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Molecular analysis of plant sugar and amino acid transporters

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Abstract

Although plants are photoautotrophic organisms, they are composed of many heterotrophic tissue systems that must import sugars and amino acids in a process known as assimilate partitioning. While the general features of assimilate partitioning were well described by the early 1980s, little was known about the various transport proteins involved in this essential activity associated with multicellular growth. In the past seven years, however, significant progress has been made in describing the transport properties and molecular genetics of these critical transport systems. Initially, these porters were well characterized using purified membrane vesicles and imposed proton electrochemical potential differences. This approach allowed for a detailed analysis of their transport kinetics, bioenergetics, and substrate specificity. Subsequently, several transporters were cloned using differential hybridization and functional complementation of yeast transport mutants. At first, isolation of transporter genes seemed to simplify our understanding by filling in gaps associated with transporter function. However, it has become increasingly clear that assimilate partitioning has many levels of complexity yet to be penetrated. This is best illustrated by the large number of carriers cloned. For example, at least 12 genes encoding putative sugar transporters in the Major Facilitator Superfamily have been identified in plants. Moreover, recent work in this laboratory has demonstrated that a sugar beet member of this superfamily is targeted to the tonoplast membrane, thus implicating that porter in intracellular sugar partitioning. Similar complexity is emerging in the number of plant amino acid transporters identified. Therefore, a detailed analysis of AAP1/NAT2 as a prototypical example of one class of amino acid carriers has been initiated. The topology of this porter is being mapped, and site-directed and random mutagenesis are being used to identify functionally important amino acid residues and protein domains. The major challenges facing this field are to determine the unique contributions made by these many transport systems, to understand their structure and function relationships and, ultimately, to identify the mechanisms which regulate activity and integrate assimilate distribution in the plant as a multicellular organism.

Key words: Symporter, plasma membrane, assimilate partitioning.

Introduction

A hallmark in the evolution of multicellular organisms was the appearance of organized tissue systems that specialize in distinct biological processes required for growth and replication. In the higher plant, for example, the leaf is the so-called 'source' tissue because it contains the photosynthetic machinery which transforms light energy into useful biological energy and because much of that captured energy is exported from the leaf in the form of sucrose and amino acids in order to satisfy the biochemical needs of the heterotrophic cells. Likewise, the heterotrophic tissues specialize in other important processes, such as nutrient acquisition (roots) or reproduction (flowers, seed, fruit). The development of specialized tissue systems generated a need for long-distance transport mechanisms which mediate nutrient flow between the various organs of the plant. This resource redistribu-

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tion process, assimilate partitioning, is a fundamental physiological activity. While the general features of assimilate partitioning were well described by the early 1980s, little was known about the transport proteins that mediate nutrient flux into cells and between organ systems. In the past seven years, however, significant progress has been made on two fronts: first, detailed biochemical descriptions of the transport properties and bioenergetics of several transporters was achieved and, second, the genes encoding these transport proteins were cloned.

The 1970s was the decade in which the notion of ion-coupled transport came of age. Convincing evidence of sodium- and proton-coupled transport was found in several prokaryotic and eukaryotic systems (Baldwin and Henderson, 1989; Etherton and Rubinstein, 1978; Hediger *et al.*, 1989; Kaback, 1987; Komor, 1977; Reinhold and Kaplan, 1984). In higher plants, perhaps the most compelling experiments in support of proton-coupled porters included those examining proton and sucrose cotransport into castor bean cotyledons (Komor, 1977) and electrophysiological measurements correlating membrane depolarization with amino acid transport (Etherton and Rubinstein, 1978). Many subsequent studies contributed further evidence supporting proton-coupled symporters as the primary sucrose and amino acid carriers in the plant plasma membrane and, thus, identified those transport systems as major contributors in assimilate partitioning (Reinhold and Kaplan, 1984, for review; Gogarten and Bentrup, 1989).

