Homologous expression in guard cells, mesophyll cells, and suspension culture cells (Ichida *et al.*, 1997; Bei and Luan, 1998; Kim *et al.*, 1998) allowed electrophysiological analyses and radiotracer flux measurements in a cell environment closely related to that of the native cell of the cloned transporter. To elaborate aspects of the physiological roles of plant transporter molecules further, it is likely that the establishment of homologous expression systems, in combination with the

development of reverse genetic approaches, allowing comparison of the transport features in the wild-type plant and in a knock out mutant (Gaymard *et al.*, 1998; Hirsch *et al.*, 1998), is now inescapable.

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