Genes and Proteins for Solute Transport and Sensing

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INTRODUCTION

Transport processes are required for nutrient acquisition, translocation in the plant and for compartmentation within the cells. The weed *Arabidopsis* occurs in many environmentally distinct locations around the world, and a significant proportion of the genome of this small plant encodes membrane proteins, especially transport proteins and putative sensors that cope with these conditions. Many of these proteins are homologous to transporters from other organisms including bacteria, fungi and animals. This chapter provides an overview of the components and mechanisms responsible for solute transport and sensing. Described are the basic principles of transport processes and the transporters of the pump and carrier classes found in *Arabidopsis*. Transport of the most prominent solutes that are taken up and distributed within the plant such as nitrogenous compounds (ammonium, nitrate, amino acids, peptides) and carbohydrates such as sugars (sucrose, glucose) or sugar alcohols (e.g. mannitol) are discussed in some detail.

### General characteristics of transport processes

Cellular plasma membranes demark the interface between life and death: they protect the highly structured and organized plant cellular interior, the cytosol, from the hostile external environment. This creates a compartment which permits biochemical reactions to be carried out within a protected domain. Such a barrier, however, aside from its protective role, must at the same time allow passage of nutrients and solutes to allow cellular functions to proceed. Acquisition of ions, transfer of metabolites and excretion of waste products, but also transport of chemoattractants, kayromones (chemicals that convey information about interactions), hormones and substances that help mobilizing nutrients (e.g. protons, organic acids and phytosiderophores), establish homeostasis between the interior and the exterior. Furthermore, the plasma membrane represents the interface to the environment and thus must play a crucial role in relaying information about the external environ-

ment. Depending on the position of a cell in the plant, this may either be the soil, or the cell wall space as the interface to adjacent or distant cells of the same or a different organism (e.g. pathogenic or symbiotic bacteria and fungi).

The plasma membrane itself is composed of membrane lipids and integral and peripheral proteins (for a detailed discussion see Gennis, 1989). Protein composition within the plasma membrane can vary in wide ranges between 20% and 80% (dry weight). Furthermore membranes may contain carbohydrates in the form of glycolipids or glycoproteins. In the aqueous environment of living cells, the hydrophobic hydrogen-carbon tails of membrane lipids avoid water contact and, although lacking attracting forces, self-assemble and cluster together into an energy minimized state. The polar headgroups orient to the surrounding water on both sides, whereas the hydrophobic tails orient towards each other on the inside leading to the formation of a bilayer consisting of two leaflets with a thickness of ~4nm (Fig. 1). Biological membranes are characterized by an asymmetric distribution of lipids between the two leaflets, i.e. restriction of phosphatidylserine to the inner leaflet (Boon & Smith, 2002; Manno et al., 2002). The lipid asymmetry may have a variety of important biological functions and a loss of asymmetry can be used as a measure for cell death (Schlegel and Williamson, 2001).

Besides the plasma membrane, the eukaryotic cell also contains intracellular compartments surrounded by internal membranes, which enclose specialized organelles and vesicles, separating functional units within a single cell. Such lipid bilayers form a diffusion barrier preventing free exchange of molecules between the in- and outside of the cellular compartments. The lipid composition of the various membranes in a plant cell can vary significantly leading to differences in the membranes properties. Furthermore the phospholipid, glycolipid and sterol composition of cellular membranes is acclimated in different environments, especially due to altered temperature in order to maintain fluidity and functionality.

The lipid bilayer forms a lipid "sea", in which molecules can normally move freely in lateral direction. In other words, individual lipid molecules and peripheral, integral and lipid anchored proteins may diffuse and distribute randomly, whereas an asymmetrical distribution of lipids
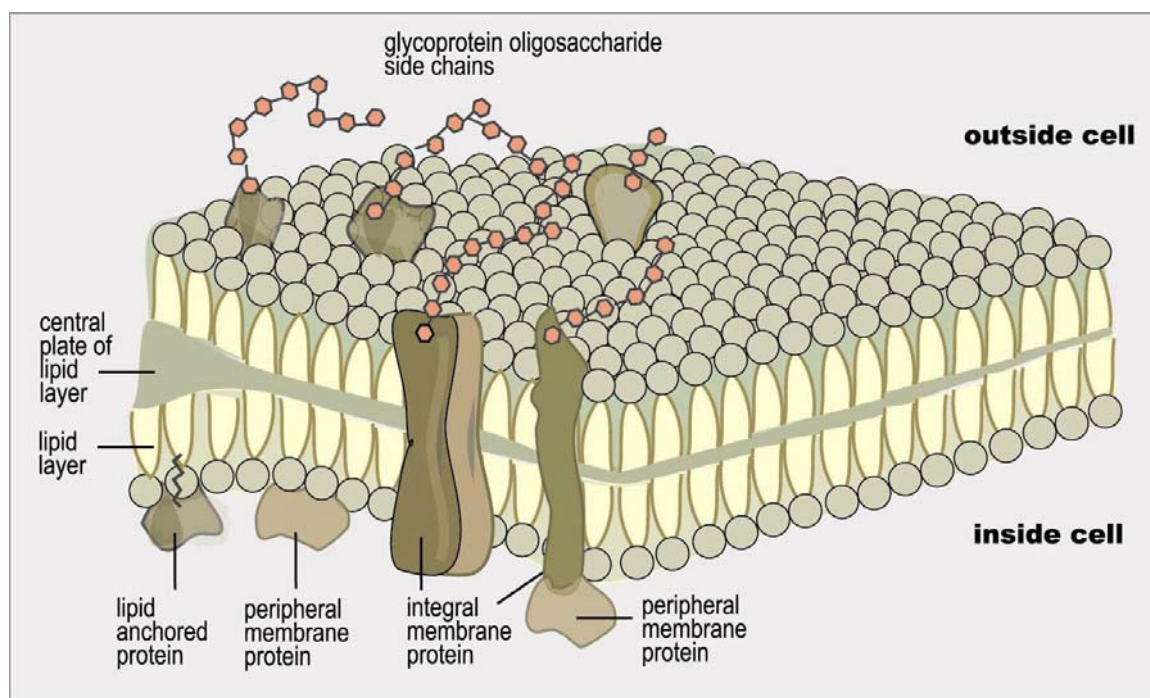
Figure 1. Model of a lipid bilayer containing both embedded transmembrane (glyco-)proteins and peripheral (glyco-)proteins associated with the membrane or anchored to it. Schematic drawing based on the Singer-Nicolson fluid mosaic model.

on the in- and outside of the membrane is generated and maintained by active processes ("flip-flop", for details confer Gennis, 1989). The accepted model for a biological membrane is that of a fluid mosaic (Fig. 1). The free diffusion of some membrane proteins, however, is restricted by cytoskeletal anchors or association with other membrane proteins, and the resulting patchy localization of proteins leads to polarized cell structures. In fungi, free diffusion of lipids and membrane proteins may be restricted by diffusion barriers, generated by septins, whereas in animals tight junctions serve to compartmentalize the plasma membrane. So far, such diffusion barriers in plants have not been identified at the molecular level, e.g. no septin homologs were found in the *Arabidopsis* genome, but it would be very surprising if plants would not contain such barriers. Although little is known about details of specific lipid-protein interactions, membrane lipids may be involved in regulation (Robl et al., 2001) and targeting (Bagnat et al., 2001) of plasma membrane proteins.

Due to the existence of specialized intercellular connections in plant cells, the plasmodesmata, the plasma membrane of most cells within a plant seems to be continuous between adjacent cells. Exceptions are specialized cells such as guard cells, which lose their intercellular connections during development, and cells or organs that are not connected by plasmodesmata, like pollen or seed. Since membrane proteins, however, are often restricted to one of two adjacent cells, barriers similar to those found in fungal or animal cells must exist that prevent free diffusion of membrane proteins between cells.

All molecules have an inherent capacity to passively traverse membranes, but permeability coefficients vary over several orders of magnitude. Usually this permeability is correlated with the solubility of the substance within the hydrophobic environment of the membrane bilayer (Gennis, 1989). Thus non-charged and hydrophobic molecules permeate more easily than ions and polar substances across a membrane. However, in most cases, passive movement is too limited to be relevant for biological processes. Thus transport proteins facilitate the movement of specific molecules across membranes, achieving rates from few molecules per second up to about $10^8 s^{-1}$, the latter value approximating the diffusional limit in aqueous solution. Although small molecules such as water and urea display relative high permeability coefficients, their transport across biological membranes is nevertheless facilitated by transport proteins, which is also the case for hydrophobic substances like lipids or cholesterol.

The composition of a given membrane is not constant, but rather the transport properties of cellular membranes are continuously adjusted to control and optimize metabolite exchange according to the cellular needs. This is achieved by acclimating the types of transporters

present at the membrane to the requirements by biogenesis of new carriers via transcriptional up-regulation on the one hand and removal of unsuitable carriers on the other hand (e.g. turnover, Kühn et al., 1997). Furthermore transport activity can be regulated very rapidly by modulating their activity by posttranslational protein modifications, by withdrawal due to endocytosis or by the release from internal compartments.

Structure of membrane proteins

Due to the difficult biochemistry of hydrophobic membrane proteins, only little is known about the exact tree-dimensional molecular structure of most membrane proteins, but integral membrane proteins are thought to contain mostly either β-barrel or α-helical domains within membrane environment (Ikeda et al., 2002; Möller et al., 2001). Solute transport proteins embedded within the lipid bilayer must have defined properties: they must form (in most cases) a partially hydrophilic "pore", through which solutes can move in a controlled manner, but must be tightly sealed against the membrane lipids to prevent passage of solutes along this interface. The few crystallized and resolved structures are mostly from bacterial origin, but strong homology suggests that the structures apply also for the eukaryotic relatives. Known structures include potassium (Doyle et al., 1998), chloride (Dutzler et al., 2002) and water channels (Murata et al., 2000; Sui et al., 2001), P-type pumps (Toyoshima et al., 2000), Na$^+$/H$^+$ antiporters (Williams et al., 1999), and ABC-transporters (Chang & Roth, 2001; Locher et al., 2002) mitochondrial proteins involved in respiration or redox reactions and the bacterial rhodopsin (Davies et al., 2001). Furthermore lower resolution data are available for a number of transporters such as EmrE, NhaA, OxlT, TetA, Band3, (for review cf. Veenhoff et al., 2002). As a general feature, major parts of these proteins are formed by α-helical domains, connected via short extramembraneous loops. A surprising diversity of functions has evolved by such an ordered alignment of amino acids and secondary structures. β-barreled structures are also observed and occur mainly in proteins from the outer bacterial membrane such as maltoporin or iron siderophore transporters (Ferguson et al., 2002; Cowan et al., 1992) but also in the outer mitochondrial membrane. Since the average thickness of the membrane is ~4 nm, the domains which span the membrane must have a length of at least 4 nm to cross the membrane. If they are tilted relative to the perpendicular angle of the surface, they must even be longer. In order to span the membrane completely, an α-helix must consist of 18-22 amino acid residues. The amino acids in such an α-helix are preferentially hydrophobic to be able to interact with the hydrophobic lipid environment in order to seal the

interface tightly. However, to the inside of the "pore", they must be hydrophilic to allow passage of hydrophilic solutes. Thus most of the helices in a membrane spanning segment must be amphipathic, i.e. hydrophilic on the inside and hydrophobic to the outside. To be able to form a selective pore in the membrane, several helices are bundled together and packed tightly. Such structures can be generated either by combining multiple polypeptides with single transmembrane spanning domains, or (as in most transporters) several such domains can be fused into a single polypeptide forming a so-called polytopic membrane protein. Most of the transporters in Arabidopsis are thought to belong to the polytopic α-helix class. This highly modular structure of amino acid composition in membrane proteins allowed the development of algorithms that predict whether a given primary gene sequence encodes for a polytopic membrane protein. Furthermore this allowed to systematically predict the presence and composition of membrane proteins in various genomes (e.g. bacteria, yeast, *Arabidopsis*). The simplest way to identify a putative transporter in the genome is to search for stretches of hydrophobic amino acids. More sophisticated, in some cases self-learning programs have been developed that enable a relative high confidence level prediction (Sonnhammer et al., 1998). Web-based prediction programs include TmHMM_v2.0 (Krogh et al., 2001), HmmTop_v2.0 (Tusnady and Simon, 1998, TMap (Persson and Argos, 1996), TopPred_v2.0 (Claros and von Heijne, 1994), TMPred (Hofmann and Stoffel, 1993), SosuiG_v1.1 (Mitaku and Hirokawa, 1999) and Eiconda_v0.9 (Schwacke, unpublished).

In the case of most solute transporters, it is often necessary to rely on such predictions, since so far no three-dimensional structures of metabolite transporters such as sugar or amino acid transporters are available. Based on the outputs of prediction programs and comparison with available structural data, one may hypothesize that membrane transporters in eukaryotes typically consist of several α-helical domains, while β-barrels predominate in the structures of porins in the outer membrane of mitochondria, reflecting their prokaryotic origin.