In spite of convincing evidence in support of the symport hypothesis, however, little progress was made in identifying the transport proteins or in characterizing them with suitable biochemical methods until the late 1980s. At that time this laboratory and others provided the first *in vitro* characterization of plant sucrose and amino acid symporters using purified plasma membrane vesicles and imposed proton electrochemcial potential differences (Bush and Langston-Unkefer, 1988; Bush, 1989; Buckhout, 1989; Lemoine and Delrot, 1989; Williams *et al.*, 1990). The results obtained demonstrated $\Delta\mu_H$+-dependent sucrose and amino acid transport. Additionally, each transport activity exhibited saturable kinetics and sensitivity to protein modification, thus implicating carrier-mediated flux. Taken together, those data provided the first biochemical descriptions of proton-coupled secondary transport systems in the plant plasma membrane (Bush, 1993, for review). Subsequent experiments using the isolated membrane vesicle approach demonstrated that these porters are electrogenic (Buckhout, 1989; Bush, 1990; Lemoine and Delrot, 1989; Williams *et al.*, 1990; Li and Bush, 1990), that the stoichiometry of the sucrose symporter is 1:1 (Bush, 1990; Slone and Buckhout, 1991), and that substrate binding involves well-defined spatial and chemical interactions (Li and Bush, 1992; Tubbe and Buckhout, 1992).

The second major advance in this area came with the successful cloning of plant sugar and amino acid transporters. The first carrier cloned was the *Chlorella* glucose transporter (Sauer and Tanner, 1989). This clone was identified using a differential screening strategy by exploiting the observation that this transport protein is inducible under heterotrophic growth conditions. Subsequently, the *Chlorella* clone was used to identify related genes in *Arabidopsis* (Sauer *et al.*, 1990). Each of these glucose transporters is a member of a superfamily of related transporters, termed the Major Facilitator Superfamily, found in all organisms (Marger and Saier, 1993). An additional achievement of the Sauer laboratory was their use of functional expression in yeast to characterize the transport properties and substrate specificity of the cloned glucose porters (Sauer *et al.*, 1990, 1994).

Yeasts have played a pivotal role in providing molecular descriptions of several plant sugar and amino acid transporters. The sucrose symporter was cloned by functionally complementing a yeast mutant with a plant cDNA library constructed in a yeast expression vector (Riesmeier *et al.*, 1992). A yeast strain was engineered that lacked both sucrose transport activity and extracellular invertase. These cells can not grow on sucrose as the sole carbon source because of these deficiencies. The sucrose symporter was successfully identified by transforming this yeast strain with a plant expression library and then screening for positive transformants that acquired the ability to grow on sucrose. Subsequently, additional sucrose symporters were cloned from different plants using hybridization screening or RT-PCR (Sauer and Stolz, 1994; Lu and Bush, unpublished data). In addition to the sucrose symporter, several amino acid transporters have also been cloned by complementing yeast strains that are amino acid auxotrophs and transport mutants (Frommer *et al.*, 1993; Hsu *et al.*, 1993; Fischer *et al.*, 1995). Indeed, one of the major observations emerging from recent molecular descriptions of plant transporter genes is that there are large families of related porters that function in assimilate partitioning. Not surprisingly, there have also been a few unexpected results. In the following paragraphs, some of the current investigations from this laboratory into plant sugar and amino acid transporters are reported.

A putative sugar transporter in the tonoplast

Several plant genes have been cloned that are members of the sugar transporter subgroup of the Major Facilitator Superfamily (MFS) of transporters (Sauer *et al.*, 1994; Weig *et al.*, 1994; Chiou and Bush, 1996). Members of this gene family are found in both eukaryotic and prokaryotic organisms, and are believed to be derived from a common, primordial transport system (Griffith *et al.*, 1992). Because of the potential significance of these porters in carbon partitioning, a RT-PCR approach was used to clone several members of this family from sugar

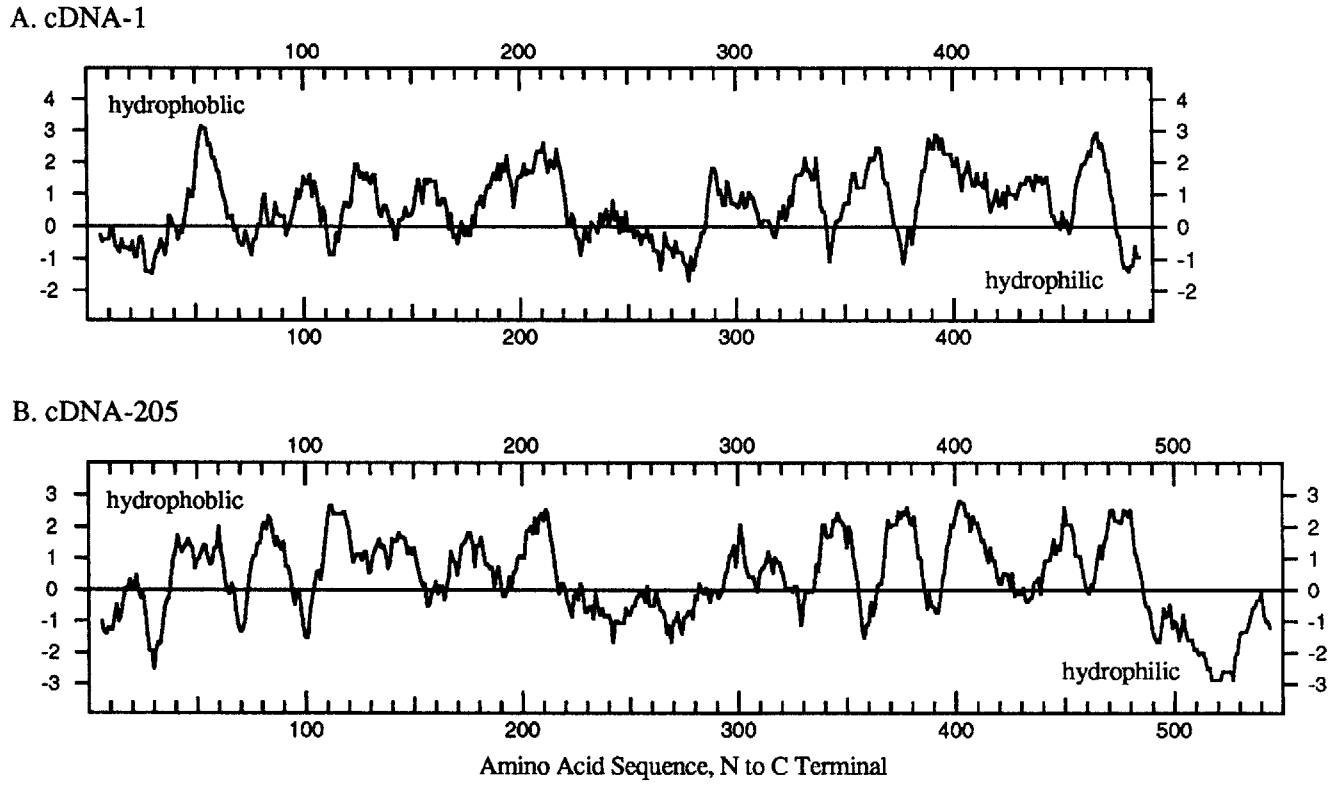

Fig. 1. Hydropathy plots predicting 12 transmembrane domains for two sugar beet cDNAs that are members of the MFS. cDNA-1 codes for a porter found in the vacuolar membrane. cDNA-205 is one of two very similar clones that are differentiated by their C-terminal sequences.

beet (Fig. 1). Interestingly, two of the three full length clones are almost identical, except for a C-terminal extension on one of them and, perhaps, the C-terminal sequence differences are important for transport regulation. These clones have been expressed in transgenic yeast as one approach to describe their transport characteristics. In spite of considerable effort, however, no transport activity has been detected when these cDNAs are expressed in transgenic yeast. These experiments included examining a variety of potential substrates and transport orientations. Therefore, a biochemical approach has been taken to learn about these porters and one clone, cDNA-1, has yielded exciting results.