Using the seven prediction programs listed above, an estimate of total integral membrane proteins in *Arabidopsis* can be given. Considering only proteins with one or two transmembrane regions (predicted by at least 6 of the 7 programs), proteins with three or more transmembrane regions (predicted by at least 5 of the 7 programs) and excluding proteins with a single predicted N-terminal transmembrane region with a cleavable signal petide, 6475 proteins (25 %) of the 25492 predicted nuclear encoded proteins can be classified as transmembrane (TM) proteins (Schwacke et al., submitted; *Arabidopsis* Genome Initiative, 2000). Typical membrane transporters have between 6-14 hydrophobic spans that may cross the membrane. About 5% of all
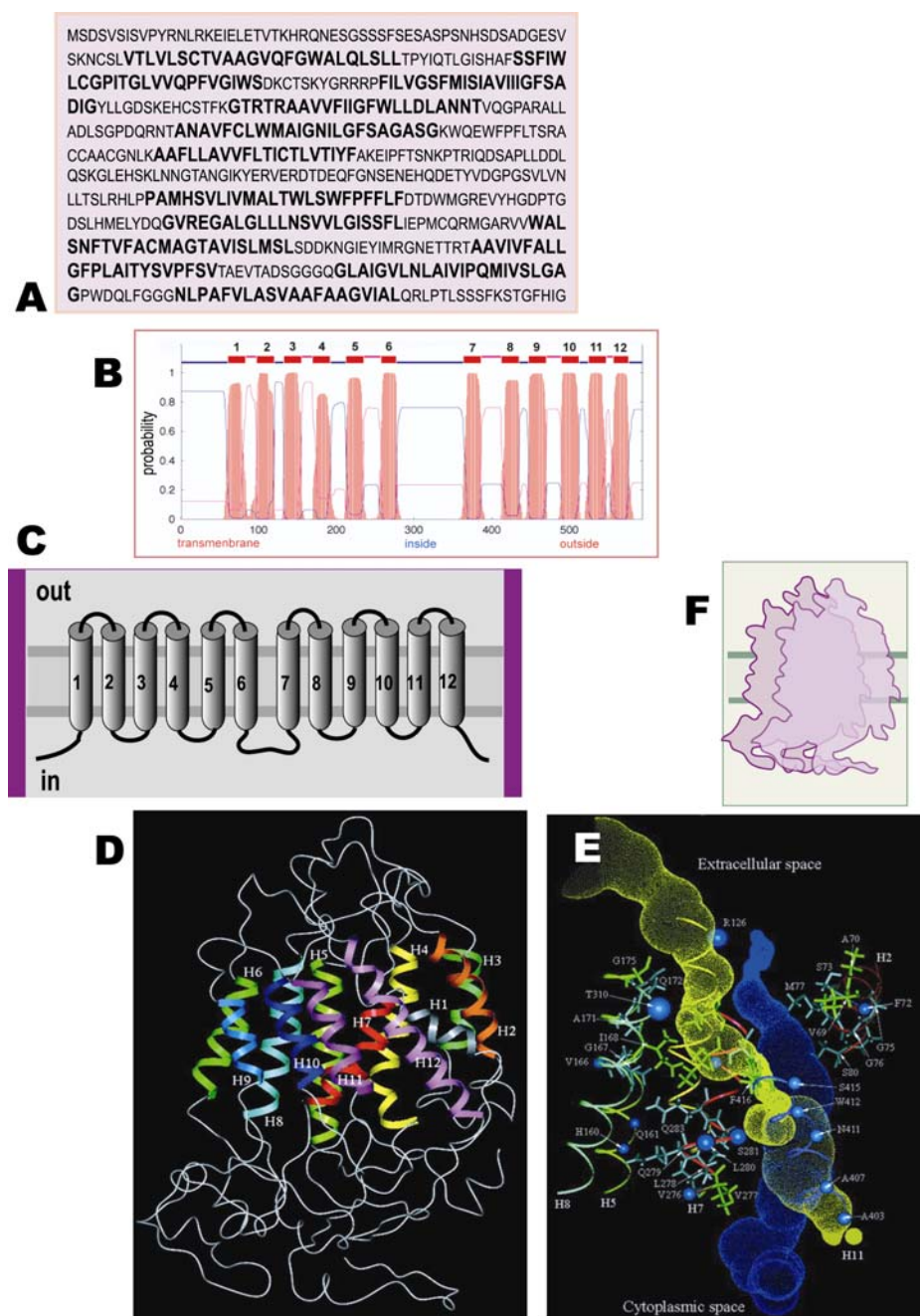
Figure 2. From sequence to structure. A: Amino acid sequence of AtSUT2 (At2g02860). Stretches of hydrophobic amino acid stretches that are predicted to be embedded into the membrane are shown in bold. B. Prediction of transmembrane topology for the sucrose transporter AtSUT2 by TmHMM v2.0. C. Schematic two-dimensional view of AtSUT2. D. Predicted 3-dimensional structure of a distant AtSUT2 homolog, the human hexose transporter GLUT1, which belongs to the MFS-family (major facilitator superfamily) (Zuniga et al., 2001). The 12 α-helical transmembrane helices are predicted to tilt slightly. E. Space filling representation of the main (in yellow) and auxiliary (in blue) substrate "pore" of GLUT1, reference as before. With kind permission from The American Society for Biochemistry and Molecular Biology. F. The functional unit of sucrose transporters may be a dimer.

genes in *Arabidopsis* are predicted to have at least six transmembrane domains. The most typical structure as originally identified in the prototype of all solute transporters, lac permease (Kaback et al., 2001) consists of 12 transmembrane helices, a topology that is found for plant sugar transporters as well (Sauer 1989; Riesmeier et al., 1992; for review cf. Veenhoff et al., 2002). Interestingly fragments of such proteins can be expressed together to reconstitute functional transporter activity (Zen et al., 1994, Wrubel et al., 1990), Reinders et al., 2002a, Xie et al., 2000), demonstrating that the packaging of the helices is mainly due to forces between the individual helices (for a detailed discussion cf. Veenhoff et al., 2002).

Membrane proteins are integrated in a defined orientation into the membrane. Prediction programs take into account differences in the amino acid distribution in helices with respect to the two sides of the membrane (e.g. "positive inside rule"), which is also reflected in the asymmetry in lipid composition between inner and outer membrane side. An example for the structural prediction of an *Arabidpsis* sucrose transporter is given in Fig. 2. The substrate recognition is somewhere inside a central "pore", accessible from both sides of the membrane. Though the transporter may have intrinsic rectification properties, directionality of transport is not created by the transporter itself, but the concentration gradients of the substrates and coupling mechanism (see below).

Transporters for specific solutes are often similar structurally and also show surprising conservation at the primary sequence level among plants, fungi,animals, and bacteria (see e.g. amino acid transport systems, Wipf et al., 2002). Thus they belong to protein families, which resemble the evolutionary and functional relationship between them. Various classification systems have been developed such as Pfam (Bateman et al., 2002), the SLC system for human solute carriers (www.gene.ucl .ac.uk/nomenclature), and a system comparable to the enzyme nomenclature, i.e. the T numbers (Saier, 2000). The system classifies membrane proteins according to five parameters: phylogenetic relationship, substrate, transport mechanism and subcellular localization. Since, however, in most cases only the phylogenetic relationship can be determined unambiguously and since for most membrane proteins the subcellular localization is still not known, it seems a bit premature to use such a system so widely. *Arabidopsis* membrane proteins have been identified using prediction programs from the genome sequence, and various databases are available providing an overview and much detailed description of the various transporter families (PlantsT: plantst.sdsc.edu, Arame mnon: Schwacke et al., submitted).

Membrane proteins in complexes and rafts

Like in other organisms, *Arabidopsis* membrane proteins are assumed to assemble in homo- or heteromeric complexes, in clusters or "rafts" (Reinders et al., 2002b; Jacobson et al., 1995). Recent findings from experiments using a variety of biochemical techniques indicate that, although monomers of a transport protein with 12 transmembrane spanning domains are mostly sufficient for the formation of the pore and thus for the transport function, most metabolite carriers seem to exist as dimers or even higher order oligomers (Heuberger et al., 2002; for recent review cf. Veenhoff et al., 2002). Mammalian glucose transporters, although classified as the most simple transport systems using facilitated diffusion (see below), exist as tetramers, in which the individual subunits communicate with each other (Cloherty et al., 2001). Similarly, also the bacterial lacS lactose transporter exists as a dimer in which the subunits interact cooperatively (Veenhoff et al., 2001). Such communication between monomers is thought to be important for controlling transport activity. Interestingly, a monomer of the lacS lactose transporter functions as a facilitator, whereas the dimer mediates proton-coupled transport (Veenhoff et al., 2001). So far, little is known about such interactions among *Arabidopsis* membrane proteins. A main reason is certainly that the biochemistry of transporters expressed in vascular cells is difficult to study and that classical methods for determining protein:protein interactions such as yeast two hybrid systems were not suitable for detecting interactions between membrane proteins. The recent development and application of the *split ubiquitin system* to plant membrane proteins provides a new tool to detect and study such interactions (Johnson & Varshavsky 1994; Reinders et al., 2002a,b, Schulze et al., 2002). The plant proton sucrose cotransporters represent distant homologues of lacS and GLUT1, and are also able to form oligomers. Three SUT paralogs are present in the same cell and can interact with each other, potentially forming heterooligomers (Reinders et al., 2002b). Also separately expressed halves of one sucrose transporter can interact with their other half or the half of a paralogous sucrose transporter leading to the reconstitution of functional transporters with new properties. The capacity to interact with domains of another molecule may suggest that chimeric transporter pores can be formed within a dimer, providing a potential explanation for the existence of a preferred cooperative interaction between two of four subunits in a GLUT1 tetramer (Cloherty et al., 2001).

Studies from other organisms have shown that transporters often form heterooligomers with structurally unrelated proteins. Classical examples are the bacterial ABC transporters, which consist of four subunits (Chang and Roth, 2001; Martinoia et al., 2002; Locher et al., 2002), whereas in eukaryotes the subunits are preferentially fused into a single polypeptide. Another example includes the mammalian Na^+/K^+-ATPase (Kaplan, 2002).

A subset of mammalian amino acid transporters function as dimers of the catalytic transporter subunit and a heavy chain, a polypeptide that contains a single membrane span and a large extracellular domain, which can either be rBAT or 4F2hc (Chillaron et al., 2001). The subunit seems to be required for targeting the transporter itself to the plasma membrane. Furthermore many processes control the activity of a given transporter, processes which require interactions with other proteins, e.g. interaction with the cytoskeleton or with other scaffolding proteins as in case of the human glucose transporter GLUT1 with the raft associated monotopic membrane protein stomatin (Zhang et al., 2001) or with the posttranslational modification machinery such as phosphorylation or ubiquitylation. Stomatins also interact with other transporters and channels (Sedensky et al., 2001).

Recent findings indicate that signaling and transport proteins are assembled into large macromolecular complexes (Huber et al., 2001; Bickel et al., 2002). In case of the light perception in rhabdomers, a scaffolding protein associates the whole signaling complex including rhodopsin, Na^+- and Ca^{2+}-channels, heterotrimeric G-proteins, phosphokinases and phospholipases into a large cluster (Huber, 2001). Since transport activities at the plasma membrane are not only optimized with respect to affinity and capacity for single substrates (see below sensing) but are also involved in the coordination of the activity between different transporters as in the case of the different PII systems in *E. coli* (Gorke and Rak, 1999) or crosstalk between lactose permease and PII system (Sondej et al., 2002), it is likely that similar tasks must also be operational in higher organisms (see below).

It has been suspected for a long time that plasma membranes contain specific domains and are organized in mosaics (Ikonen et al., 2001, Bagnat et al., 2001; Simons and Toomre, 2000; Barenholz, 2002). Extraction of membrane preparations with the detergent Triton X-100 demonstrated enrichment of certain lipids and membrane proteins in the insoluble fraction. Using modified green fluorescent protein (GFP) halves that are unable to dimerize and by attaching lipid anchor attachment sites, the sorting of proteins into rafts could be followed directly (Zacharias et al., 2002). The availability of new membrane protein purification systems in combination with TAPing and mass spectrometrical analyses will enable analyses of complexes and rafts in much more detail and promises to give novel insights into membrane function and regulation of transport (Ho et al., 2002).

The role of metabolite sensing in regulation of transport

At first sight, it seems sufficient for a plant to have a single carrier for each substrate. Furthermore, if one would assume that metabolic reactions are the major determinants of metabolism, transport might just be constantly available, and thus does not require significant regulation. However, today it is becoming clear that an organism uses the full complement of possibilities to acclimate metabolism adequately. Most obvious is the necessity to regulate uptake of nutrient metals, many of which can be cytotoxic at high concentrations (Connolly et al., 2002). Furthermore external availability of nutrients can vary significantly. Thus a plant cell will have to acclimate membrane properties by exchanging or regulating transporter activities. Sugar metabolism and transport seem to be also highly regulated in plants (Hellmann et al., 2000).

Instructive examples of how organisms deal with such requirements come from bacteria. Interaction of ferric citrate with its transporter at the outer membrane induces a number of conformational changes that finally induces transcription of its own operon (Braun, 1997; Ferguson et al., 2002). Bacterial PTS transport systems are well known to provide important information about nutrient availability and uptake to regulate metabolism (Saier and Reizer 1994; Lengeler and Jahreis, 1996). The phosphorylation status is used to monitor the state of the various PTS sugar availabilities in order to hierarchically tune expression of the different PTS operons in a physiologically meaningful way. Thus, the PTS represent a highly integrated signal transduction network in carbon catabolite control (Gorke and Rak, 1999). Furthermore, the unphosphorylated form of the PTS glucose transport system IIAGlc also plays a role in the inhibition of transport of non-PTS sugars such as lactose, maltose, melibiose, and raffinose (termed inducer exclusion). In the case of lactose, IIAGlc binds to lac permease to inhibit the lactose transport (Sondej et al., 2002). UDP glucose uptake into *E. coli* and other bacteria is controlled by a pair of highly related proteins, a transporter UhpT and a sugar sensor UhpC (Schwoppe et al., 2002). Similar systems have been described in yeast, where SSY1, an amino acid transporter-like protein is involved in amino acid sensing at the cell surface and in regulation of amino acid transporters (Van Belle & André, 2001; Forsberg and Ljundahl 2001). In case of glucose transport, even two transporter-like sensors exist which serve as high and low affinity monosaccharide sensors and which control the many monosaccharide transporters of yeast in a complex network (Özcan & Johnston, 1999; Forsberg and Ljundahl 2001; Rolland et al., 2001; Van Belle & André, 2001). Similar to yeast sugar sensing, the mammalian glucose transporter GLUT2 is suspected to serve as a pancreatic glucose sensor (Thorens, 2001). The cell surface is thus obviously an ideal site to sense nutrients or metabolites in order to control nutrient uptake. Since the sensors from yeast are characterized by features such as extended cytosolic domains for interaction with the signaling machinery, are weakly expressed and have a low codon bias, one of the sucrose transporters from *Arabidopsis* and some members of the *Arabidopsis* monosaccharide

transporter family have been proposed to serve as plant sugar sensors (Lalonde et al., 1999; Barker et al., 2000). Candidate amino acid sensors are the putative glutamate receptors, homologs of ionotropic glutamate receptors in animals (Chiu et al., 1999). Direct proof for the existence of such sensors in plants is however still lacking.