The cDNA-1 clone codes for a protein with 490 amino acids and an estimated MW of 54 kDa. The deduced amino acid sequence exhibits significant similarity with members of the sugar porter subgroup of the MFS in both predicted membrane topology and sequence homology, 20–30% identity and 60–70% similarity. This suggests that cDNA-1 is a member of the sugar transporter family and that it may encode a sugar carrier in plants. RNA gel blot analysis revealed that this putative sugar transporter was expressed in all vegetative tissues, and expression increased with development in leaves. In order to investigate the expression and function of cDNA-1 in more detail, antibodies directed against small peptides representing the N- and C-terminal domains of the cDNA-1 protein were generated. In transgenic yeast, an additional 40 kDa polypeptide was observed in protein blot analysis of the microsomal membrane fraction of cDNA-1 transformed cells versus the non-transformed controls. This band was diffuse and smaller than that predicted from the deduced molecular weight, but was very similar to the *in vitro* translation product of cDNA-1. Moreover, antibodies directed against either the N- or C-terminal domain recognized the same protein. Protein blot analysis identified the cDNA-1 protein in sugar beet and transgenic tobacco membrane fractions containing golgi, ER, and tonoplast membranes. This was a surprising result because transporters in the MFS are almost exclusively found in the plasma membrane. To identify to which membrane system the cDNA-1 protein is targeted, the transporter's distribution across linear sucrose gradients was examined and showed that it co-migrated with tonoplast membrane markers (Plate 1). Additional experiments with flotation density gradients confirmed these results (Chiou and Bush, 1996). It is concluded that this carrier is located on the tonoplast membrane and that it may mediate sugar partitioning between the vacuole and cytoplasmic compartments.

Structure and function analysis of an amino acid symporter

The first plant amino acid symporter cloned was identified by functional complementation of yeast amino acid trans-