Inactivation and degradation of transporters

In the context of acclimation of transport, besides biogenesis of new transporters also regulated retrieval and degradation of existing transporters is required to prevent that the membrane accumulates too many unnecessary transporters and to exclude unwanted compounds from the cell. Cycling of transporters between endocytic compartments and the plasma membrane is a well established phenomenon in yeast and mammals (Pelham, 2001). Examples of proteins which may be inactivated by endocytosis and degradation are the yeast uracil permease and the yeast general amino acid permease (Dupré et al., 2000; De Craene et al., 2001). Phosphorylation and ubiquitination seem to play an important role in inactivation, endocytosis and cycling of these transporters.

Also in mammals, transporters are known to cycle. Glucose transporters such as GLUT4 can be transiently stored in vesicles close to the plasma membrane (Bryant et al., 2002). Upon receiving a signal from insulin, vesicles are released to the plasma membrane to allow rapid uptake of glucose into the cells (Al-Hasani et al., 2002). On the other hand, rafts seem to be involved in subsequent internalization of GLUT4 (Ros-Baro et al., 2001). Interestingly, GLUT4 interacts physically with ubiquitin-related proteins involved in degradation and stabilization (Lalioti et al., 2002). Finally, transporter activity can be regulated directly by physical factors as e.g. in case of transporters involved in osmoregulation (Poolman et al., 2002).

In plants much less is known about regulation of transporters at the posttranslational level and on cycling. As an example, sucrose transport activity is affected by sucrose and sucrose transporters may be regulated by phosphorylation (Chiou and Bush, 1998; Roblin et al., 1998). Furthermore the sucrose transporter SUT1 is turned over very rapidly, suggesting that active mechanisms are also involved which provide a means to control sucrose transporter activity also by degradation (Kühn et al., 1997).

How transporters are targeted to their destined cellular location

As cellular compartments have different functions, so do the membrane proteins differ within membranes delimiting these organelles. Each protein is targeted to the respective membrane by a specific address label (ZIP code) to allow delivery to the correct compartment, while some proteins may be targeted to more than one organell (Peeters and Small, 2001). In some specialized cases, there maybe even protein transport to adjacent cells, thus the cell-cell connecting plasmodesmata have to recognize specific ZIP codes.

Plasma membrane proteins contain specific information that directs their targeting via ER and Golgi to their destination in the plasma membrane (Goder and Spiess, 2001; Pelham, 2001). Briefly, a hydrophobic signal sequence in a polytopic membrane protein may serve as a signal for the signal recognition particle (SRP). After initiation of translation upon ribosome binding to the transcript, the SRP/ribosome/nascent chain complex (SRP-RNC) docks with the SRP receptor on the cytoplasmic face of the ER. Thereafter, each transmembrane domain (TMD) is translated on the ribosome and may be inserted cotranslationally into the ER membrane. Concurrently and/or subsequently, one or more transmembrane domains at a time presumably move laterally to the periphery of the translocon and into the bilayer. From there the membrane protein traffics to the Golgi. Loading of the vesicles may involve cargo specifiers (Gilstring et al., 1999; Antonny and Schekman, 2001). Within the Golgi, proteins are either sorted into vesicles destined to the plasma membrane or to the vacuole (Pelham, 2001). Thus membrane proteins use a pathway similar to those of secreted soluble proteins, but they always remain integrated into the membranes to reach their target membrane. However, in contrast to soluble protein ZIPs, which are normally cleaved within the endoplasmic reticulum (ER), the signal sequences of polytopic membrane proteins are usually not cleaved. It is often assumed that the N-terminus and/or first transmembrane spans serve as ZIP codes (for review cf. Chin et al., 2002).

Since ER import of transmembrane proteins is related to membrane sorting in bacteria, one may use the wealth of information generated to extrapolate from knowledge available for *E. coli* transporters. Interestingly, the length of the central loop of lac permease seems crucial for correct integration of the transmembrane helices into the membrane (Weinglass & Kaback 2000). The functional reconstitution of separately expressed sucrose transporter halves in yeast suggests that both halves of the sucrose transporters contain signal sequence information to enter the ER. Since, in ER-targeted soluble proteins, the cleavage product provides experimental access to identify and characterize signal sequences, ZIP codes of soluble proteins are far better understood as compared to membrane proteins.

Various algorithms and programs have been developed that allow prediction of the destination of a query protein (Schwacke et al., submitted). However the prediction for

membrane proteins so far is not very reliable (Bannai et al., 2002; Emanuelsson et al., 2000; Schwacke et al., submitted). Proteins destined for the tonoplast must also use specific signal sequences for sorting into vesicles fusing with the vacuole. However, a detailed comparison of the sequencewise highly related tonoplast and plasma membrane aquaporins has not yet allowed the identification of such signal sequences (Johanson et al., 2001). Membrane proteins that transport solutes across organellar membranes also use ZIP codes for posttranslational sorting. In the case of the prototype of plastidic transporters, the triosephosphate translocator (TPT), a cleaved signal sequence has been identified (Flügge et al., 1989). Interestingly, a member of the plasma membrane hexose transporter family is responsible for plastidic glucose transport and contains a signal similar to that of the triose-phosphate translocator for targeting to plastids (Weber et al., 2000). Similarly, as sulfur metabolism is predominantly found in chloroplasts, a member of the plasma membrane sulfate transporter family containing a signal sequence has been localized to plastids (Takahashi et al., 1999). Some inner mitochondrial membrane proteins also have a clear signal sequence, while others, although known to be localized to these membranes, do not contain obvious signal sequences. To date, reliable information about the subcellular localization of proteins can only be obtained experimentally. However, even the available methods may be erroneous due to mistargeting upon overexpression or fused reporter molecules (Okumoto et al., unpublished). So far, very little is known about these targeting sequences, although prediction programs can give an idea about subcellular localization (see for example Schwacke et al., submitted).

Transporters for a wide range of substances

Within living species, specific transporters for probably all classes of substances exist – many can serve as nutrients for *Arabidopsis*, suggesting that a wide spectrum of carriers on the root surface and rhizodermis exists. Many plants form a symbiosis with mycorrhiza, but not *Arabidopsis*. This plant must acquire all these nutrients on its own. Therefore it is an ideal model system to understand principles of nutrient uptake. In order to evaluate the function of a plant transporter, the substrate specificity is of prime importance. In addition, the intracellular localization (plasma or organellar membrane) and cell-type specificity within a tissue determine its function. Expression in the vasculature suggests a function in long distance transport and in partitioning between tissues of supply (sources) and tissues of demand (sinks). Although historically plant membrane transport has often been investigated in species other than *Arabidopsis*, the ease of identification of mutants, completion of the genome sequence, the many genetic

and molecular tools available, and a large scientific community promise that *Arabidopsis* will be the plant to uncover many fundamental plant transport processes in the future. For a general reading of plant membrane transport, students are recommended to read the respective chapters in textbooks as Taiz and Zeiger (1998) or Buchanan, Gruissem and Jones (2000).

General furnishing with proteins in every higher plant may be similar, and the specificity of a given plant may arise from specific regulation of a small set of genes. For example, in some legumes like pea and bean, besides amines or amino acids, ureides are a major transport form of fixed nitrogen in the phloem. Ureide transporters are also found in the genome of *Arabidopsis* (Desimone et al., 2002), although ureides have not been detected in the phloem sap so far. Some plants, however, clearly have distinct transporter types, as the LCT1 calcium transporter from wheat has no homologs in *Arabidopsis* (Schachtman et al., 1997). Transport proteins for prominent solutes are members of multigene families. The reason for the apparent redundancy is not clear, but fine tuning is probably involved. It can be expected (and has been shown for amino acid transporters (AAPs) and sugar transporters SUTs) that many transporters have overlapping substrate specificities. Due to the methods how the transporters were identified, the true substrate (or some of the substrates) in the plant may not have been identified. The competition of amino acids on binding to amino acid transporters has been shown for AAPs expressed in yeast (in growth assays) and directly expressed in oocytes (Fischer et al., 2002).

Imaging of metabolites in Arabidopsis

Compartmentation of metabolic reactions and thus transport within and between cells can only be understood if their subcellular distribution has been determined by non-destructive dynamic monitoring techniques. Currently, methods are not available for *in vivo* metabolite imaging at cellular or subcellular levels. Limited information derives from techniques requiring fixation or fractionation of tissue (Farré et al., 2001; Borisjuk, 2002). Such static analysis of metabolite composition in organs, tissues and cellular compartments involves cell disruption. Thus most techniques neither measure metabolite changes in real-time nor account for likely variation in local metabolite concentration at the cellular level. Furthermore, these methods have low resolution and are prone to artefacts, e.g. contamination by other cell types or subcellular compartments. Little is known about the dynamic changes in concentration of metabolites such as sugars and amino acids at the site of transport, i.e. at the loading site of the phloem in plants, but also concerning the distribution of different sugars within cellular subcompartments. To better understand

metabolism and compartmentation, non-invasive techniques would be of significant advantage, such as the FRET (fluorescence resonance energy transfer) system to image calcium fluctuations (for review cf. Tsien 1998). Using a similar approach, novel nanosensors for sugars were developed by transforming substrate-induced hinge-bend movements upon substrate binding into changes in fluorescence resonance energy transfer between two different fluorescent proteins (YFP and CFP; Fehr et al., 2002). The backbone of the nanosensors are bacterial periplasmic solute binding proteins. The nanosensors were shown to function *in vitro* and detect sugars in complex mixtures. Furthermore, uptake of sugars into yeast cells expressing the nanosensors in the cytosol was visualized in real time. Given that periplasmic binding proteins exist for a wide spectrum of substrates, such strategy may provide a basis for the development of a variety of transgenic plants for analysis of ion and metabolite concentrations, transport and sensing.

Different organs of *Arabidopsis* are connected by vasculature

Many nutrients and metabolites such as sugars, sugar alcohols, amino acids, organic acids and nucleotides are translocated between different organs within plants preferentially in the vasculature. In addition, hormones and some secondary metabolites are also transported between organs via these conduits. Plants including *Arabidopsis* use two major routes for long distance transport, i.e. the phloem and the xylem (see below), which can be compared to the highways for long distance transport. On top, "slower country roads" exist, that allow long distance transport by cell-to-cell movement (e.g. polar auxin transport in the xylem parenchyma). Many of these compounds may be imported into the vascular systems via specific transporters, while others may be co-transported in concert with other substances by the same transporter. Metabolite profiling of phloem and xylem exudates will lead to an expansion of our view of which solutes are transported in the vascular saps (Fiehn et al., 2000). Many of these transported substances may have both nutrient and signal function. The presence of a specific set of transporters thus controls which substances can move in the phloem or xylem saps. Thus transporters allow uptake of sucrose, but not glucose into the phloem stream. Nevertheless, several studies have indicated that the transport systems involved have a limited selectivity for their substrates. Amino acid transporters transport a wide spectrum of different amino acids and derivatives (Fischer et al., 2002). Sucrose transporters transport phenylglucosides and even biotin (Ludwig et al., 2000). Thus many xenobiotics may be able to traffic in the plant by means of such low-selective transport systems. The extended selectivity for the

substrate may even allow to design large substrate conjugated molecules that act as xenobiotics which are imported into the vascular system (Deletage-Grandon et al., 2001).

Para- and transcellular pathways for solute transport

Arabidopsis as an organism composed of a wide variety of cell types does not only require transport systems allowing uptake and release of compounds into and from individual cells, but in addition has to exchange solutes between cells. This becomes most apparent when considering the specific requirements of different organs such as roots and leaves. Leaves produce assimilates such as sucrose, whereas all the essential mineral nutrients are almost exclusively taken up by the root. Thus both systems are fully dependent on exchange of solutes via the vascular systems. But even within an organ, different cells types fulfil different functions and even biosynthetic pathways may be compartmentalized between different cell types within the same tissue (St-Pierre et al., 1999). Two principal routes exist for solute exchange between neighboring cells: (i) the paracellular pathway, in which solutes pass through the cell wall,
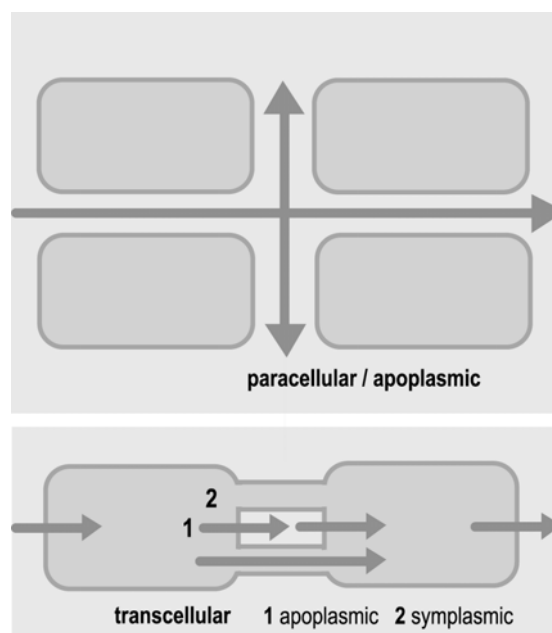
Figure 3. Schematic view of para- and transcellular solute flow. During paracellular solute flux in the aqueous cell wall space, the apoplasm, solutes neither cross membranes, nor enter the cells. Transcellular solute movement is characterized by initial solute influx into the cell. Further flux may include efflux into the apoplasm and subsequent reuptake by the neighbouring cell or symplasmic movement.

a space named apoplasm and (ii) the transcellular pathway where solutes move via the cytoplasm, defined as the symplasm (Fig. 3).

Intercellular translocation can occur by movement through the apoplasm, as long as the continuity of cell walls is not interrupted by barriers such as cutinized/suberinized layers, as e.g. in case of the Casparian strip, which separates the rhizodermis from the stele. Xylem vessels are formed by dead cells thus they belong to the apoplasm since they are continuous with the cell wall space and are not separated by a membrane from each other. Thus translocation in the apoplasm can have a direction and it can be much more rapid than diffusion if water moves due to root pressure, capillary pressure, transpiration or other factors, e.g. in the leaf apoplasm. Directionality is given here by the direction of water flux through the plant. In case of transcellular movement via the symplasm, translocation occurs via the plasmodesmata, which are highly specialized intercellular connections not found in animals. Almost all *Arabidopsis* cells are interconnected by plasmodesmata. The best-known exceptions are guard cells, in which plasmodesmatal connections are eliminated during development. Plasmodesmatal structure and function still is only poorly understood. So far, no direct evidence has been presented for movement of small solutes through plasmodesmata. However chemical compounds such as dyes, but also RNA, proteins and even virus can move between cells, we generally assume that small molecules can also freely be exchanged between neighboring cells either through the desmotubules or through the space between plasma membrane and ER strands within a plasmodesma. If the hypothesis of free movement of ions and other small solutes through plasmodesmata holds true, the availability of open plasmodesmatal macrochannels would have important implications: the membrane potential can not be regulated by individual cells, energization is delocalized. The H^+-ATPase activity in one cell may energize a process taking place in the adjacent cell. Solutes may move simply by diffusion from cell to cell, along gradients established by metabolic reactions in different cells connected by plasmodesmata. This provides a unique pathway through what is called the symplasm, since the individual cytoplasm of the different cells is interconnected. Obtaining direct proof for symplasmic transport of solutes is complicated due to the existence of cell walls and the complexity of plasmodesmata. Double patch clamp studies similar to those used for analysis of mammalian gap junction and novel imaging techniques are required to prove directly that small molecules can move freely. The relative role of sym- and apoplasm routes is obviously also important for questions relating to long distance translocation of solutes within a plant.

Long distance translocation in the vasculature

Nutrients may be taken up directly at the root surface, e.g. into the root tip cells or root hairs and then move symplastically or may move in the apoplasm up to the suberinized endodermis, which in *Arabidopsis* is located only two layers below the rhizodermis (Barton 2001). After entering the central cylinder, inorganic ions as well as organic molecules have to be exported into the apoplasm near the xylem vessels in order to be transported to the shoot. The vectorial transport from root to shoot is not only due to the driving forces acting on xylem translocation, but also to cell specific expression of carriers, e.g. in xylem parenchyma. The transport pathway for nutrient redistribution to plant parts distant from the soil is the xylem, the main long distance transport pathway for mineral nutrients and water. Flow in the xylem is essentially unidirectional, from root tips to the sites of water evaporation.

The driving forces, however, are not yet fully understood (Beevers and Tanner, 2001). Although consisting mainly of dead cells, the xylem contains living xylem parenchyma that is probably not only involved in generating driving forces for xylem translocation, but also plays an important role in regulating flux (Zwieniecki et al., 2001). The water transported in the xylem is either evaporated via stomata, secreted via hydathodes or recycled via the phloem. The hydathodes in leaves are connected to open ended xylem vessels, covered by a cap structure called epithem. Secretion occurs through specialized stomata. Hydathodes are related to nectaries and thus are supposed to be able to secrete specific substances while retaining others. In broad terms hydathodes can thus be compared to kidneys that must contain a specific set of carriers to fulfill these retrieval functions. Growing and expanding leaves, as well reproductive organs that are low in transpiration receive little supply by the xylem. Similarly, optimal supply of photoassimilates to storage and developing tissues is provided by long distance translocation in the phloem. However, since the solute movement in the phloem is driven by the sites of solute production (sources) and utilization (the sinks), the direction is under continuous changes driven by the different demands during development (Busse and Evert, 1999a,b). The actual conduits are the sieve elements, peculiar cells lacking a nucleus in the mature stage and tightly associated with companion cells (Van Bel and Knoblauch, 2001; Van Bel et al., 2002; Lalonde et al., 2001). An instructive example is the expression of a sucrose transporter during transition of a leaf from sink to source (Fig. 4).

The vascular bundle holds four to more than 20 independent sieve elements in parallel, thus movement
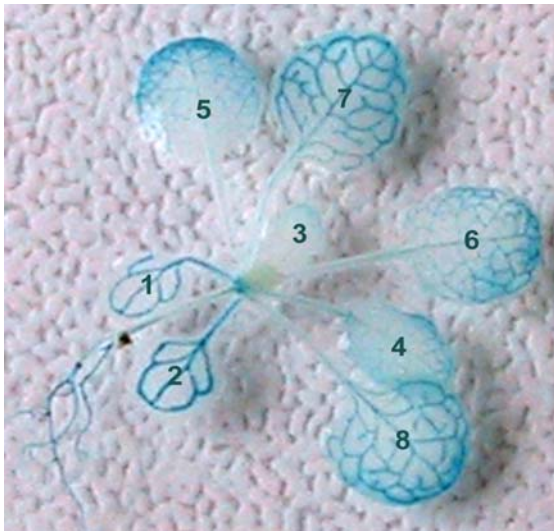
Figure 4.•β-Glucuronidase assay of the localization of promoter activity of the source specific AtSUC2, a companion cell specific sucrose transporter. The picture shows promoter activity in vascular bundles of leaves, stems and roots. Cotyledons (leaf No. 1,2) GUS staining follows the transition from sink to source and is strongest in old leaves and increases in young leaves from bottom to top. (Courtesy by W. Schulze)

within a vascular bundle can be uni- and bidirectional, but due to the general rule from high pressure to low pressure, flow cannot be bi-directional in the same sieve element. Though transport may be mediated by osmotic generated pressure flow (OGPF), the specificity has to be established by specific transporters, which again have to be expressed in a cell specific manner. The definition of sink and source status thus is defined by the major osmolytes that enter the phloem, mostly by active transport. Since in many plants the major osmolyte is sucrose, followed by amino acids and potassium, directionality is created by the loading and unloading of these substances. Therefore the terminology is used preferentially in the context of sugar movement, whereas in case of other solutes, e.g. amino acids, the terminology of sink and source is less useful, especially since movement occurs in phloem and xylem and directionality of amino acid translocation is controlled by different mechanisms.

Sugars, which are produced by photosynthesis must move from the sites of synthesis, i.e. the mesophyll to the phloem. Theoretically, movement to the phloem can be symplasmic, as 1-10 plasmodesmata/μm^{-2} connect mesophyll cells with the companion cell/sieve element complex (Haritatos et al., 2000). This plasmodesmatal frequency and the lack of distinct "intermediary cell" morphology in minor vein companion cells classifies *Arabidopsis* as a type 1-2a species (Gamalei, 1991). Morphologically, *Arabidopsis* represents thus an intermediate species between typical type 1 symplasmic

loaders and pure apoplasmic loaders (type 2). As expected for a type 1-2a species, the main transport sugar in *Arabidopsis* is sucrose, but also raffinose and traces of galactinol are transported (Haritatos et al., 2000). *Arabidopsis* may therefore be an excellent model system to analyze the putative contribution of symplasmic loading and raffinose transport in addition to sucrose transport in long distance translocation. In contrast to the morphological data, the phenotype of mutant plants with a deletion of a single companion cell sucrose transporter, AtSUC2, classifies *Arabidopsis* as an apoplasmic loader (Gottwald et al., 1999). Complete knock-out of the transporter gene resulted in stunted growth, retarded development and sterility. Source leaves contained great excess of starch, and sugars were only minimally transported to sink organs as roots and inflorescences (Gottwald et al., 1999). The AtSUC2 transporter is expressed in the phloem of source tissues (Truernit and Sauer, 1995; Fig. 4). It is expressed in major veins in leaves that act as sinks, and in all veins when a leaf is a clear exporter of sucrose. A gradual transition from a sucrose importing leaf located at the tip to a sucrose exporting leaf at the base can be followed by equivalent expression of SUC2 (Fig. 4). For sucrose, a concentration gradient is created by proton-coupled sucrose transport at the sieve element/companion cell complex (SECC, see below). The concentration of sucrose (and other osmolytes) in the phloem sap creates water potential leading to water influx from the surrounding tissue, including the xylem. This results in a positive pressure in the phloem. Translocation is driven by mass flow if sucrose is unloaded in the sink organs. Only little efflux is observed along the path by using phloem mobile dyes (Wright and Oparka, 1997; Imlau et al., 1999). In most cases, sieve element unloading is assumed to proceed via bulk flow through plasmodesmata. Because of the large pressure gradient involved, this first step is essentially irreversible. The phloem is unloaded in root tips, and unloading of carboxyfluorescein and green fluorescent protein from jellyfish suggests that the size of the plasmodesmatal pores connecting *Arabidopsis* plant cells have size exclusion limits larger than the typical 800Da, suggesting greater conductivity, but the size exclusion limit for plasmodesmata may vary upon environmental stimuli. Uptake into seeds requires apoplasmic transport steps since endosperm and embryo are symplasmically isolated. Both cellular exporters and import systems in the endosperm and the cotyledons of the embryo contribute to phloem unloading (Roche et al., 2002). The physiology of unloading processes has been described elsewhere in detail (Patrick and Offler, 2001; Lalonde et al., 2002).

Hormones

Plant hormones (auxin, cytokinin, ethylen, abscisic acid,

jasmonic acid, giberrellin, brassinosteroids, peptides, salicylic acid) play a key role in controlling the growth and development of plants. Plant hormones are organic compounds synthesized in one part of the plant and in most cases are translocated to another organ. They generally act at very low concentrations (often <10µM) by eliciting a physiological response at their target site. Thus inorganic ions, like K^+, PO_4^{3-} or Ca^{2+}, or metabolites, although transported within the plant and with clear physiologic responses at target tissues, are not considered as hormones. Accordingly, specific transport proteins as for metabolites and nutrients are responsible for the transport of hormones within the plant. These proteins often have similar structure to metabolite transporters and share the basic mechanisms of transport. As the auxin indole 3-acetic acid has structural similarity to the amino acid tryptophan, it is not too surprising that the auxin transporter AUX1 is also structurally related to amino acid transporters of the ATF-family (Bennett et al., 1995). Another example is the nucleobase transporter PUP1 that not only transports purines, but also structurally similar cytokinins (Bürkle et al., unpublished). Molecularly identified putative auxin efflux transporters include the PIN proteins (8 members; Luschnig et al., 1999) and ABC-type transporters that are related to animal multidrug resistance (MDR) genes (Noh et al., 2001). However, although an *in planta* role in auxin efflux carriers has been identified, direct transport of auxin by these proteins awaits to be shown.

Principle transport mechanisms

The compartmentation of water-soluble molecules by lipid membranes is the key event during evolution of life. Only hydrophobic molecules dissolve in the lipid membrane, and the passage and exchange of solutes and especially ions is diminished to very low levels.

The electrochemical potential is fundamental for understanding solute fluxes across membranes. At temperatures relevant for the plant, all ions, metabolites, proteins and lipids are in constant thermal agitation and movement. For such undirected "brownian" motion of many molecules, the overall tendency of the system can be summarized as a tendency to equilibrate. The equilibrium is the energy-minimized state. Differences between compartments separated by a semipermeable membrane can be calculated quantitatively. The electrochemical energy μ_1 of a mole of particles is proportional to the natural logarithm of the concentration c_1 of particles: $\mu_1 \sim RT \ln[c_1]$, with the Boltzmann constant R and the absolute Temperature T. When a ratio of concentrations is considered (c_1/c_2), for a concentration gradient across a membrane, the difference in electrochemical potential is considered by the following term, the Boltzmann equation: $\mu_1 - \mu_2 = \Delta\mu = RT \ln(c_1/c_2)$. The

electrochemical potential (or energy) can be viewed as the energy needed to concentrate metabolites as they cross the membrane. If the substance is electrically charged (an ion), the electrical field across the membrane has to be considered additionally: $\mu_{el} \sim zF\Delta E$, and the corresponding energy of that ion is proportional to the ion valence z (e.g. z=+2 for Ca^{2+} and z=-1 for Cl^-) and the membrane potential ΔE (which is measured in volt). F is Faraday`s constant: the amount of electrical charge in a mole of electrons. This term only contributes for charged substances, and equals zero for all neutral substances. Thus, the electrochemical potential difference is a sum of a chemical and an electrical term: $\Delta\mu = RT \ln(c_1/c_2) + zF\Delta E$. With a large membrane potential applied to the membrane, energetic passive downhill flow for an ion may as a consequence be from low concentration to high concentration. An important term used to characterize transport is the reversal potential. It is the potential at which inward fluxes of a charged substance equal outward fluxes. Membrane potential and induced current can be measured electrophysiologically (Ward, 1997).