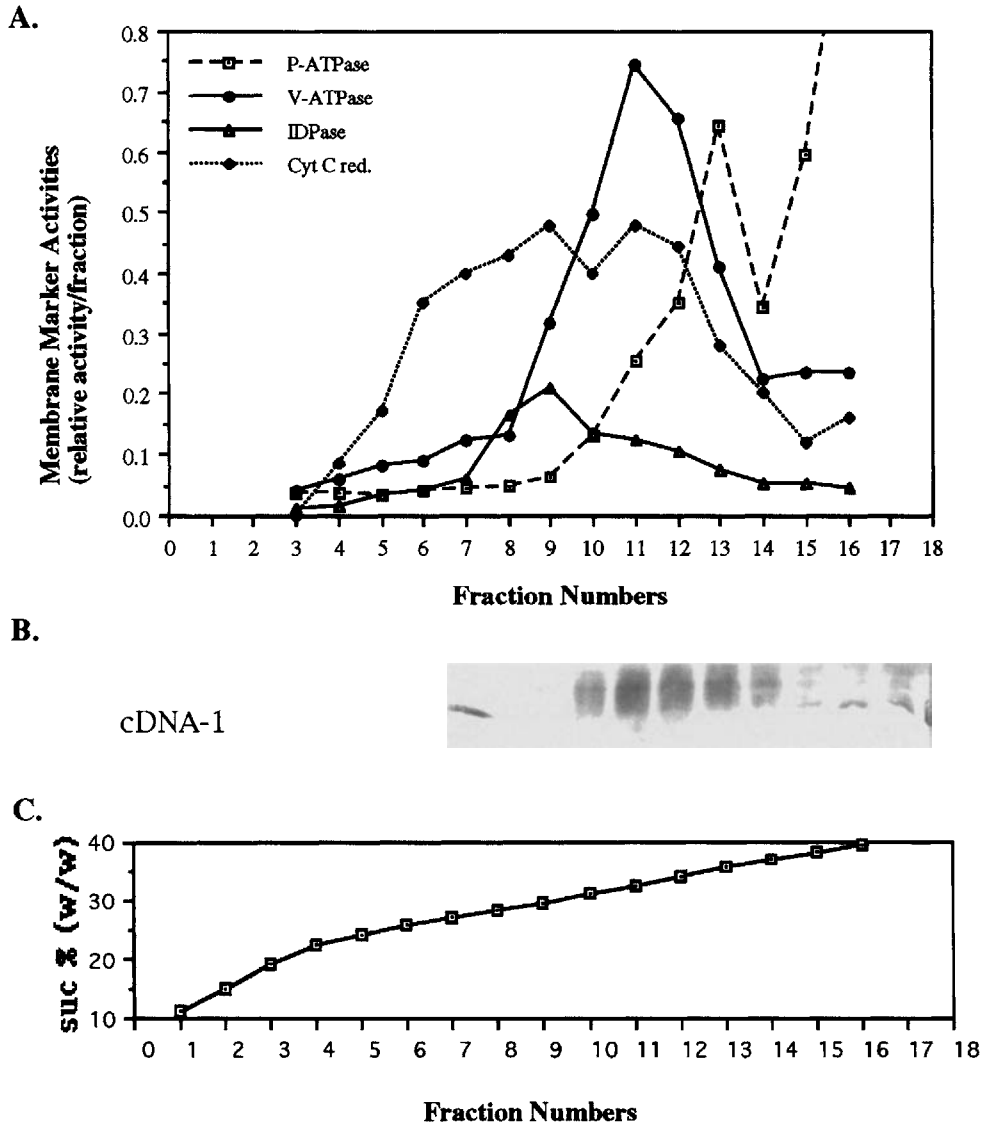

Plate 1. Gradient distribution of enzyme markers and cDNA-1 protein in sugar beet membranes. Membrane vesicles were separated on a linear sucrose gradient (20–40% (w/w)). Membrane marker activities and cDNA-1 protein were detected for each fraction. (A) Distribution of membrane marker activity, P-ATPase: VO_4^--sensitive P-type H^+-ATPase, a marker for plasma membrane and ER; V-ATPase: NO_3^--sensitive V-type H^+-ATPase, the marker for tonoplast; IDPase: Triton-stimulated inosine diphosphatase, the marker for Golgi membrane; Cyt *c* red: NADH-dependent, antimycin A-insensitive cytochrome *c* reductase, the marker for ER. (B) Protein gel blot analysis of cDNA-1 protein (anti-4C). (C) Sucrose concentration for each fraction.

port mutants with plant cDNA expression libraries (Frommer *et al.*, 1993; Hsu *et al.*, 1993). This symporter is currently termed AAP1/NAT2. It moves a variety of amino acids, but appears preferentially to translocate neutral amino acids. The deduced amino acid sequence contains 486 amino acid residues with a calculated molecular mass of 52.9 kDa and three sites of potential N-linked glycosylation. Hydropathy analysis suggests this is an integral membrane protein with 10–12 membrane-spanning α-helices. Subsequent screens of the yeast transport mutants identified at least five additional amino acid symporters that are closely related to AAP1/NAT2. Indeed, it is now clear that this is a large family of symporters in the plant (Fischer *et al.*, 1995). They are differentiated by subtle variation in transport substrate and in expression patterns. Since these transporters appear to be present in all plant tissues, it is considered important to examine the structure and function of this class of porters in detail. To achieve that goal, AAP1/NAT2 is being targeted as a prototypical example of this gene family because it is widely expressed and because it transports many of the major amino acids found in the phloem translocation stream.

Previous work in this laboratory had shown that this transporter is sensitive to chemical modification by diethyl pyrocarbonate (DEPC) (Li and Bush, 1990). DEPC

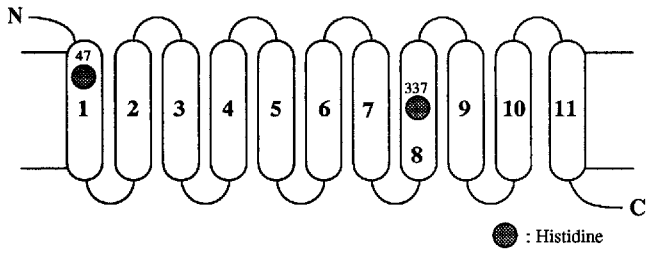

Fig. 2. Predicted topology of AAP1/NAT2 and relative location of His47 and His337.

forms a covalent bond with the imidazole ring of protein histidine residues. Additional experiments defining the time-dependent inactivation of this symporter by DEPC demonstrated substrate protectable inactivation kinetics, suggesting DEPC binds at, or binding is conformationally linked to, the substrate binding site. Together, these observations are consistent with a histidine residue functioning in the transport reaction mechanism. When the distribution of histidine residues in AAP1/NAT2 was examined, two residues were identified in putative transmembrane domains that are potential candidates for the DEPC-sensitive histidine (Fig. 2).