The plasma membrane H^+-ATPase is the principle primary active transport system in the plant plasma membrane. Plants and fungi use P-type ATPases to pump protons out of the cytosol and thus form a large electrochemical potential. Consequently, plant secondary transporters are typically coupled to protons rather than to sodium, as most animal symporters. The transmembrane electrochemical gradient for protons is called the proton motive force (pmf). The pmf is a measure for the free energy stored in the transmembrane electrochemical gradient for H^+. It is the sum of two components: the chemical potential of the proton gradient and the electrical potential across the membrane. It is measured in volts. At a typical plant plasma membrane the •pH will be around two pH units (cytosol pH•7.5 and apoplasm pH•5.5) and the membrane potential V_m is in the order of -150 mV. A 10-fold gradient in the permeant ion (H^+) is energetically equivalent to 59mV of membrane potential, thus the pmf is around -268mV. The proton gradient across plant membranes is the principle actively maintained gradient across the membranes. Proton gradients are also found across intracellular membranes as the thylakoid membrane or vacuolar membrane. The electrochemical energy stored in the proton gradient is energetically equivalent to chemical energy in the phosphate bond of ATP, and both energy forms are interconvertable ("chemiosmotic theory").

Active and passive transporters or carriers

Transporters are often classified as active or passive (Fig.5). Passive transport catalyses the downhill movement of a substance from higher to lower electrochemical potential. As discussed above, this does
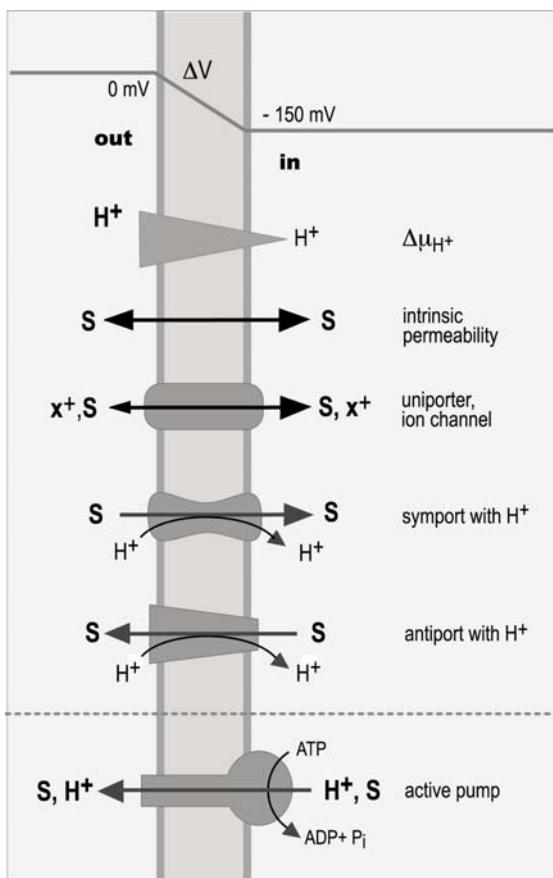
Figure 5. Schematic representation of transport mechanisms and direction of transport. Left side outside, right side inside.

not necessarily mean that a substance is moving from higher to lower concentration, as the sum of both the electrical and the chemical term add up to the electrochemical potential. Active transport is used to describe the uphill movement of a substance against its electrochemical potential.

Primary active transporters couple light energy or the energy released upon hydrolysis of a chemical bond to the conformational change of a membrane protein, and by that couple transport of a substance against its electrochemical potential. The P-type H^+- and metal pumps, the organellar proton pumps and many ABC proteins belong to that class.

Secondary active transporters use the free energy difference stored in the electrochemical gradient of protons or other solutes across the membrane to drive simultaneous movement across the membrane of another substance. In effect this energetically adds up to a net downhill flow. Depending on the direction of transport and whether a coupling ion is used, **symport** and **antiport** are discriminated. Such transporters are symporters (often also simply considered as co-transporters) when two

dissimilar solutes (substrates) are transported together or sequentially within a transport cycle into the same direction across the membrane. Transport of the two solutes is obligatorily coupled. In vivo, secondary active plant co-transporters are generally H^+-coupled systems, so that the large electrochemical potential for protons, generated by primary active transporters, is coupled to the uphill movement of another substance. The transport is considered as antiport, when the direction of both substances is opposite. The conformational changes associated with the transport do not require hydrolysis of chemical bonds, but solely rely on the electrochemical gradients for both substrates.

Examples of **passive transporters** are channels, like water or potassium channels. Other passive transporters are uniporters that transport only one substance passively downhill an electrochemical gradient. Although the distinction may be arbitrary, membrane passage through uniporters or carriers is often assumed to involve conformational changes within the transport cycle, or even diffusion of the carrier with bound or without substrate through the lipid membrane, while channel mediated transport does not involve conformational rearrangements, although channels may open and close.

Polarity of transport processes

It is obvious that transport is a vectorial process. In a multicellular organism, solutes have to be transported from one cell type or organ to the other. Directionality can only be achieved if mechanisms are at hand to drive transport in a certain direction. Besides polarity within a transport system (e.g. by coupling to a defined gradient, i.e. proton gradient), it is essential that transporters are expressed only in a certain cell type. For example, the coexistence of cotransporters for the same substrate in adjacent cells will lead to competition between the cells rather than directional transport. In contrast, if only one of the two cells expresses the transporter gene, whereas the other contains a uniporter or an exporter (e.g. a proton antiporter), flux will be directed from one cell to the other. Therefore many transporters have highly cell-specific expression patterns. This also means that in order to increase flux, it is not possible to overexpress the transporters under control of general cell-non-specific promoters like the CaMV 35S promoter as is normally done for increasing enzyme activities, since this should collapse directionality and thus may be counter-productive. For transcellular transport, as e.g. in microvillar cells in the mammalian intestine, uptake and release carriers may be transported to specific domains in the polarized cell. Provided that at least one of the carriers is primary or secondary active, this polarization allows unidirectional flux across the cell. A typical example for plants is the polarized distribution of auxin influx and

efflux carriers permitting polarized transcellular hormone fluxes (Friml and Palme, 2002).

Molecular identification of transporters

Our knowledge about the true substrate of a given plant transporter is often limited. For example, transporters for amino acids often transport many different amino acids, and according to the availability, the true substrate in the plant may differ from the substrates that the transporter was initially isolated for. Sucrose transporters are able to transport additionally other glucosides and even structurally unrelated biotin. Although sucrose transport may quantitatively be the pivotal function, transporters may be involved also in transport of many other substances. Therefore, the widespread different strategies used to identify transporters will be shown and advantages and limitations will be briefly discussed.

The most widely used and probably most successful approach to identify plant transporters for a given substrate is suppression cloning in heterologous hosts, especially yeast (for reviews cf. Frommer and Ninnemann 1995; Barbier-Brygoo et al., 2001). The basis of this approach is a yeast lacking a specific transport capacity, either because yeast does not have such a system or because the endogenous system has been inactivated, e.g. by supply of a suppressing agent (ammonium for general amino acid permease GAP1), or by mutating (in most cases deleting by homologous recombination) the transporter gene(s) or by manipulating the strain in a way that it becomes dependent on an uptake system. This strain is transformed with a plant cDNA library, which can
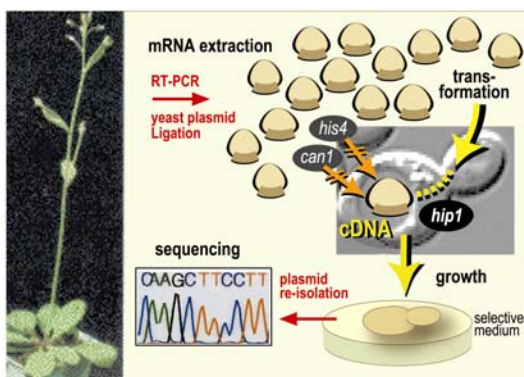
Figure 6. Representation of a functional complementation approach to identify plant transporters for specific substrates. mRNA has to be extracted from the plant, and cDNA is synthesized by RT-PCR. Size fractionated cDNAs are ligated into a yeast expression vector. Plasmids are transformed into yeast, e.g. a histidine auxotroph (JT16) mutant. Growth on selective media allows only yeast to grow that express a transporter or enzyme that circumvents the auxotrophy. After re-isolation of the plasmid the encoding cDNA is sequenced.

be expressed under control of a yeast promoter, and in the simplest case, yeast mutant growth is monitored under selective conditions. Selective conditions may be e.g. media without the substance that the yeast is auxotroph for. Only if the transfected cDNA provides the message to express a transport protein that ameliorates the auxotrophy, yeast can grow, and the cDNA carrying plasmid is isolated and sequenced (Fig.6). This approach has the enormous advantage that no other information is necessary about the transporter, and the isolated cDNA automatically encodes a functional transporter by itself. This method has been used to isolate plasma membrane and organellar transporters. Functional complementation of simpler systems as bacteria has not been as successful, possibly due to toxic effects of heterologous membrane protein expression on *E.coli* growth (Frommer and Ninnemann, 1995). However, mammalian COS cells have also been used to identify heterologously expressed plant aquaporins (Kammerloher et al., 1994). The yeast complementation system identifies thus transporters for a given substrate, but if this substrate is indeed the true substrate within the plant is not known. In addition, only transporters that are functional as single polypeptides will be isolated by that approach, but some plant transporters may be encoded by multisubunit complexes (see above). Transporters may even be false targeted to other membranes in heterologous systems, thus a plasma membrane transporter in yeast may not necessarily encode a plasma membrane transporter in plants.

Many of the advantages and disadvantages also apply to a related approach: expression cloning in frog oocytes. Here radiotracer uptake or ionic conductances induced by expression of plant mRNAs are monitored in single oocytes, and starting from simultaneous injection of a whole library into single oocytes, the activity induced by a single cDNA clone is isolated. This approach has been extremely successful for identification of mammalian ion channels and transporters, but was less widely used for plant transporters, although many plant transporters can in principle be expressed in oocytes (Boorer et al., 1996ab; Zhou et al., 1997; 1998; Fischer et al., 2002; Desimone et al., 2002). This approach has so far only yielded the cloning of a syntaxin, which may indirectly affect endogenous ion channels in the oocytes (Leyman et al., 1999).

Another highly successful method to isolate transporters is the screen of plant mutants involved in specific transport tasks within the plant. Starting from EMS mutagenized or T-DNA tagged lines, morphological mutants were often easily identifiable as being involved in plant hormone transport. A disadvantage of this approach is that, as transporters are often members of multigene families, other transporters may compensate for the loss of a specific transporter. This approach may be especially promising with plant hormone transporters, since altered transport of hormones may lead to easily observable phenotypes.

Among the advantages of that method is that it directly defines the functional role within the plant, as the loss is already observable in the tissue where the transporter is normally expressed. However, in a complicated system such as the multicellular plant, a regulatory effect on a transporter can often not be excluded. In the absence of transport function in a heterologous system or when biochemically isolated, these proteins have to be considered as putative transporters.

A strategy that currently is widely used with the sequencing and analysis of other related organisms, is candidate cloning by homology. This approach is simple, but needs a "lucky guess" on the substrate and subcellular localization. Other methods, like differential screening of a cDNA library have been used (e.g. for identification of a algal sugar transporter HUP1; Sauer et al., 1989), or labelling by specific substrate or inhibitor binding, purification, reconstitution, sequencing of protein (Flügge et al., 1989). If a highly abundant transporter recognizes highly specifically its substrate, such a strategy may be advantageous, but may fail due to many experimental difficulties. Thus nearly in all approaches the methods may not identify the true substrates, even if one substrate is identified.

For a given nutrient, often multiple transport systems coexist within a single cell to assure uptake over a broad range of substrate concentrations. These systems are frequently referred to as high and low affinity systems. The quantitative parameter is the K_m that determines the affinity of the substrate concentration at which the transport flux (J_{max}) is half-maximal (in analogy to enzyme kinetics). Similar to enzymes, many transporters have affinities in the millimolar and micromolar range. The K_m often reflects the order of magnitude of substrate the transporter is facing. This has the advantage that the transporter is not saturated, and the substrate concentration can easily be kept constant. If it would be saturated all the time a little bit more would lead to accumulation, if the affinity of the transporter would be much lower than substrate concentration, accumulation would be much less effective, especially in the competition with another binding site. Binding sites of different organisms might compete about substrates.

Using mainly suppression cloning in yeast, of the ~6475 membrane proteins from *Arabidopsis*, about 50% have been assigned a putative function. The rest is unknown and classified as hypothetical protein (Schwacke et al., submitted).

Proton pumps

Directionality in transport is not only due to differential expression of transporter proteins, but may also be due to directionality of the transport process itself. Membranes are not only used to enclose compartments, but also to generate potentials that determine which compounds move in which direction. *Arabidopsis* uses mainly proton gradients to generate membrane potential (Sze et al., 1999). This potential sums up from an electrical and a chemical component. Cell walls are therefore often acidic, and the membrane potential is in the range between -120 and -250mV. These two components can be used to drive transport of other compounds in a certain direction, e.g. into the cell in a concentrative manner. The key components of the cellular machinery responsible for creating these gradients are pumps that extrude protons into the cell wall space or into the vacuole.

Active transmembrane proton transport is mediated by proteins that couple the energy released by cleaving a high energetic bond, e.g in ATP, to conformational changes in a protein (Sze et al., 1999). This conformational change then mediates uphill transport of the substrate. With the exception of the pyrophosphatase, all plant pumps share homologs or orthologs in all domains of life, including humans. This is also true for several passive transporters and underscores how the fundamental transport processes are similar in all organisms.

P-type pumps

These pumps derive their name from the presence of a covalent pump-phosphate transition state during the reaction cycle that distinguishes P-type from F-type ATPases. During the reaction cycle, the •-phosphate of ATP becomes transiently, but covalently, bound to a cytoplasmic aspartyl residue. While H^+-binding sites within the protein are exposed to alternate sides of the membrane, the hydrolysis of this acyl-phosphate bond supplies the driving force for the conformational change that translocates one cytoplasmically bound proton against its driving force. Later protein rearrangements lower the external proton binding site affinity and permit H^+ dissociation. P-type ATPases are specifically blocked by the structural analog orthovanadate.

P-type pumps have typically 10 TMs, a molecular mass •100 kDa and are encoded by 45 genes in *Arabidopsis*. They may be grouped into 5 subfamilies. Several members of a subgroup of 12 proteins (P3A) have been functionally shown to encode plasma membrane H^+-pumps. An overview can be found in Axelsen and Palmgren (2001). Other subfamilies are heavy metal pumps (P(1B), 7 members), calcium pumps (P(2A) and P(2B), 14 members). The last subgroup (P(4), 12 members) has been implicated in aminophosholipid flipping, but this may be an indirect effect of pump activity. P-type pumps do not necessarily localize to the plasma membrane. All P-type pumps actively transport the substrate uphill, from the cytoplasm into the apoplasm or organelles.

Vacuolar, mitochondrial and chloroplastic H⁺-pumps

The vacuolar-type (V-type) proton pump is localized both on the tonoplast and in endomembranes. It actively pumps protons from the cytoplasm into the endomembrane lumen, like the endoplasmatic reticulum, into Golgi vesicles and into the vacuole (Sze et al., 2002). The pump is a multisubunit complex that is composed of two functionally distinct major components: the membrane integral V_o that mediates the proton translocation, and the cytoplasmically oriented hydrophilic headgroup V_1 that mediates ATP-hydrolysis. Both components are connected and stabilized by additional subunits, but are easily dis- and reassembled *in vitro*. The functional significance of the de- and reassembly is not yet fully clear. The membrane intrinsic part is composed of multiple copies of the extremely hydrophobic subunit c (therefore called proteolipid). These subunits form a transmembrane pore for H⁺-translocation. The center of the pore is filled by the γ subunit, which is also bound to the center of V_1. The headgroup is mainly composed of a three-fold alternating arrangement of α- and β- subunits. Reversible and ordered binding, ATP-hydroplysis and ADP release at the three binding sites for nucleotides drives rotation of the central γ-subunit and transport of H⁺ in the pore. Normally two H⁺ are translocated per hydrolysed ATP, but the ratio may be influenced by the luminal pH. Several subunits are represented only once in the *Arabidopsis* genome, while others are identified with multiple, exchangable copies. The proton "pump" found at the chloroplastic thylakoid membrane, the CF-type ATP synthase, is a distant relative of the V-type ATPase. Although structurally related, the *in vivo* function is essentially reverse (inverse) to that of the vacuolar pump: the CF-type ATP synthase generates ATP from ADP and P_i, by using the energy stored in the proton gradient across the thylakoid membrane. Again, the pump has similar multisubunit structure and is composed of a hydrophilic ATP-hydrolyzing head group (F1) and the hydrophobic proton channel (F0). The photosynthetic proton accumulation in the thylakoid lumen, induced by the light-stimulated electron transport chain, develops a large proton and electrical gradient across the thylakoid membrane. The protons passively flow downhill from the stroma according to their electrochemical potential through the proton channels conferred by the CF-type ATPase. During H⁺-flow, they induce a rotary movement of the central subunit of the proton pump and drive the conformational changes responsible for ATP-synthesis.

A similar proton motive force is established across the inner mitochondrial membrane. However, it is not light induced, but redox-potential driven. In that membrane, a distinct but structurally similar F-type pump acts as an ATP-synthase. Thus, physiologically both proton "pumps" act in a reverse mode. They synthesize one ATP per translocation of 3-4 protons. The reaction cycle of the chloroplastic and mitochondrial pump is, in contrast to the vacuolar pump, reversible, thus ATP hydrolysis can establish a proton gradient across the respective membranes. Though specific parts of the pumps are homologous, the genes encoding plastidic proton pumps are distinct from the endomembrane pump.

Pyrophosphatase

Another H⁺-pump (H⁺-PPase) that couples hydrolysis of inorganic pyrophosphate to proton translocation is found specifically in vacuoles, but probably not in endomembranes. Homologs are encountered only in planta, microorganisms, archaebacteria and bacteria. In contrast to the V-type ATPase, the H⁺-PPase is composed of a single polypeptide of • 85 kDa with 14-16 TMs, and the active form is thought to be a homodimer.

Pyrophosphate is an unwanted by-product of several biochemical pathways involved in cellulose, nucleic acid and protein metabolism that are especially active in immature tissues. In these tissues the H⁺-PPase may be more active than the V-type ATPase. Thus the H⁺-PPase may function to hydrolyze excess PP_i and store the released energy in a large vacuolar proton gradient. The H⁺-ATPase is represented by 3 members in the *Arabidopsis* genome.

ABC transporters

In plants many secondary, often plant specific metabolites, like flavonoids, anthocyanins or breakdown products of chlorophyll are sequestered into vacuoles, often for defense purposes. In addition, vacuolar sequestration serves as a mechanism to detoxify the cytoplasm from xenobiotics-synthetic compounds such as herbicides. Vesicles made from vacuoles can import these compounds in an ATP-dependent manner, but this import is not sensitive to protonophores that dissipate the vacuolar proton gradient. Thus this transport, although active and energy requiring, is independent of the vacuolar H⁺-pumps and the proton motive force across the tonoplast. Transport is not sensitive to V-type pump inhibitors like bafilomycin, but is rather sensitive to valinomycin.

The molecules that mediate the transport of such diverse substances by coupling the release of chemical energy stored in ATP to substrate translocation are called ATP-binding cassette (ABC) proteins. Although many homologs of this class of proteins are found in bacteria, archeabacteria, yeast and mammals, the microscopic details of substrate recognition, transport mechanism and energy coupling are still almost obscure. Only a few plant transporters and their substrate specificities have been

analysed in detail (Martnoia et al., 2002). The ABC-transporter class is known for an extraordinary broad substrate spectrum: in mammals and yeast some ABC-transporters confer "multidrug resistance" by exporting stucturally diverse drugs and peptides, others transport lipids or heavy metals in a phytochelatin-dependent manner. The human homolog CFTR (cystic fibrosis transmembrane regulator) is a chloride channel, while another ABC-protein, the sulfonylurea receptor, acts as a regulator of ion fluxes, by specifically regulating a potassium channel. In bacteria, some ABC-transporters import amino acids or mineral nutrients.

At least four plant ABC-transporters (AtMRP1-3,5) function in vacuolar sequestration of flavonoids and xenobiotics after their conjugation with the thiol-tripeptide glutathione: they are localized to the tonoplast and act as glutathione S-conjugate pumps (Martnoia et al., 2002). AtMRP2 additionally transports linear tetrapyrols, breakdown products of chlorophyll. AtMRP1 and AtPGP1, which have been implicated in auxin transport and are related to animal multidrug resistance (MDR) genes localize to the plasma membrane (Noh et al., 2001).

The prototype of these proteins contains two nucleotide-binding folds (NBFs) and two integral membrane domains with multiple TMs, separated by each other. ABC-proteins in *Arabidopsis* appear to be constructed in a modular manner, as is the case for other organisms: the genome disposes several half-molecules, containing a single ATP-binding domain fused to a membrane intrinsic domain with multiple TMs. In addition, similar as in bacteria, soluble proteins with NBFs, but without TMs are encountered. Each NBF is composed of • 200amino acid residues containing a Walker A motif, a signature sequence and a Walker B motif. More than 100 genes encode ABC-proteins in *Arabidopsis*, and about 80% contain contiguous transmembrane spans. A complete summary of *Arabidopsis* genes belonging to this class of transporters can be found in Sanchez-Fernandez et al. (2001).

Notable features of the *Arabidopsis* ABC superfamily are the presence of a large yeast-like PDR (pleiotropic drug resistance) subfamily, and the absence of genes encoding bona fide cystic fibrosis transmembrane conductance regulator, sulfonylurea receptor, and heavy metal tolerance factor 1 (HMT1) homologs.
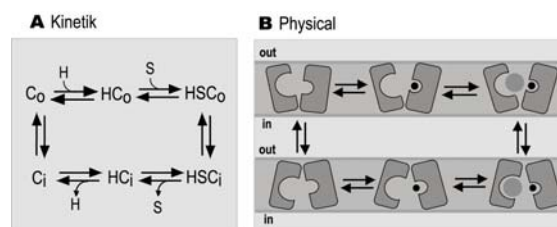
Passive transporters

Transporters that passively equilibrate the respective concentrations across membranes are also sometimes called carrier or permeases, without clear distinction in the transport mechanism.

A common feature of transporters is that the transport is saturable at high substrate concentrations. This is easily understood when transport of a substrate is followed step by step (Fig.7). In the simplest scheme the substrates are bound to externally exposed binding sites, the transporter rearranges by a conformational change and releases the substrates in an ordered or unordered fashion on the internal side. Binding of a substrate, translocation to the other membrane side and release reminds of enzyme reaction kinetics, where a substrate is bound, modified and the new product is released. As long as intracellular concentrations are low, transport and intracellular release are basically irreversible like an enzymatic reaction. The rate constant for binding and dissociation on the external side, and the transport rate step are under these conditions the moist relevant rate constants. The similarity in the underlying reaction is manifested in the fact that transporters, like enzymes, show Michaelis-Menten kinetics. The Michaelis constant K_m in enzyme kinetics is defined as the substrate concentration, where the catalytic reaction is half-maximal. At high, saturating substrate concentration the maximal reaction capacity v_{max} is reached. Similarly, net uptake rate by a transporter is maximal at J_{max} and half-maximal at Michaelis constant K_m. The uptake rate is resultant of influx and efflux. The half-maximal transport capacity of a transporter is one basic functional characteristic that can be easily determined (Fig. 8).

The kinetics can be easiest studied when the respective transporter is biochemically isolated or functionally expressed in heterologous systems. In the plant, where generally many transporters contribute to net uptake or release, metabolism has to be considered additionally. Thus the measured kinetics in living organisms have to be taken with caution: enzymatic consumption or subsequent uptake into an internal compartment such as the large vacuole in plant cells may misrepresent the actual plasma membrane uptake rates. For actual in vivo uptake kinetics see for example Marschner (1993).

The transport mechanism of some transporters has been analysed and described by kinetic rate models. In these simplified models the physical states of the transporter are represented and transitions between these states are the rates that kinetically describe the transport (Fig. 7). Detailed model evaluations have been done for hexose transport in AtSTP1(Boorer et al., 1994), sucrose transport

Figure 7. Schematic representations of reaction schemes for cotransporters. Kinetic (A) and physical (B) models to describe the transport of a substrate (S) and a proton (H) to the carrier with its binding sites exposed to the external side (C_o) or to the internal side (C_i)
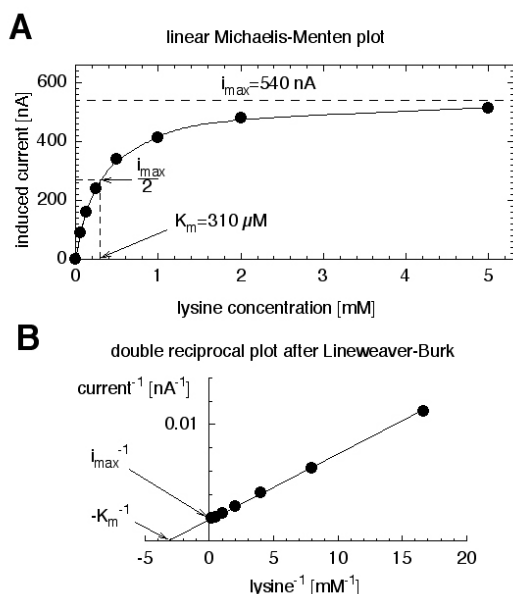
Figure 8. Dependence of transport activity of AAP5 on substrate (lysine) concentration A Lysine induced current versus concentration in a linear Michaelis-Menten plot, B double reciprocal plot. (unpublished)

by AtSUC1 and StSUT1 (Boorer et al., 1996; Zhou et al., 1998) and amino acid transport by AtAAP1 and AtAAP5 (Boorer et al., 1996; Boorer and Fischer, 1998). Since none of the rate constants can be measured in isolation, the models must generally extract kinetic information from steady state situations. The simplest model for a H^+/symporter involves 6 states and 12 rate constants, which obviously leads to some flexibility of a single model to describe the data (Fig. 7). Such flexibility is problematic when considering whether a specific model with defined rate constants exclusively describes the mechanism in one transporter. Although it may be arbitrary for the physiology of the plant if a transporter is binding its substrates in an ordered fashion or not, it is very instructive to understand the principles of transport. Both transporters act as proton symporters with 1:1 stoichiometry, and interestingly, StSUT1 may have a "slip" modus, with sucrose transported without protons (Boorer et al., 1996). Maltose is the only sugar transported with high efficiency (about half as good as sucrose), but monosaccharides or higher order sugars are not transported. Some cooperativity and voltage dependent binding of uncharged sucrose is observed. The data have been interpreted in two slightly different ways: AtSUC1 binds external protons and sucrose in non-ordered fashion, but on the internal side the sugar dissociates first (Zhou et al., 1997). StSUT1 binds protons before sucrose, both ligands are transported simultaneously across the membrane and sucrose dissociates first (Boorer et al.,

1996). Although both sucrose transporters may function differently, the multiplicity of parameters in such models may impair simple conclusions. Even a model with 6 states may be only a minimal approximation of the real situation.