Table 1. *Site-directed mutants of His337 and their ability to complement amino acid transport deficient yeast*

His337 changed to	Complementation
Leucine	No
Proline	No
Cysteine	No
Phenylalanine	No
Tyrosine	No
Tryptophan	No
Arginine	No
Glutamine	No

To test the hypothesis that one or both of these histidines is important for function, site-directed mutagenesis was used to replace each residue with a variety of other amino acids. Significantly, all AAP1/NAT2 constructs containing single amino acid changes at His337 have lost amino acid transport activity (Table 1). It is concluded, therefore, that His337 is an essential contributor to amino acid transport function. His47 has also been changed, but these site-specific mutants have not been scored yet for transport activity. These directed-mutagenesis experiments have provided the first molecular information about essential amino acids that contribute to AAP1/NAT2 structure and function. While this is an important step forward, it is necessary to identify additional residues that contribute to porter structure and function before the building of predictive models of the folded protein can be considered. Therefore, random mutagenesis to examine AAP1/NAT2 structure and function is also being used.

Random mutagenesis can be applied here because simple screens exploiting the yeast transport-deficient mutants have been developed to identify functionally important mutations. Briefly, randomly mutagenized AAP1/NAT2 is transformed into the yeast strain and those cells are scored for growth under defined conditions that identify transformants that exhibit altered transport properties, such as modified K_m, V_{max}, or coupling to the pmf (Plate 2). By selecting for mutants with modified transport properties, functionally important residues and protein domains can be identified without the benefit of prior experimental evidence, such as the DEPC results used in the site-directed experiments. The aim is to use

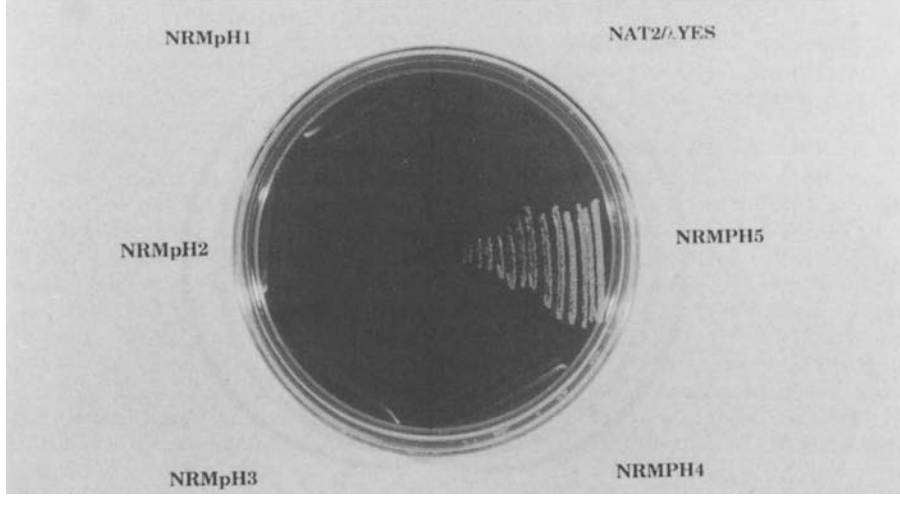

Plate 2. Plate screen of randomly mutagenized AAP1/NAT2 selecting for mutations that render the porter less sensitive to high pH. Randomly mutagenized AAP1/NAT2 was transformed into yeast transport mutants and screened for growth at pH 8.0. This phenotype is of interest because it may report on domains associated with pmf coupling and/or proton binding. Note that growth of the NAT2/λYES control was inhibited under these conditions.

these results, in combination with complementary experiments mapping the topology of this protein in the bilayer, to build an experimentally based molecular model of AAP1/NAT2. Moreover, it is hoped to identify regulatory domains that modulate transporter activity.

Considering the large number of sugar and amino acid transporters identified thus far, the major challenges facing this field are to determine the unique contributions made by these many transport systems, to understand their structure and function relationships and, ultimately, to identify the mechanisms which regulate activity and integrate assimilate distribution in the plant as a multicellular organism.

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