An overview over metabolite transport systems in Arabidopsis

The most advanced system regarding knowledge of the transportome (i.e. the complete set of transporter proteins) of a eucaryote is the yeast *Saccharomyces cerevisae*, which serves as a model system for many of the studies in *Arabidopsis* (van Belle and André, 2001). Completion of the genomic sequence of *Arabidopsis* has allowed to generate membrane protein databases providing an overview over the potential number of *Arabidopsis* transporters (Ward 2001; Schwacke et al., submitted). According to the most recent analysis, the *Arabidopsis* genome contains ~6475 proteins with at least one membrane span or ~ 1976 proteins with at least four membrane spans (Schwacke et al., submitted). One may assume that a large proportion of the proteins with at least four membrane spans are involved in transport. Regarding functional assignment, transporters for the
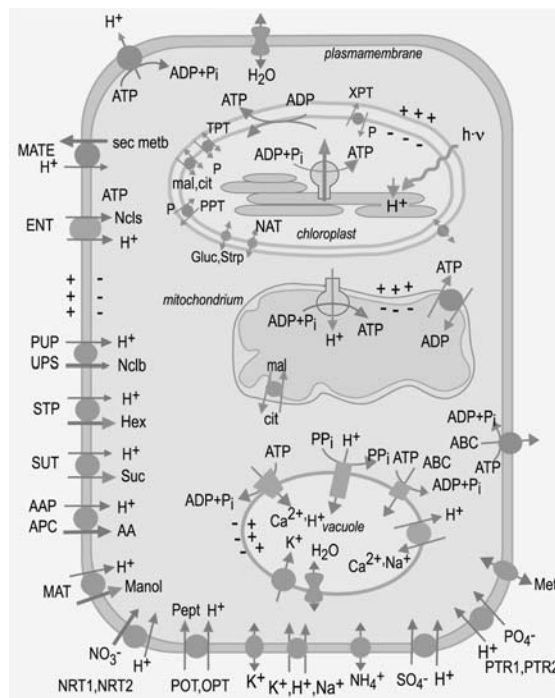
Figure 9. Schematic view of a cell with intracellular compartments. Molecularly identified nutrient and metabolite transporters are drawn in their respective membranes.

most nutrients and many metabolites have been identified in *Arabidopsis* at the molecular level. Most transporters are members of multigene families, and only in rare cases all similar or homologous transporters have been investigated in detail. The uptake transporters for nutrients and metabolites face a general problem: usually cell has to accumulate nutrients and metabolites against a concentration gradient. On the other hand the cells have to excrete waste products. If the cell would allow facilitated diffusion, the quickly reached equilibrium would impede further uptake. Only if the metabolite concentration within the cell decreases steadily, by active assimilation or metabolism, the cell can act as a sink and would maintain a steady influx.

Nutrient transporters

The uptake of nutrients in plants is of principle importance for optimal growth and development. Besides the elements C, H and O, several more elements are essential at high quantities, and thus plants have developed robust and efficient uptake systems for the macronutrients (N, K, Ca, Mg, P, S) from the rhizosphere. Of the micronutrients Cl, B, Fe, Mn, Zn, Cu, Mo, Ni, Na (Marschner, 1993) only trace amounts, generally about 100-10.000 fold less than macronutrients, are necessary for plant growth. However, secondary nutrient transporters are also involved in water balance, osmolyte equilibration and turgor generation. The cell wall allows the plant cells to survive in a dilute medium without bursting, which is in contrast to animal cells that strictly regulate the turgor in a small range by uptake and excretion of osmolytes.

Transporters for all macronutrients have been identified in *Arabidopsis* (Fig. 9). For e.g. potassium uptake, a cation critically involved in turgor generation and establishment of the membrane potential, many unrelated transporters with diverse coupling mechanisms have been identified. An overview about transporter families involved in anorganic cation transport can be found in Maser et al. (2001), and will not be detailed here.

Several organic compounds and metabolites can be taken up by roots from the rhizosphere and may serve as nutrients. In the case of nitrogenous compounds, the primary nitrogen sources in most soils are anorganic nitrate and ammonium. Ammonium and nitrate are acquired by use of high and low affinity transporters (Nigel M. Crawford and Brian G. Forde; this book). Although ammonium may be preferred due to the lower energetic cost needed for assimilation (Gazzarrini et al., 1999), nitrate is often the main nitrogen source for plants. After concentrative, electrogenic H^+-coupled uptake into the symplasm by epidermal and cortical cells, it may be stored in vacuoles, where concentrations up to several tens of mM can occur. Nitrate is subsequently reduced in a two-step process to ammonium, or mobilized to the xylem, and later reduced to ammonium in shoots. Both low and high affinity transporters have been molecularly identified. They belong to two structurally distinct families, the NTR1 (52 members, Tsay et al., 1993) and NTR2 (7 members, Trueman et al., 1996) families, both with 12 TMs. Interestingly, one barley homolog of the NTR1 family transports histidine in addition to nitrate (Zhou et al., 1998), and one *Arabidopsis* homolog transports dipeptides and histidine (Rentsch et al., 1995). This may indicate that within a single protein family the requirement for nitrogen leads to evolution of a capacity to transport several nitrogenous compounds. However, the details of substrate recognition in such transporters remain obscure. High affinity acquisition of ammonium is due to AMT transporters (6 members), which facilitate NH_4^+ diffusion across membranes (Ninnemann et al., 1994; Ludewig et al., 2002). These proteins form multimers with each subunit composed of 11 TMs. Nitrogen uptake and metabolism is highly regulated and tightly linked to carbon metabolism (Coruzzi and Bush, 2001, Corruzzi and Zhou, 2001).

Other macronutrients, like sulfur and phosphorous may be also taken up in form of organic molecules, but in most soils sulfur will be acquired by sulfate transporters, that are secondary active and import SO_4^{2-} by cotransport with three protons, giving a net positive charge transport into the cytoplasm. 12 members of that family with 12 putative TMs are identified in the *Arabidopsis* genome (Takahashi et al., 2000).

Phosphate (PO_4^{3-}) is, unlike nitrate and sulfate, not reduced in plants during assimilation. It remains in the oxidized state and forms phosphate esters in many organic molecules. Many factors, including poor solubility, high sorption to soil particles, and competition for phosphate in the soil lead to low availability of phosphate to the plant. Thus plants have evolved regulated high affinity systems to cope with typical soil concentrations of below one µM. In addition, some plants modify the rhizosphere to increase free inorganic phosphate by release of organic acids and protons or exudate phosphatases to release organic bound phosphate. Phosphorous uptake (as PO_3^-) is mediated by secondary active co-transport with protons. Transporters of the high-affinity AtPT family (9 members) encode major facilitator superfamily proteins with 12 TMs and show significant homology with the yeast Pho84 H+-Pi cotransporter (Muchhal et al., 1996). Most H^+-P_i cotransporters cloned in plants are expressed preferentially in the roots, with transcript abundance increasing after Pi starvation.

A single homolog of the animal Na-P_i cotransporters was identified in the genome of *Arabidopsis* and encodes a low-affinity H^+-P_i cotransporter expressed preferentially in leaves (Pht2;1; Daram et al., 1999). Pht2;1 is structurally unrelated to the other phosphate cotransporters, although also having 12 membrane-spanning regions. A putative P_i efflux transporter PHO1, involved in P_i release into the xylem has been identified after map based cloning in the

pho1 mutant (Hamburger et al., 2002). PHO1 has 7 TMs and 10 homologs in the genome. Interestingly, the proteins are related to receptor proteins in other species that are involved in G-protein associated signal transduction. The N-termini share similarity to several proteins involved in the regulation of phosphate transport, including the putative phosphate level sensors PHO81 from *Saccharomyces cerevisiae*.

Metabolite carriers

Sucrose

In *Arabidopsis*, as in most mainly apoplasmic loaders, sucrose is the major long distance transported sugar derived from photoassimilation. In all plants sucrose is produced in the cytosol of mesophyll cells of mature leaves. Sucrose is the first nonphosphorylated sugar that is produced after triosephosphates are exported from chloroplasts. Among other metabolites, Fru-6-P is produced from exported triosephosphates and together with UDPGluc forms Suc-6-P, which is digested by sucrose phosphatase to sucrose and phosphate. Sucrose is then exported via the sieve elements in the phloem. The exact path of sucrose from the mesophyll cells to the actual conduits, the sieve element companion cell complex (SECC), is still not understood. It still remains obscure how sucrose effluxes into the cell wall. In contrast, the subsequent step, i.e. the import of sucrose into the SECC has been elucidated in very much detail. Biochemical approaches had originally identified a 42 kDa protein in sugar beet as the membrane protein responsible for sucrose transport (Li et al., 1992; 1994). However final cloning of the gene was achieved by suppression cloning in an artificial yeast mutant (Riesmeier et al., 1992; 1993). Such a mutant has been an excellent tool to isolate further functional *Arabidopsis* transporters (Sauer and Stolz, 1994). Sucrose transporters are proton symporters (Boorer et al., 1996; Zhou et al., 1997). Antisense repression demonstrated unambiguously that sucrose transport mediated by this membrane protein represents the major translocation pathway for sucrose supplying the sink organs with carbon skeletons and energy (Riesmeier et al., 1994; Lemoine et al., 1996; Kühn et al., 1996). In addition, solanaceous plants contain at least two other sucrose transporters, SUT2 and SUT4 (Barker et al., 2000; Weise et al., 2000; Meyer et al., 2000). Whereas SUT1 serves as a high affinity transporter, the affinity of SUT2 and SUT4 are one order of magnitude lower (Schulze et al., 2000; Weise et al., 2000).

The *Arabidopsis* genome encodes 9 proteins homologous to the previously identified sucrose transporter SUT1 (Riesmeier 1992; 1994). The *Arabidopsis* sucrose transporter family can phylogenetically be divided into three subgroups: the high affinity SUC2 and the low affinity transporters SUT2 and SUT4. In addition, AtSUC1, which is similar to the SUT1 paralog NtSUT3 in tobacco (Bürkle et al., 1998; Lemoine et al., 1999), is specifically expressed in pollen. In contrast to SUT1 in solanaceous species, which is localized in the enucleate sieve elements (Kühn et al., 1997; Reinders et al., 2002), its *Arabidopsis* ortholog is found exclusively in companion cells (Stadler and Sauer, 1996). Thus orthologous transporters may obviously have different localizations and functions in different plants.

Disruption of the transporter responsible for phloem loading of sucrose thus is expected to massively disturb growth and development. Indeed, analysis of T-DNA insertion mutants in the gene for the specifically phloem companion cell-expressed SUC2, impairs much of the overall sucrose transport (Gottwald et al., 2000). This is very similar to other plants, where antisense repression of SUT1, the corresponding (orthologous) gene impairs normal development (Riesmeier et al., 1994; Lemoine et al., 1996; Kühn et al., 1996, Schulz et al., 1998). However, plants survive without that major loader, and this indicates that other sucrose transporters exist and may take over part of the function in mutant plants. Organ specific overexpression can lead to higher sucrose accumulation in that tissue, as shown in pea (Rosche et al., 2002). Sucrose transporters are probably multimers, as shown by use of the split-ubiquitin assay, that reports if membrane proteins are in close proximity (Reinders et al., 2002a). All analysed sucrose transporters have distinct tissue localization suggesting specific and nonredundant functions, with SUT2 expressed in all tissues at low level. In both tomato and *Arabidopsis*, some genes such as pollen specific AtSUC1 (Stadler et al., 1999) and AtSUC5, probably derive from recent gene dublications.

Interestingly, the structurally unrelated Biotin is also transported by AtSUC5 and PmSUC2 (Ludwig et al., 2000), but the surprisingly low cross inhibition by both substrates may indicate that both substances bind to different substrate recognition sites.

Other carbohydrates

A number of plant species transport raffinose-type oligosaccharides in the phloem. Raffinose has also been found in leaves of *Arabidopsis* (Hariatos, 2000). Whether raffinose is actually transported in *Arabidopsis* remains to be shown. Competition experiments for sucrose uptake mediated by SUT sucrose transporters indicates that none of the known SUTs is able to transport raffinose (Riesmeier et al., 1992; Schulze et al., 2000). Thus one may speculate whether other transporters, potentially also members of the MFS family may be responsible for transport of these compounds.

Table 1. Sucrose transporter genes in tomato and *Arabidopsis*

Tomato	*Arabidopsis*	code	K_m*A.th.*	Cell type tomato	Cell type *A.th.*	Remarks
LeSUT1	AtSUC2	At1g22710	1 mM	SE	CC	
[NtSUT3]*	AtSUC1	At1g71880	1 mM	pollen	pollen	
	AtSUC5	At1g71890				Biotin
		At2g14670				
		At5g43610				
		At1g66570				
		At5g06170				
LeSUT2	AtSUT2/ AtSUC3	At2g02860	11 mM	SE	phloem	
LeSUT4	AtSUT4	At1g09960	8 mM	SE	phloem	

* So far only found in tobacco

Instead of importing sucrose, phloem cells can accumulate carbohydrates via monosaccharide transporters after extracellular hydrolysis by cell wall-bound invertases into fructose and glucose.

The prototype of these transporters, AtSTP1, functions as a high affinity monosaccharide H$^+$-symporter (Boorer et al., 1994) and is expressed in leaves and other organs including stems, flowers and roots (Sauer et al., 1990). STPs contain twelve transmembrane spanning domains and also belong to the MFS family. A mutant deleted in AtSTP1 suggests that this transporter is of major importance for monosaccharide uptake in seedlings (Sherson et al., 2000). However, although monosaccharide uptake in AtSTP1 deletion mutant seedlings was strongly reduced, growth was not obviously impaired (Sherson et al.,2000). Another transporter, AtSTP2, is expressed exclusively during the early stages of pollen development where it may be responsible for carbohydrate import into the male gametophyte (Truernit et al., 1999). AtSTP3 is a low-affinity transporter found in leaves (Büttner et al., 2000). AtSTP4 is expressed sink-specific and is regulated in response to environmental factors, such as wounding or pathogen infection (Truernit et al. 1996). Quite surprisingly, *Arabidopsis* encodes more monosaccharide than disaccharide transporters, although long distance translocation is mainly in the form of sucrose. In total, 14 putative monosaccharide transporters exist in *Arabidopsis*, which have 12 TMs and belong to the MFS-family (major facilitator superfamily) of transporters (Büttner and Sauer, 2000).

Several plant species also transport sugar alcohols such as mannitol or sorbitol in the phloem. A mannitol transporter has been identified in celery, where mannitol is, besides sucrose, the main long distance transported sugar (Noiraud et al., 2001). The celery transporter AgMaT1 is highly homologous to seven *Arabidopsis* transporters, that are distantly related to sugar transporters and belong to the major facilitator superfamily (MFS). Furthermore myo-inositol transporters have been identified, belonging also to the MFS family

(Chauhan et al., 2000). This MFS superfamily in *Arabidopsis* encodes 86 proteins in total and may contain other carbohydrate transporters. Although organic acids are abundant in the phloem, no plasma membrane transporters have been identified so far.

Amino acid transporters

Amino acids are the major transport form of organic nitrogen for most plants. In contrast to sucrose, which is transported almost exclusively in the phloem, amino acids cycle in the plant vascular system and are found in both phloem and xylem (for review cf. Fischer et al., 1998; Delrot et al., 2000). Exchange is facilitated by specialized morphological structures and close proximity of the vessels. Therefore the use of terms like sink and sourc is problematic and should be restricted to sucrose and sugar transport. All proteinogenic amino acids are found in the two vascular systems. The relative abundance of the amino acids transported over long distances varies during the day with the availability of carbon skeletons from photosynthesis, but glutamine, glutamate, aspartate and asparagine generally dominate (Lam et al., 1995). Assimilation of nitrogen (in the form of ammonium) almost exclusively occurs via glutamine synthetase with glutamate as substrate, while glutamate dehydrogenase is thought to play only a minor role (Coruzzi and Bush, 2000). With tight coupling to the citric acid cycle, glutamate synthase and aspartate aminotransferase transfer assimilated ammonium to other "transport amino acids". Since all other nitrogenous metabolites derive from these primary amino acids, the abundance may provide a simple explanation for the abundance of glutamine and other transport amino acids. This focus on certain amino acids may also allow the plant to control total amino acid metabolism at a few key regulatory enzymes and links nitrogen assimilation tightly to carbon metabolism (Coruzzi and Bush, 2000).

Similar to sucrose and ammonium transporters, amino acid transporters were initially identified by functional yeast complementation (Frommer et al., 1993; Hsu et al., 1993). A large number of putative amino acid transporters exists in the genome of *Arabidopsis*: at least 46 members of the amino acid transporter family (ATF1), with 9-11TMs (Chang and Bush, 1997), and 14 members of the amino acid polyamine choline transporter family (APC), containing 12-14 TMs (for an overview of the family and their predicted structure cf Wipf et al., 2002).

The substrate specificity of some amino acid transporters (AtAAP1-6 of the ATF1 class) has been studied in detail. Although all have different specificities, each AtAAP is capable of transporting all proteinogenic amino acids (Boorer et al., 1996; Boorer and Fischer, 1997; Fischer et al., 1995, 2002). Although negatively charged glutamate and aspartate are abundant in the vasculature, these amino acids are only transported in their uncharged, zwitterionic form in AtAAP transporters (Fischer et al., 2002). AtAAP1-5 transport neutral amino acids with an affinity in the millimolar range, only AtAAP6 has an about tenfold higher affinity. Only AtAAP3 and AtAAP5 efficiently transport positively charged lysine, ornithine, arginine and histidine, but the higher abundance of other amino acids in the apoplast may minimize uptake of these positively charged amino acids (Fischer et al., 2002). Other, but homologous amino acid transporters have a narrow substrate specificity and may participate in accumulating preferential amino acids to specific organs (Schwacke et al., 1999). Proline, glycine betaine, and γ-amino butyric acid are known as compatible solutes, accumulating during drought and salt stress. Several transporters, the ProTs that belong to the ATF1 class, have preferences for these amino acids and may regulate and participate in organ specific accumulation of compatible solutes (Rentsch et al., 1996; Schwacke et al., 1999; Breitkreuz et al., 1999).

All analysed amino acid transporters have functionally been described as proton-coupled import systems, thus being preferentially involved in accumulation, but not release of amino acids. Amino acid concentrations in the phloem vary between 100-200 mM, while concentrations within the xylem are ~10-times lower. Amino acids are found at lower concentrations, but similar composition also in the apoplasm, a consequence of apoplasmic phloem loading discussed earlier for sucrose. When considering the pathway to the symplasmically isolated seeds and pollen, similarly transporters for release and subsequent uptake have to be considered (Lalonde et al., 2002; Patrick and Offler, 2001). AtAAP1 is expressed in seeds, suggesting a role in supply of developing embryos, whereas AtAAP2 is found in vascular tissue of seeds, potentially acting as xylem and phloem trans-loader (Hirner, 1998). Proline importers may be important for pollen loading (Schwacke et al., 1999). All investigated transporters show specific expression patterns (Chen and Bush, 1997; Chen et al., 2001; Fischer et al., 1995;

Frommer et al., 1995; Kwart et al., 1993; Rentsch et al., 1996). With the large number of still un-analyzed putative amino acid transporters and unknown cell specific expression of the known transporters, it remains unclear how these amino acid transporters contribute to cell and organ specific loading of amino acids and to cycling of amino acids (Atkins, 2000).

Peptide transport

Two protein families have been implicated in peptide transport in plants: the POT transporters for di- and tripeptides with 52 homologs has a general 12 TM architecture (Stacey et al., 2002) and the unrelated OPT family for tetra- and pentapeptide transport, with 9 members containing 12-14 TMs (Koh et al., 2002). The large number of putative plant peptide transporters is somewhat surprising, as peptide transport in plants had been only implicated in embryo development previously (Stacey et al., 2002). *Arabidopsis* has more predicted peptide transporters compared with other sequenced organisms. Many of them may play a role in rapid mobilization of nitrogen from proteolysis, e.g. during germination, flowering, seed filling and senescence. In addition transport of peptides may have other roles than nutrition. A potential substrate is the tripeptide glutathione, which is one of the major redox buffers in most aerobic cells. It may be implicated redox signalling and cellular homeostasis. The substrate specificity, however, of the peptide transporters has to be explored in more detail, as distant POT homologs transport nitrate, single amino acids, and dipeptides (Zhou et al., 1998; Rentsch et al., 1995).

Heterocyclic N-transporters

Nucleobases play a central role in DNA and RNA metabolism. They are precursors of secondary plant compounds like cytokinins, some alkaloids and of ureides. Ureides are a major long distance nitrogen transport form in legumes. In many plants nucleobases, nucleosides, but also derivates such as allantoin, uric acid, xanthine, caffeine and cytokinins are transported. As these compounds have a high nitrogen/carbon ratio and are precursors of genetic information, these metabolites may be especially important during germination, may be accumulated in pollen, but may also simply serve a role in nitrogen nutrition.

The *Arabidopsis* genome contains at least four types of nucleobase/nucleoside transporters: PUPs (purine permeases), UPS (ureide permeases), ENT/CNT (equilibrative nucleoside transporters) and NAT (nucleobase ascorbate transporters). The PUP family

contains 20 members with 9-11 TMs, currently three are characterized. The first PUP transporter was identified by complementation of an adenine transport deficient yeast strain (Gillissen et al 2000). PUP transporters accumulate their substrates in a pH-dependent manner and can also transport the plant signaling molecules cytokinin and caffeine (Frommer et al., unpublished). PUP transporters are expressed in hydathodes and phloem (Frommer et al., unpublished). Five transporters of the second family, UPS, with 10 TMs are identified in the genome of *Arabidopsis*. UPS transport allantoin and other N-heterocyles by proton co-transport (Desimone et al., 2002). The third characterized nucleotide transporter family has 10-12 TMs and 11 members in Arabidopsis. A maize homolog encodes a high-affinity transporter for the oxidized purines xanthine and uric acid (Argyrou et al., 2001). Intrestingly, this transporter is necessary for proper chloroplast development in maize. Nucleotide transport into organelles like the peroxysome may be important during DNA catabolism, and such peroxysomally localized transporters have been identified in yeast (Palmieri et al., 2001). The first transporter of the ENT family mediates proton coupled high affinity nucleoside import and has 11 TMs (Möhlmann et al., 2001). Other so far uncharacterized putative nucleotide/nucleoside transporters found in the genome are three homologues to uracil/allantoin transporters and a homolog of a concentrative nucleobase transporter family. A detailed characterization of the phloem sap of Arabidopsis using "metabolome" analyses may shed new light on the requirements regarding transport activities of the phloem.

Efflux transporters

Little is known about primary metabolite efflux proteins, but recently several members of the multidrug and toxic compound extrusion (MATE) family of transporters have been analysed and found to function in export. The MATE proteins are characterized by the presence of 12 putative transmembrane segments. MATEs are believed to function as proton-dependent efflux transporters, based on experimental evidence from the plant transporters and family members in other organisms (Li et al., 2002). MATEs transport lipophilic cations and related compounds, such as norfloxacin, ethidium bromide or berberine, a common alkaloid of plants and also cadmium, probably in a chelated form (AtDTX1; Li et al., 2002). Knock-out of another transporter of that family, transparent TEST12 (AtDTX41), altered the seed coat color, which is largely effected by accumulation of flavonoids in the vacuole. This suggests that AtDTX41 plays a role in sequestration of flavonoids into the vacuole of the seed coat endothelium (Debeaujon et al., 2001). Another member of that family (ALF5 =AtDTX19) was identified in a genetic screen to be involved in lateral root

formation and mediates the efflux of e.g. tetramethylammonium (Diener et al., 2001). Positional cloning of the gene involved in low accumulation of salicylic acid in a pathogen-hypersusceptible mutant revealed that another MATE transporter (EDS5 =DTX47) an essential component of salicylic acid-dependent signaling (Nawrath et al., 2002)

Organellar transporters

The photosynthetic electron transfer in chloroplasts generates ATP and energy-rich reductants in order to assimilate carbon, nitrogen and sulfur. Plastids contain an outer and inner membrane layer, which are both selectivity barriers and function in concentrating the precursor molecules and exporting the end products. Several solute channels have been molecularly identified in the outer envelope membrane. These porin-like proteins in the outer envelope were formerly thought to be quite unspecific, but have now been shown to exhibit significant substrate specificity and to be highly regulated (Bolter and Soll, 2001).

Of fundamental importance for carbohydrate and sugar metabolism are phosphate translocator (PT) proteins in the inner plastid membrane, which all are thought to function as antiport systems (Weber and Flügge, 2002). They exchange inorganic phosphate or phosphorylated C3 and C6 compounds. Whereas the triose phosphate/phosphate translocator TPT (Flügge et al., 1989; Riesmeier et al., 1993; Fischer et al., 1994) is predominantly active in photosynthetically active tissues, the phosphoenolpyruvate/phosphate translocator (PPT) appears to be ubiquitously expressed and mediates plastidic phosphoenolpyruvate import, which is used as a substrate for the formation of aromatic amino acids via the shikimic acid pathway leading to a series of secondary compounds (Fischer et al., 1997). The Glc-6-P/phosphate translocator (GPT) is found in heterotrophic tissues, transports both Glc-6-P and triose phosphates, which are used either for the syntheses of starch and fatty acids or is fed into the plastidic oxidative pentose phosphate pathway (Kammerer et al., 1998). Another translocator is the plastidic pentose/phosphate translocator that transports some C5 ketose sugars, but no hexoses (Eicks et al., 2002). All phosphate translocator proteins belong structurally to a large superfamily with 100 proteins with 6-9 TMs, but almost all proteins are still uncharacterized. A distant homolog is golgi-localized, involved in cell wall formation and transports GDP-mannose (Baldwin et al., 2001). A plasidic glucose exporter that mediates glucose export from plastids was identified as a member of MFS-hexose transporter family and contains a N-terminal signal peptide for chloroplast import (Weber et al., 2002).

Nitrogen assimilation is in part also performed in the chloroplasts, and metabolite transporters involved include

the 2-oxoglutarate/malate transporter. It has 3 homologs and 12 putative transmembrane domains (Weber et al., 1995). Two plastidic ATP/ADP antiporters contain 12 putative TMs (Neuhaus et al., 1997). A large number of 58 putative mitochondrial carriers (Laloi, 1999) of the MCF-type (mitochondrial carrier family) are mostly uncharacterized. The first molecularly characterized transporter of that type encodes a transporter for di- and tricarboxylates (Picault et al., 2002). Thus, although intracellularly localized and often experimentally difficult to approach, many proteins corresponding to plant plastidic transport activities have been already molecularly identified.

Conclusion

Suppression cloning in yeast and electrophysiological analysis have provided tools for rapid progress in the insights into the Arabidopsis transportome (Ward et al., 2001; Schwacke et al., 2002). However still many of the membrane proteins have not been characterized. For a full understanding of the transport processes it is necessary to characterize subcellular localization and cell specific expression pattern of the transporters in conjunction with metabolite mapping *in vivo* using nanosensors (Stitt, 2002). Furthermore *knock out* mutants and RNAi approaches, preferentially under the control of regulated systems will help us to understand the physiological role of the transportome much better. The interaction of transporters in rafts and complexes and with soluble proteins will be another major advance required for a full understanding of transport processes. The combined knowledge gained by the different approaches will then provide a basis for biotechnological manipulations aiming at an optimization of transport in the context of improved quality of products in harvest organs and for improving agrochemical delivery to sites of action (Kunze et al., 2002).

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